Mediterranean Marine Science

Vol 20, No 2 (2019)

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doi: 10.12681/mms.18660

Research Article

Mediterranean Marine Science
Indexed in WoS (Web of Science, ISI Thomson) and SCOPUS
The journal is available on line at http://www.medit-mar-sc.net
DOI: http://dx.doi.org/10.12681/mms.18660

Seasonal and plant-part isotopic and biochemical variation in *Posidonia oceanica*

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Handling Editor: Athanasios ATHANASIADIS

Received: 2 October 2018; Accepted: 18 March 2019; Published on line: 28 June 2019

Abstract

Posidonia oceanica is an iconic and highly productive Mediterranean seagrass. As most studies have focused on the fate of its production, temporal and plant part-specific variations of isotopic composition and biochemical content were overlooked. Combined seasonal and plant-part stable isotope composition and biochemical concentrations were measured at the lower depth limit of a *P. oceanica* meadow (~ 25 meter depth), and explained on the basis of previous knowledge of the specific metabolic functioning of each part. The predominance of compounds with complex chemical structure was reflected by the high concentrations of insoluble carbohydrates, high C/N ratios and high $\delta^{13}C$ values. Plant parts clustered in 3 groups with similar isotopic or biochemical features and metabolism: rhizomes and juvenile leaves, intermediate and adult leaves, senescent and drifting leaves. This result agrees with the vegetative phenology of the plant. The biochemical composition and the isotopic composition of the plant parts were consistent with previous knowledge regarding the photosynthetic activity and its seasonal variation. Correlations were found between N-linked descriptors ($\delta^{15}N$ and protein content), and between $\delta^{13}C$ and insoluble carbohydrate concentration. Epibiont values differed considerably from those of the leaf, as this community is taxonomically diverse and seasonally variable. Biochemical and isotopic composition measured confirmed that the current complex metabolism of *P. oceanica* results from adaptations to the specific features of life in a marine oligotrophic environment.

Keywords: *Posidonia oceanica*; Mediterranean Sea; stable isotopes; biochemical composition.

Introduction

Seagrasses are emblematic marine primary producers, widely distributed in the global ocean, fulfilling important ecological and economic functions, and are strongly affected by human activities (Cambridge & McComb, 1984; Bell & Pollard, 1989; Short & Wyllie-Echeverria, 2000; Heck Jr., Hays & Orth, 2003; Boudouresque *et al.*, 2009; Waycott *et al.*, 2009; Coles *et al.*, 2013; Ourgaud *et al.*, 2015). In the Mediterranean Sea, five seagrass species can be found, with *Posidonia oceanica* (Linnaeus) Delile being the most common in open sea. *Posidonia oceanica* is an iconic species of the Mediterranean coasts, mostly due to its endemism and the numerous ecosystemic functions it fulfills (Bell & Harmelin-Vivien, 1982; Harmelin-Vivien, *et al.,* 1995; Jiménez *et al.,* 1996; Mateo *et al.,* 1997; Boudouresque *et al.,* 2012, 2014; Pergent *et al.,* 2012). *P. oceanica* meadows are included in the group of low nutrients/high chlorophyll ecosystems (Boudouresque *et al.,* 2014), being some of the most productive ecosystems of the planet despite the oligotrophic nature of the Mediterranean Sea. Annual net primary production can reach 1 500 g dry mass m^{-2} a^{-1} for leaves and 900 g dry mass m^{-2} a^{-1} for the epibiotic community in shallow meadows (Libes *et al.,* 1983; Pergent-Martini *et al.,* 1994; Cebrià*n et al.,* 1997; Cebrià*n* & Duarte 2001; Romero 2004; Vela *et al.,* 2006). Analysis of the biochemical concentrations in plant part types enabled the resolution of this paradox through the identification of fluxes of nutrients and organic matter from the environment and within *P. oceanica* part types. It revealed several physiological adaptations that enable *P. oceanica* meadows to efficiently uptake nutrients from the environment, to store excess production in dedicated tissues and to recycle organic compounds from senescent leaves (Augier *et al.,* 1982; Pirc 1989; Pirc & Wollenweber 1988; Alcoverro *et al.* 2000, 2001; Lepoint, *et al.* 2000, 2002; Romero 2004; Boudouresque *et al.,* 2006). The seasonal and plant part-type variations of photosynthetic activity were also investigated with biochemical measurements. Previous studies identify the youngest leaves as the most photosynthetically active whereas the growth is reduced

in older ones (Alcoverro *et al.,* 1998). This high primary production is also due to the juxtaposition of two types of primary production, leaves and epibionts (Boudouresque *et al*., 2006). Epibionts can be considered as high nutrient/high chlorophyll in eutrophic systems (Boudouresque *et al.,* 2014).

Understanding the fate of this massive production has also been the focus of numerous studies, investigating notably the organization of trophic networks, organic matter (OM) fluxes within the trophic networks of *P. oceanica* meadows and the actual ability of invertebrates and teleosts to directly graze on leaves or epibionts. The epibiont biomass is considered as an important food source for invertebrate and teleost grazers (Shepherd 1987; Verlaque 1990; Havelange *et al.,* 1997; Tomas *et al*., 2005; Tomas *et al.,* 2006; Prado *et al.,* 2007), whereas living leaves are poorly consumed. Less than 10 % of the leaf biomass production is considered as directly grazed. The vast majority of this production is turned into necromass and then (1) buried in sedimentary pools (Pergent *et al.,* 1994; Pergent, Rico-Raimondino & Pergent-Martini 1997; Papadimitriou *et al.,* 2005; Cresson *et al.,* 2012; Personnic *et al.,* 2014; Boudouresque *et al.,* 2016), (2) integrated in complex detritus-feeder pathways (Lepoint *et al.,* 2006; Costa, Mazzola & Vizzini 2014; Michel *et al.,* 2015), or (3) exported to other marine or terrestrial ecosystems (Pergent, Rico-Raimondino & Pergent-Martini 1997; Romero 2004; Colombini *et al.,* 2009; Boudouresque *et al.,* 2016). In contrast, epibionts are classically considered as the main trophic source of grazers. The differential consumption of these two adjacent primary producers is explained by their different biochemical composition, that drive a differential nutritional interest for grazers (Ott & Mauer 1977; Shepherd 1987; Verlaque 1990; Prado, Alcoverro & Romero 2010; Prado & Heck Jr. 2011). The presence of structural compounds and chemical repellents makes the leaves unpalatable for the vast majority of herbivores (Boudouresque *et al.,* 2006; Tomas *et al.,* 2006; Prado *et al.,* 2007; Prado *et al.,* 2010). The generalized use of C and N stable isotope measurement represented a major breakthrough in this field, and confirmed the preferential assimilation of epibiotic biomass (Lepoint *et al.,* 2004; Tomas *et al.,* 2006; Fourqurean *et al.,* 2007; Vizzini 2009; Prado *et al.,* 2010). Since leaves and autotrophic epibionts use different photosynthetic metabolisms, their isotopic composition is different. Measuring the isotopic composition of a grazer can provide the means to determine the relative importance of leaves or epibionts in their diet and to confirm the fluxes of organic matter (*eg.* Dauby 1989).

Nevertheless, in most studies, C and N isotopic composition were measured in adult leaves only, and possibly for epibionts. Adult leaves predominate in the shoot and are thus a useful proxy (Scartazza *et al.,* 2017), notably when the aim of the study is to assess the fate of shoot production. However, some leaf-type specific functioning, metabolism and phenology may be missed if only adult leaves are considered, as leaves of different ages and metabolisms coexist within the same shoot (Giraud 1979; Pergent *et al.,* 1989; Boudouresque *et al.,* 2012). Previous results have demonstrated that several biochemical, metabolic or environmental factors affect the carbon isotopic ratio (hereafter referred as $\delta^{13}C$), such as growth rate, leaf thickness, inorganic C concentration in water, depth, light irradiance or pH (Cooper & DeNiro 1989; Lepoint *et al.,* 2003; Fourqurean *et al.,* 2007; Scartazza *et al.,* 2017). Similarly, nitrogen isotopic ratio (hereafter $\delta^{15}N$) of marine primary producers is commonly used as a proxy of anthropic nitrogen releases (Costanzo *et al.,* 2001; Vizzini & Mazzola 2004; Vizzini *et al.,* 2005; Pérez *et al.,* 2008; Lassauque *et al.,* 2010; Vermeulen *et al.,* 2011), but recent results indicated that $\delta^{15}N$ could be used to track fluxes of matter within the shoot (Scartazza *et al.,* 2017). Thus, isotopic differences between plant part types might be expected, since the physiology, metabolism and environmental context of the *P. oceanica* meadow change between plant-part types and seasons. To our knowledge, seasonal variation has barely been investigated, and plant part type variation only once (Vizzini *et al.,* 2003). In this paper, one storage organ (rhizomes), and several leaf types were considered, so as to track the biochemical and isotopic changes associated with creation, growth, senescence and drift of leaves, and seasonal cycle of primary production. Earlier studies also demonstrated that biochemical composition differed between leaves (*e. g.* Pellegrini 1971; Augier *et al.,* 1982; Pirc & Wollenweber 1988; Lawrence *et al*., 1989; Pirc 1989), and proposed that the biochemical variations might lead to isotopic differences (Lepoint *et al.,* 2003; Vizzini *et al.,* 2003), but no study combining the two approaches has been performed so far to verify this hypothesis (but see Scartazza *et al.,* 2017).

Consequently, the aims of the present study were firstly to combine isotopic and biochemical analyses performed on the same samples in order to document plant part type and seasonal variations of those parameters in a deep *P. oceanica* meadow. Even if the photosynthetic metabolism was not specifically determined in the present paper, results were analyzed in relation with the literature with regard to this aspect, hypothesizing that seasonal and plant part type specific variation of photosynthesis intensity and of nutrient availability might drive the patterns observed.

Material and Methods

Sampling

Several *Posidonia oceanica* live shoots (~5-10) were collected seasonally in March, June, September and November 2012 at the lower depth limit (~ 25 meter depth) of a meadow in the bay of Marseille (France, Mediterranean Sea; Fig. 1). The sampling site is located in the vicinity of an artificial reef system monitored since 2010 to understand in particular what organic matter fuels artificial reefs food webs (Cresson, Ruitton & Harmelin-Vivien 2014; Cresson *et al.,* 2019), and how artificial reefs may alter the density and lower depth limit of the meadow (Astruch *et al.,* 2015). In the laboratory, each shoot

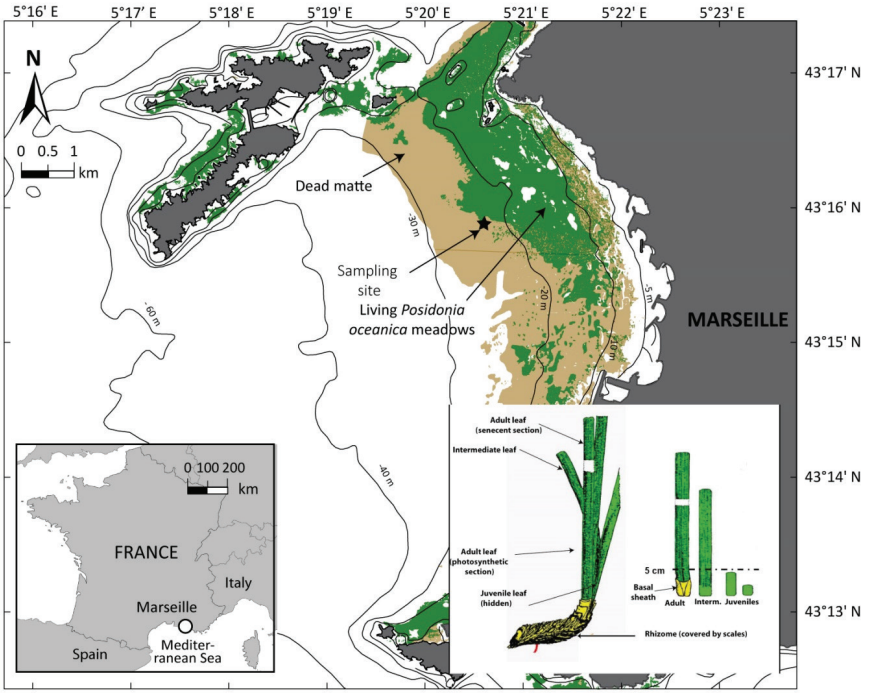

Fig. 1: Map of the sampling site (based on data from Andromede Océanologie, 2014)). The organization of a *Posidonia oceanica* shoot is represented in the lower-right panel (redrawn from Boudouresque *et al.* (2012).

was separated among different leaf types depending on their age following the classification of Giraud (1979): juvenile (less than 5 cm long, with intact leaf tip), intermediate (more than 5 cm and without basal sheath) and adult (more than 5 cm, with a basal sheath). Adult leaves were subsequently divided between the basal green part without the sheath (photosynthetically active, hereafter adult leaf) and the apical brown section (senescent leaf). In addition, dead *P. oceanica* leaves drifting away were collected at random close to the meadow, to investigate the subsequent changes in isotopic and biochemical parameters of the *P. oceanica* leaves. As dead leaves are also predominant contributors of sediment necromass (*e.g.* 70% of leaf production is directed toward sediment, Boudouresque *et al*. 2016), assessing their biochemical composition may be useful to accurately assess detrital fluxes in seagrass meadows (Boudouresque *et al.,* 2016). All leaves were cleaned and their epibionts removed by gently scraping with a razor blade. Leaf epibionts were preserved for isotopic and biochemical analyses. A small apical section (~3 cm) of rhizome (belowground storage plant part) was also collected on each shoot and included in the analyses, after the removal of the persistent basal leaf sheath (scales). All samples were stored frozen and freeze-dried. The amount of matter needed for successful replicated isotopic and biochemical analyses required the pooling of several leaves of the same type collected on several shoots at each site and in each season, even if this procedure precluded detection of individual variation. They were integrally used and homogenized prior to analyses with a mechanical grinding mixer mill. The resulting powder was used for both isotopic and biochemical analyses.

Isotopic and biochemical analyses

Prior to stable isotope measurement, powder resulting from leaf epibiont grinding was divided into two parts. Since carbonate can represent a bias for $\delta^{13}C$ determination, one subsample was acidified following classical procedure (e.g. Bosley & Wainright 1999; Jacob *et al.,* 2005). Briefly, powder resulting from epibiont grinding was repeatedly immersed in 1% HCl until no more CO_2 was released, then rinsed with deionized water and dried. The effect of acidification on $\delta^{15}N$ composition is questioned but might represent a bias, thus this analysis was run on the untreated subsample.

Stable isotope composition was determined using a continuous-flow isotope-ratio mass spectrometer (Delta V Advantage, Thermo Scientific, Bremmen, Germany) coupled to an elemental analyzer (Flash EA1112 Thermo Scientific, Milan, Italy). Results were expressed with the δ notation, where $\delta X = \left(\frac{R_{sample}}{R_{standard}} - 1\right) \times 10^3$, with X = ^{13}C or ^{15}N and R the isotopic ratio $^{13}C/^{12}C$ or $^{15}N/^{14}N$ respectively. Standards used were V-PDB for carbon, and atmospheric N_2 for nitrogen. For both $\delta^{13}C$ and $\delta^{15}N$, measurement precision is <0.1‰ (replicate measurements of internal laboratory standards, acetanilide).

Biochemical concentrations (soluble and insoluble carbohydrates and lipids) were determined with spectrophotometric methods and based on replicated analyses of *P. oceanica* samples. Briefly, these methods are based on the specific reactivity of the biochemical molecules with reagents, and by the production of solutions of which the color intensity and light absorption at a specific wavelength are proportional to the concentration. Comparison

of the solution absorption with values measured for calibration standards of known concentration enables the determination of the solution concentration. Soluble (SC) and insoluble (IC) carbohydrates concentrations were determined following the method of Dubois *et al.* (1956) and expressed as glucose equivalent. Soluble carbohydrates were extracted from samples with distilled water (100°C, 20 min) and insoluble carbohydrates from the residual solution. Lipid concentrations were determined following Bligh & Dyer (1959) and were expressed as tripalmitic acid equivalent. Two methods were used for protein determination. For leaf epibionts, protein content was determined with the method of Lowry *et al.* (1951), recommended as the most appropriate for most marine algae (Barbarino & Lourenço 2005). Since this method is known to interfere with phenolic compounds produced in high concentrations by *P. oceanica* tannin cells (e. g. Cuny *et al.,* 1995), it is not well-suited for leaves and rhizomes. Consequently, the protein content in leaves and rhizomes was calculated from the %N, considering a conversion factor between %N and protein concentration. This technique is currently being called into question. Recent studies calculated a nitrogen-to-protein conversion factor lower than the theoretical 6.25 value, and observed major differences between species and taxonomic groups (eg. Lourenço *et al.,* 1998; Diniz *et al.,* 2011). To our knowledge, no dedicated study has investigated this conversion factor for *P. oceanica* or for any other Magnoliophyta. Nevertheless, a conversion factor of 4.28 was calculated from previous results (Augier *et al.,* 1982) as the ratio between protein concentration (calculated as the sum of the total amino-acids) and % N of *P. oceanica* adult leaves collected at 30 m depth at the Port-Cros National Park (~90 km east of Marseille). Prior to actual chemical analyses, several tests with increasing amounts of sample were performed. The aim was to determine the most appropriate mass of sample for efficient quantification, *i.e.* the amount of sample that would produce a solution the absorption of which would be within the most effective range of the spectrometer. The amount of matter used was dependent on the expected quantity of each biochemical class in plant part-type, and was ~1 mg for carbohydrates, ~ 10 mg for lipids and ~ 60 mg for proteins. All biochemical concentrations were expressed in mg g^{-1} dry mass. Finally, the inorganic matter content of the samples was determined as the ash content determined by weight loss after combustion in a muffle furnace (500°C, 5 h). Due to the amount of matter needed for ash content determination, only one analysis per plant part type and season was performed, precluding the use of those results in statistical analyses. No ash content was determined for juvenile leaves in spring and summer (as not enough juvenile leaves were found in the shoots in this period), or for leaf epibionts in all seasons.

Numerical analyses

After checking for normality and homogeneity of variances, two-way ANOVAs, followed by Student's Least Square Distance post-hoc tests when significant, were performed to assess the effect of season and plant part type on stable isotope composition and biochemical content. If prerequisites were not reached, non-parametric Kruskall-Wallis ANOVAs were performed. The effect of acid on the $\delta^{13}C$ composition and %C of epibionts was assessed with a non-parametric Mann Whitney test. Finally, PCA analyses were performed on seasonal mean isotopic composition, biochemical concentrations and ash content to identify similar plant parts, including or not epibionts in the analysis. All statistical analyses were performed using R software with "FactoMineR" package (Lê, Josse & Husson 2008; R Core Team 2018).

Results

Isotopic composition

Values measured for *P. oceanica* plant parts (i. e. leaves and rhizomes) ranged between -17.60 ± 0.13 ‰ and -13.98 ± 0.22 ‰ for $\delta^{13}C$ and between 2.77 ± 0.02 and 6.42 ± 0.23 ‰ for $\delta^{15}N$ (Fig. 2). Leaf epibionts exhibited a significantly lower $\delta^{13}C$ value than leaves and rhizomes (ANOVA $F_{(1,88)}$=465.50, p-value < 0.0001), but a similar $\delta^{15}N$ value (ANOVA $F_{(1,83)}$ = 1.17, p-value = 0.19). Juvenile leaves and rhizomes exhibited the highest annual average $\delta^{15}N$ composition (4.98 ± 0.94 ‰ and 5.00 ± 0.28 ‰, respectively; Table 1). Adult and intermediate leaves had similar mean $\delta^{13}C$ values (-15.97 ± 0.89 ‰ and -15.97 ± 1.05 ‰, respectively). Senescent and drifting leaves exhibited rather similar mean $\delta^{15}N$ values, lower than those of other parts. As expected, acidification has a significant effect on both $\delta^{13}C$ (Mann Whitney Z = 4.36, p < 0.001) and %C (Mann-Whitney Z = 4.37, p < 0.001) of leaf epibionts (Fig. 3). Acidification resulted in a ~3-fold division of %C (15 to 18 % for untreated samples, 5.4 to 6.6 % for acidified samples) and in a 7 ‰ diminution of $\delta^{13}C$ values (between -15.52 to -14.77 ‰ for untreated samples, -21.52 to -20.64 ‰ for acidified samples). The trend was less pronounced in spring (16.79 to 8.9 % for %C, -17.13 to -22 ‰ for $\delta^{13}C$) than in other seasons.

Seasonal variations for the whole plant (leaf epibionts excluded) were only detected for $\delta^{15}N$, with lowest values measured in winter and spring (Table 2). This trend persisted when plant parts were considered separately, except for juvenile and dead drifting leaves. Juvenile leaves exhibited higher $\delta^{15}N$ values in spring and summer and lower values in winter. Regarding $\delta^{13}C$, seasonal variations were only detected when considering each plant part separately, with no consistent pattern among them (Table S1).

Biochemical concentrations

Insoluble carbohydrates were the predominant biochemical compounds detected in leaves and rhizomes, as they always represented ~20 – 30 % of the sampled dry mass (i.e. the mass of insoluble carbohydrates scaled to

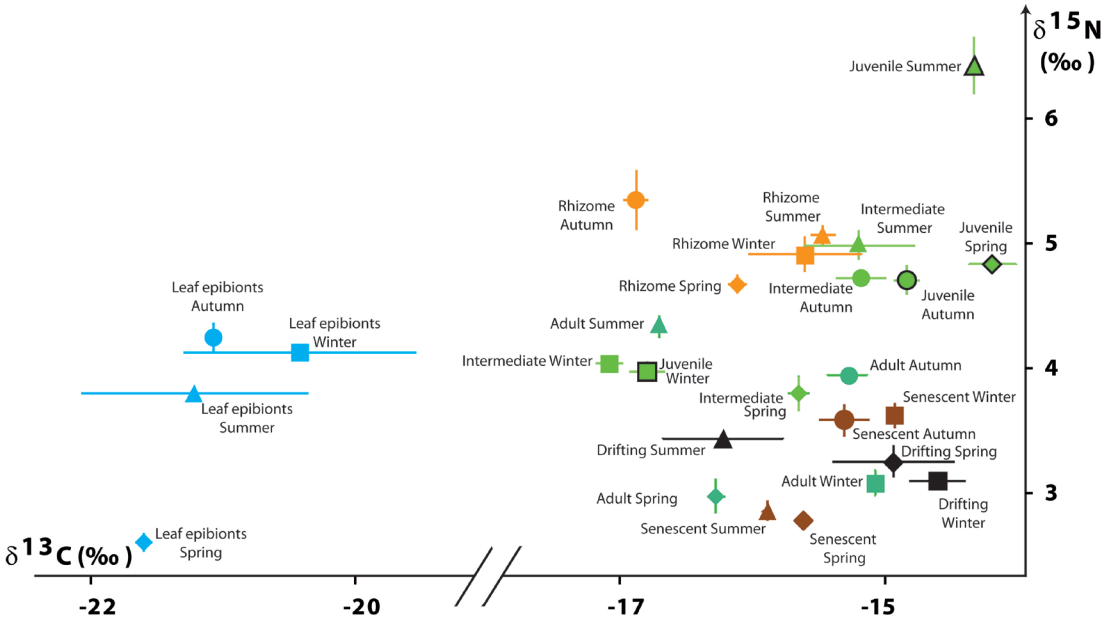

Fig. 2: Seasonal variation of isotopic values ($\delta^{13}C$ and $\delta^{15}N$, ‰, mean ± sd) of the shoot components, with colors standing for the plant part (leaf epibionts: blue, drifting dead leaves: black, senescent leaves: brown, adult leaves: dark green, intermediate leaves: light green, juveniles leaves: light green with black border, rhizome: orange) and symbols for season (spring: diamonds, summer: triangle, autumn: circles, winter: squares). For graphic purposes, the x-axis is cut between -17 and -20 ‰. For interpretation of the references to color in this figure legend, the reader is referred to the online version of the paper.

Table 1. Average (mean ± sd) of isotopic and biochemical parameters of different plant part types. SC: Soluble Carbohydrates, IC: insoluble carbohydrates. Sum: sum of all biochemical concentrations. "Stats" line reports the results of ANOVA mean comparison tests performed separately for each parameter (***: p-value < 0.0001), with significant differences assessed by LSD post-hoc tests marked with different letters. No statistical tests were performed on protein concentration, since it results from %N. Leaf epibionts $\delta^{13}C$ and %C values were measured on acidified samples. nd: no data. Since the number of replicates is not similar for all analyses, sum of the means for each column may be slightly different from the means of the sums displayed in the two last columns. SC: soluble carbohydrates, IC: insoluble carbohydrates.

	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	C/N	%C	%N	SC (mg g^{-1})	IC (mg g^{-1})	Lipids (mg g^{-1})	Proteins (mg g^{-1})	Ash content (mg g^{-1})	Explained part
Rhizome	-16.17 ^{b} ± 0.70	5.00 ^{d} ± 0.28	16.84 ^{d} ± 6.69	39.11 ^{d} ± 3.99	2.66 ^{d} ± 1.03	227.01 ^{f} ± 33.05	267.93 ^{b} ± 47.12	18.26 ^{a} ± 6.30	113.88 ± 44.10	87.11 ± 7.72	71.4 %
Juvenile	-15.04 ^{c} **± 1.37**	4.98 ^{d} **± 0.94**	13.41 ^{d} **± 2.28**	32.45 ^{c} **± 7.80**	2.51 ^{d} **± 0.79**	114.83 ^{e} **± 29.62**	214.56 ^{b} **± 56.02**	27.65 ^{bc} **± 4.04**	107.46 ± 33.83	165.01 **± 8.48**	63.0 %
Intermediate	-15.97 ^{b} ± 1.05	4.38 ^{c} ± 0.52	15.18 ^{d} ± 2.82	32.75 ^{c} ± 1.33	2.22 ^{c} ± 0.33	93.46 ^{d} ± 35.33	278.64 ^{b} ± 116.70	36.68 ^{d} ± 11.63	94.87 ± 15.09	209.12 ± 19.90	71.3 %
Adult	-15.97 ^{b} ± 0.89	3.54 ^{ab} ± 0.60	19.96 ^{c} ± 4.16	30.67 ^{c} ± 1.48	1.60 ^{b} ± 0.36	84.24 ^{c} ± 19.37	251.77 ^{b} ± 86.83	35.86 ^{d} ± 11.08	68.68 ± 15.22	262.53 ± 6.75	70.3 %
Senescent	-15.54 ^{bc} ± 0.48	3.21 ^{a} ± 0.42	28.50 ^{b} ± 8.73	27.40 ^{bc} ± 3.45	1.09 ^{a} ± 0.50	52.04 ^{b} ± 19.29	308.01 ^{b} ± 47.87	28.95 ^{c} ± 11.73	46.79 ± 21.47	349.32 ± 81.68	78.5 %
Drifting	-15.22 ^{c} ± 0.94	3.26 ^{ab} ± 0.16	30.40 ^{b} ± 6.92	26.13 ^{b} ± 5.12	0.90 ^{a} ± 0.28	20.27 ^{a} ± 5.10	265.98 ^{b} ± 69.31	21.34 ^{ab} ± 6.20	38.52 ± 11.83	399.87 ± 32.57	74.6 %
Leaf epibionts	-21.27 ^{a} ± 0.91	3.69 ^{b} ± 0.68	4.62 ^{a} ± 0.82	8.51 ^{a} ± 8.28	1.88 ^{b} ± 1.69	25.41 ^{a} ± 13.15	65.23 ^{a} ± 88.30	17.37 ^{a} ± 3.96	62.08 ± 16.86	nd	10.7 %†
Stats	F = 88.7 ***	F = 22.4 ***	F = 42.8 ***	F = 5.8 ***	F = 20.3 ***	F = 105.9 ***	F = 11.0 ***	F = 11.0 ***	-	-	

†: cumulative results not complete, due to the impossibility to determine ash content of epibionts.

1 g, as expressed in Fig. 4). Soluble carbohydrates were mainly detected in juvenile leaves and rhizomes, where they represented 12 and 23 % of the dry mass respectively, whereas they represented less than 10 % in all other plant parts. Soluble carbohydrate concentrations varied seasonally, whether considering all plant parts together or

Table 2. Seasonal variation of isotopic and biochemical parameters with all plant parts pooled. Epibionts were not included in this analysis. Letter in the stats column stands for the test used (H: Non-parametric Kruskall-Wallis ANOVA, F: parametric ANOVA). Seasons are abbreviated by their first letters; SC: soluble carbohydrates, IC: insoluble carbohydrates.

Parameter	Stats	p-value	Post-hoc
$\delta^{13}C$	$H_{(3,72)} = 1.06$	0.782	
$\delta^{15}N$	$F_{(3,69)} = 5.23$	0.003	Spr = Win < Sum=Aut
%C	$H_{(3,73)} = 3.63$	0.304	
%N / Proteins	$H_{(3,73)} = 5.00$	0.172	
C/N	$F_{(3,69)} = 4.94$	0.073	
SC	$H_{(3,71)} = 9.32$	0.025	Spr = Sum = Win < Aut
IC	$F_{(3,67)} = 0.28$	0.842	
Lipids	$F_{(3,66)} = 11.04$	<0.001	Win = Aut < Spr < Sum

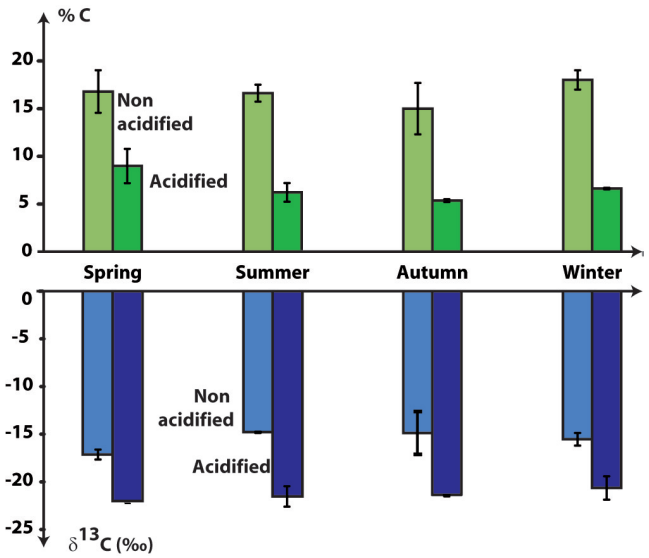

Fig. 3: Effect of acidification on leaf epibionts %C (green bars, above panel) and $\delta^{13}C$ ratios (blue bars, below panel). Values represented are mean ± standard deviation. Darkest bars represent values measured in acidified samples.

separately (excluding epibionts), with maximum values in summer and autumn (Table 2, Fig. 5). Percentage of carbon (%C) was the only descriptor showing no seasonal variation, whether considering all plant parts together or separately, with the exception of senescent leaves.

The lowest inorganic matter content (inferred from ash content) was measured in rhizomes (~9 %), and followed an increasing trend according to the age of leaves, with less than ~16% in juvenile leaves, ~21% in intermediate leaves and ~ 26% in adult leaves. The highest values were found in senescent and drifting dead leaves (~35 and 40 % respectively). The percentage of matter detected by the analyses (the sum of biochemical compounds as a proxy of organic matter plus ash as the inorganic matter) ranged between 63 and 79 % of the total compounds of plant parts, when all analyses could be performed. The other part could be attributed to the non-reactive organic molecules not detected with the chemical methods used. For leaf epibionts, lipids, proteins and both classes of carbohydrates represented 17 % of the total biomass in all seasons but spring. Values measured in spring represented 41 % of the total mass, mostly because of the high protein and insoluble carbohydrate concentrations (Fig. 5). The undetermined part might be attributed to inorganic

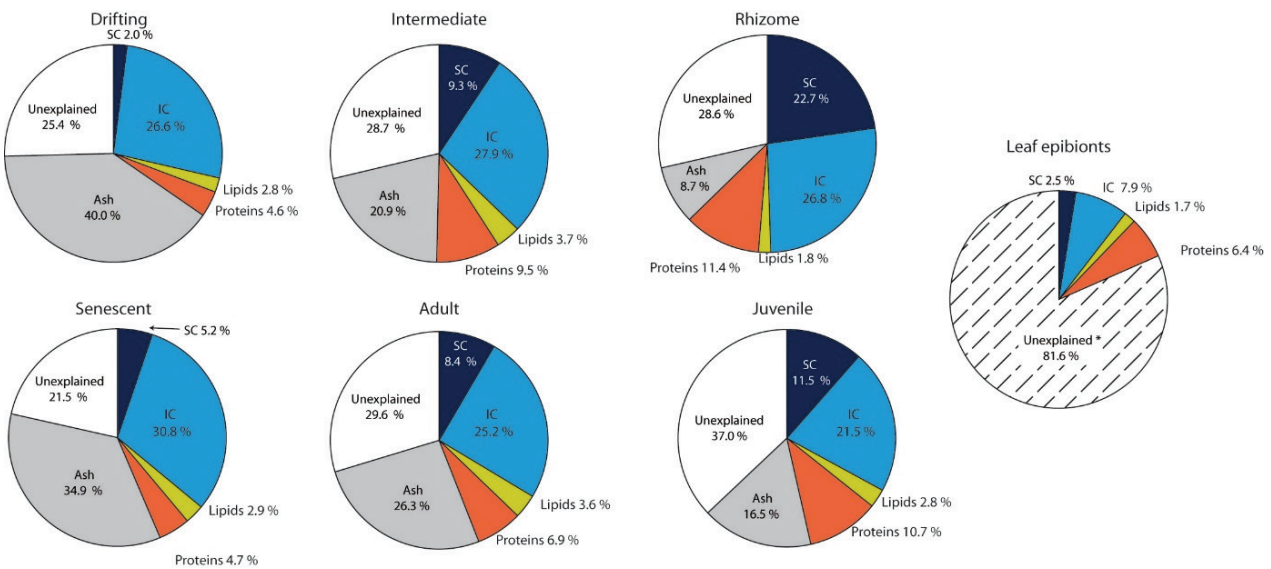

Fig. 4: Average proportions of biochemical compounds (SC: soluble carbohydrates, IC: insoluble carbohydrates) and ash content for the different components of the shoot. Ash content was not determined for leaf epibionts.

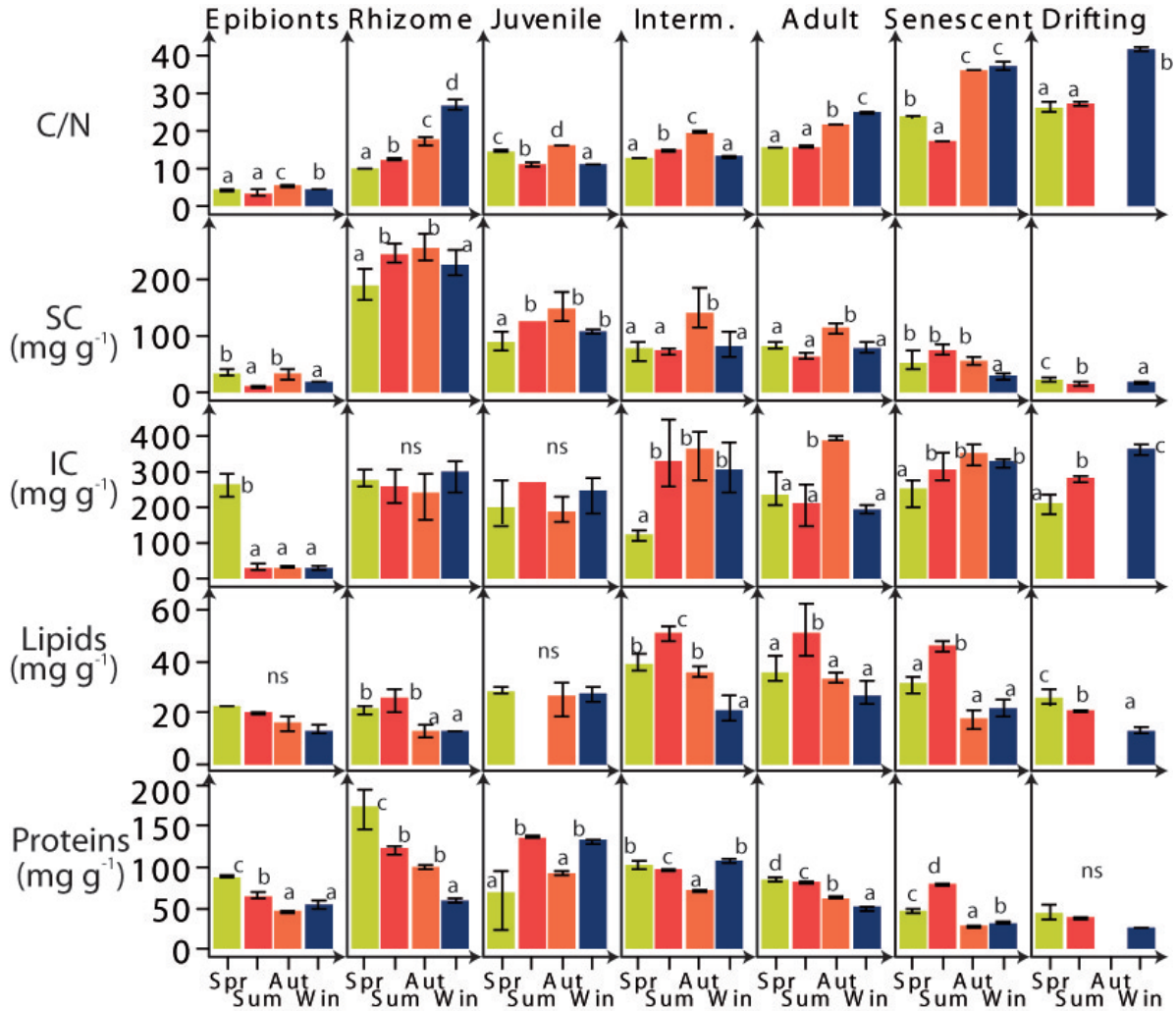

Fig. 5: Seasonal variation of C/N ratios and biochemical concentrations (mean ± sd). For graphic purposes, seasons (Spr: spring, Sum: Summer, Aut: Autumn, Win: Winter) and biochemical compounds (SC: soluble carbohydrates, IC: insoluble carbohydrates) are abbreviated. Letters above bars denote differences in post-hoc tests, bars with similar letters are not significantly different (ns: no significant difference between all seasons). Parameters of the statistical tests are provided in Table S1.

matter (mostly calcium carbonate), since no ash content measurement could be performed.

The PCA combining isotopic composition and biochemical concentrations indicated that more than 70% of the variance of data was explained by the first two axes when epibionts were included (Fig. 6a), and more than 50% without the epibionts (Fig. 6b). The PCA with epibionts confirmed the major difference between this community and the shoot. The higher protein content of epibionts separated this group from shoot components on the horizontal axis of the first PCA. The pattern observed for the shoot was nonetheless similar in both analyses: juvenile and intermediate leaves and rhizomes occurred in the same zone of the PCA plot (lower part of the first plot, right part of the second) due to their high and similar $\delta^{15}N$ composition, and protein and soluble carbohydrate concentrations. In contrast, senescent and drifting leaves occurred in the opposite part of the plots, in particular as their ash content and $\delta^{13}C$ composition were higher. This analysis also offered confirmation of correlations between biochemical and isotopic parameters: as expected $\delta^{15}N$ and proteins were strongly correlated, but the different pattern of correlation between the two PCA may demonstrate differences in drivers of N isotopic composition between leaves and epibionts. Similarly, $\delta^{13}C$ was always strongly correlated with insoluble carbohydrate concentration.

Discussion

Functioning of Posidonia oceanica *shoots and influence of the environment*

The first biochemical result observed in the present study is the predominance of insoluble carbohydrates, consistently with previous knowledge (Table S2), according to the taxonomic position (Magnoliophyta, kingdom Archaeplastida) and the terrestrial origin of *P. oceanica* (Larkum & Den Hartog 1989; Waycott & Les 2000; van der Heide *et al.,* 2012). Values are notably higher than for some Chlorophyta (*e.g. Codium* spp., *Caulerpa* spp.) or Rhodophyta species (*e.g Gracilaria* spp.) that exhibit insoluble carbohydrate concentrations lower than

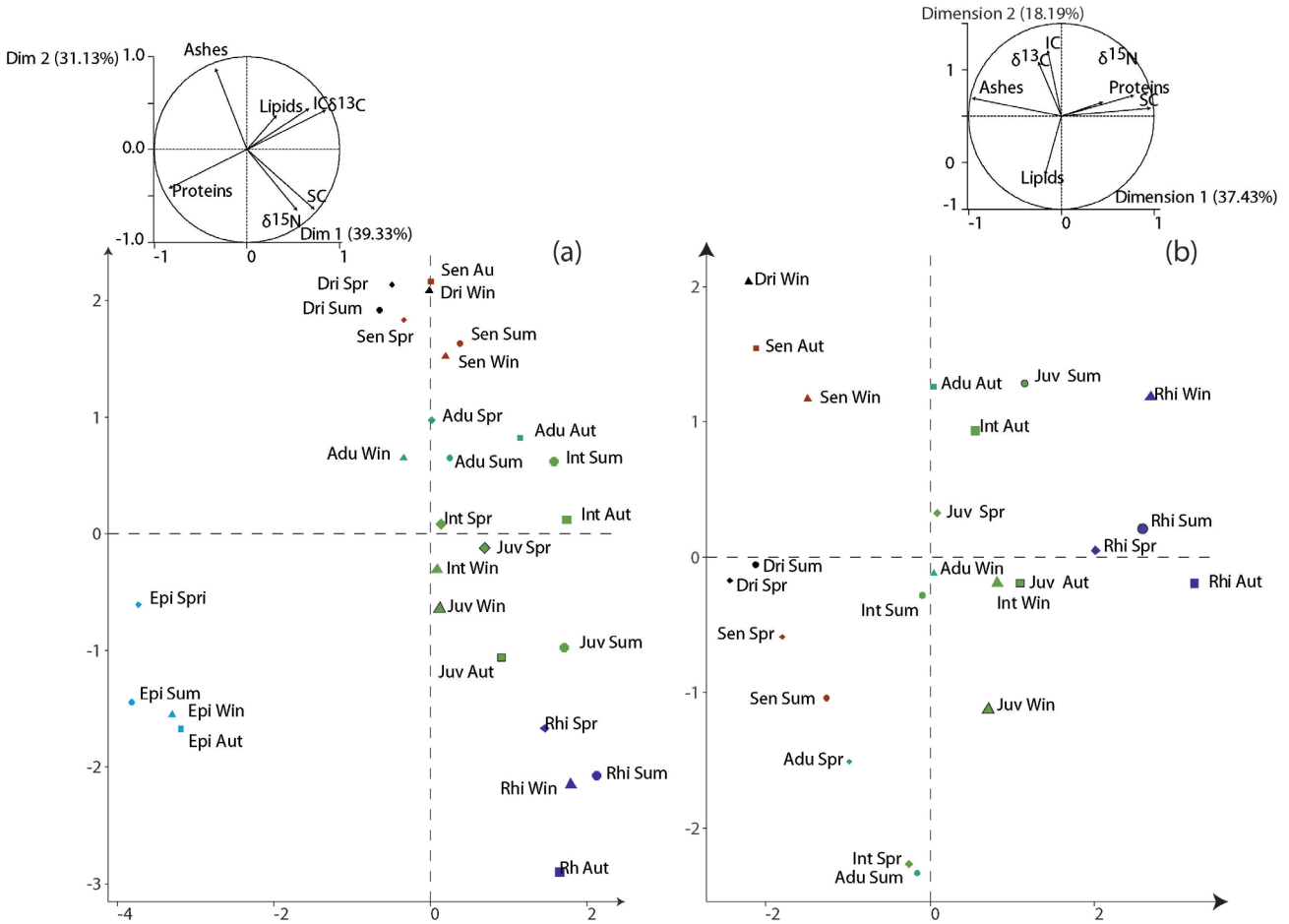

Fig. 6: First plane of the PCA performed on mean seasonal isotopic ratios, biochemical concentrations and ash content, with colors standing for the plant part (leaf epibionts: blue, drifting dead leaves: black, senescent leaves: brown, adult leaves: dark green, intermediate leaves: light green, juvenile leaves: light green with black border, rhizome: orange) and symbols for season (spring: diamonds, summer: triangle, autumn: circles, winter: squares), with (left panel) or without (right panel) epibionts. Points are referred to by the three first letters of the plant part and of the season. For interpretation of the references to color in this figure legend, the reader is referred to the online version of the paper. Correlation circles are superimposed above each plot.

200 mg g^{-1} (McDermid & Stuercke 2003, Table 3). In *P. oceanica*, the high concentrations of insoluble carbohydrates might be linked to the predominance of cellulose, hemicellulose and lignin, a legacy of its terrestrial origin (Ott & Mauer 1977; Vitale & Chessa 1998; Coletti *et al.,* 2013; Scartazza *et al.,* 2017). These high concentrations also induce the high C/N ratios usually measured in *P. oceanica* (Pirc & Wollenweber 1988; Fourqurean *et al.,* 2007; Scartazza *et al.,* 2017). In addition, %C, lignin and cellulose do not vary seasonally in all plant parts except senescent leaves and are not affected by environmental stress such as water acidification (Fourqurean *et al.,* 2007; Scartazza *et al.,* 2017). In contrast, starch and sucrose (*i.e.* soluble carbohydrates) content decreases when pH decreases (Scartazza *et al.,* 2017). These results confirm that the structural role of insoluble carbohydrates is a strongly constrained feature and a legacy of the terrestrial origin of *P. oceanica.* In the same way, low lipid content is recorded in all tissues sampled in the present and previous studies (Table S2). In addition, lipids and chlorophyll may interfere during extraction through the Bligh & Dyer method, leading to an overestimation of lipids (Archanaa, Moise & Suraishkumar 2012). Actual lipid values could then be even lower than the values presented here.

The range of $\delta^{13}C$ values measured for leaves and rhizomes was also consistent with the classical trend of higher $\delta^{13}C$ values in seagrasses than in other marine primary producers. Even if seagrasses are considered to use mainly a C3 photosynthetic metabolism, the coexistence of intermediate C3-C4 metabolisms or of a C4-like metabolism has been widely debated (Beer & Wetzel 1982; Larkum & James 1996; Beer *et al.,* 2002; Touchette & Burkholder 2000a; Raven, Cockell & De La Rocha 2008). In addition, the $\delta^{13}C$ values also trace the predominant role of inorganic Carbon Concentrating Mechanisms (CCM). CCM are mechanisms acquired by primary producers to saturate rubisco with inorganic carbon and limit its photorespiration activity (Griffiths 2006; Raven *et al.,* 2008). Thermodynamic properties of gas diffusion in water increase the need for such mechanisms for marine producers. The ability to use HCO_3^-, the predominant dissolved form of inorganic carbon in marine waters, *via* the activity of surface carbonic anhydrase is considered as the predominant CCM for marine producers (Giordano *et al.,* 2005; Raven *et al.,* 2008). For *P. oceanica*, more

Table 3. Comparison of ranges of biochemical concentrations observed in *Posidonia oceanica* and in different marine and terrestrial primary producers, regardless of the method used to calculate or measure the concentrations and separating calcified red algae. *: only the components of the plant are considered here (not the litter).

Taxonomic group	Lipids	Proteins	Ash	Carbohydrates	Fibers	Species	References
Phaeophyta (Stramenopiles)	8 to 324 mg g^{-1}	40 to 150 mg g^{-1}	179 to 350 mg g^{-1}	60 to 123 mg g^{-1}	371 to 560 mg g^{-1}	*Dictyota* spp. (3 species) *Durvillaea antarctica* *Fucus* spp. (2 especes) *Halidrys siliquosa* *Halopteris* sp. *Himantalia elongata* *Laminaria* spp. (2 species) *Padina pavonica* *Sargassum* spp. (4 species) *Undaria pinnatifida*	Munda, 1962; Fleurence *et al.,* 1994; Herbreteau *et al.,* 1997; McDermid & Stuercke, 2003; Ortiz *et al.,* 2006; Dawczynski *et al.,* 2007; Schaal *et al.,* 2010; Murakami *et al.,* 2011; Shams El Din & El-Sherif, 2012
Seagrasses (Archaeplastida)	18 to 37 mg g^{-1}	46.79 to 113.88 mg g^{-1}	87-349 mg g^{-1}	215 to 308 mg g^{-1}	not measured	*Posidonia oceanica**	Present study
Chlorophyta (Archaeplastida)	3 to 137 mg g^{-1}	70 to 270 mg g^{-1}	110 to 640 mg g^{-1}	45 to 400 mg g^{-1}	150 to 380 mg g^{-1}	*Caulerpa* spp. (3 species) *Codium* spp. (2 species) *Halimeda tuna* *Flabellia petiolata* *Ulva* spp. (5 species)	Fleurence *et al.,* 1994; Herbreteau *et al.,*1997; McDermid & Stuercke, 2003; Ortiz *et al.,* 2006; Shams El Din & El-Sherif, 2012
Rhodophyta (Archaeplastida)	11 to 19 mg g^{-1} (calcified) 6 to 33 mg g^{-1}	69 to 309 mg g^{-1} (calcified) 120 to 310 mg g^{-1}	830 to 859 mg g^{-1} (calcified) 14 to 26 mg g^{-1}	50 to 500 mg g^{-1}	186 to 467 mg g^{-1}	*Chondrus crispus* *Ellisolandia elongata* *Gracilaria* spp. (2 species) *Grateloupia turuturu* *Osmundea pinnatifida* *Lithophyllum incrustans* *Mastocarpus stellatus* *Palmaria palmata* *Porphyra* spp. (2 species) *Rhodymenia ardisonnei*	Fleurence *et al.,* 1994; McDermid & Stuercke, 2003; Jacquin *et al.,* 2006; Dawczynski *et al,.* 2007; Denis *et al.,* 2010; Schaal *et al.,* 2010; Shams El Din & El-Sherif, 2012
Terrestrial plants, vegetables (Archaeplastida)	2 to 29 mg g^{-1}	9 to 156 mg g-1	6 to 242 mg g^{-1}	455 to 856 mg g^{-1}	7 to 309 mg g^{-1}	*Amaranthus caudatus, Abelmoschus esculentus, Brassica rapa, Lathyrus aphaca, Raphanus sativus, Solanum melongena*	Rehman et al. 2014
Cereals (Archaeplastida)	9 to 42 mg g^{-1}	88 to 194 mg g^{-1}	6 to 29 mg g^{-1}	536 to 779 mg g^{-1}	19 to 221 mg g^{-1}	*Triticum aestivum* (hard and soft grains), *Hordeum volgare*, *Pennisetum glaucum* , *Secale cereale*, *Sorghum bicolor*	Ragaee et al., (2006)

than 50 % of the inorganic carbon used in photosynthesis is fixed by surface carbonic anhydrase, one of the highest percentages measured in marine Magnoliophyta (Invers *et al.,* 1999; Touchette & Burkholder 2000a). The presence of an aerarium, a lacunar structure that runs from leaf tips down to the rhizomes and harbors a gas complex, enables *P. oceanica* to integrate gaseous inorganic carbon instead of dissolved carbon (Boudouresque *et al.,* 2006). All these biochemical reactions are associated with isotopic discrimination (*i.e.* modification of the $^{13}C/^{12}C$ ratio) and are likely to be a cause of the higher $\delta^{13}C$ values measured in *P. oceanica* than in other marine benthic primary producers. The values measured in the present study (-17 to -14 ‰) seem slightly lower than the classical $\delta^{13}C$ values (-15 to -5 ‰) generally reported for seagrasses (Bricout *et al.,* 1980; Vizzini *et al.,* 2003; Lepoint *et al.,* 2003; Fourqurean *et al.,* 2007). This discrepancy might be linked to the depth of our sampling (lower limit of the meadows), while most studies are conducted in shallow meadows. Previous results demonstrated that depth influences *P. oceanica* isotopic composition – the deeper the meadow, the lower the $\delta^{13}C$ value – as light intensity and photosynthetic activity decrease with depth (Lepoint *et al.,* 2003; Fourqurean *et al.,* 2007). Regarding $\delta^{15}N$, measured values also range within the values previously measured. As previously stated, $\delta^{15}N$ values are commonly considered as an effective proxy of anthropic contamination. In the NW Mediterranean, $\delta^{15}N$ values measured for *P. oceanica* range between 2 ‰ in rather pristine sites to 7 ‰ in polluted sites (Lepoint *et al.,* 2000; Vizzini & Mazzola 2004; Papadimitriou *et al.,* 2005; Vizzini *et al.,* 2005; Tomas *et al.,* 2006; Pérez *et al.,* 2008; Lassauque *et al.,* 2010). The intermediate values measured in the present study confirmed a moderate anthropic effect already detected in suspended and sedimentary organic matter pools at that site (Cresson *et al.,* 2012).

Isotopic and biochemical features: proxies of plant part-specific functioning

The comparison of isotopic and biochemical analyses enabled the separation of the plant into several groups with similar features, and thus potentially sharing similar functioning. The separation on the basis of age is clearly apparent on the PCA plot. Juvenile leaves and rhizomes share similar biochemical features, notably a high amount of soluble carbohydrates. In *P. oceanica*, soluble carbohydrates are mainly stored as sucrose, a compound highly synthesized during fast-growth periods (Pirc 1985, 1989; Touchette & Burkholder, 2000a; Alcoverro *et al.,* 2001; Scartazza *et al.,* 2017). Glucose and fructose also represent important soluble carbohydrates but in lower concentrations (Pirc 1989; Scartazza *et al.,* 2017). The maximum soluble carbohydrate concentrations in summer or autumn, and in rhizomes and juvenile leaves, were consistent with previous results (Pirc, 1989), with the high photosynthetic activity in juvenile leaves (Alcoverro *et al.,* 1998) and with the storage of the summer excess production of the whole shoot in the rhizomes afterwards (Alcoverro, *et al.,* 2000, 2001). This is also consistent with trends observed in the rhizomes of several other seagrass species in Florida or in India (Dawes & Lawrence 1980; Pradheeba *et al.,* 2011), and with the similarity between juvenile leaves and rhizomes previously detected (Pirc 1985). Low values in spring may also result from a shading effect of the abundant epibiotic community in this season, potentially explained by a massive development of brown algae (see below). The effect of epibiont cover on leaf production was considered negligible (Tomas, Turon & Romero, 2005), but this work was performed in a shallow meadow (5-6 m depth) where light might be less limiting than at ~30 m depth. Interestingly, higher concentrations of structural compounds within older leaves were also observed for terrestrial oaks *Quercus pubescens* and *Q. ilex* (Damesin, Rambal & Joffre, 2002). These authors also observed a link between $\delta^{13}C$ values and the use of reserve carbon compounds. This seems to demonstrate that photosynthetic activity and storage mechanisms are well-conserved within marine Magnoliophyta, another legacy of the terrestrial origin of this group. In contrast, low values of soluble carbohydrates in the rhizomes in winter and spring would reflect the use of stored carbohydrates to support the early growth of juvenile leaves (Romero, 2004). Rhizomes and juvenile leaves also share similarities regarding N-linked descriptors, mainly high $\delta^{15}N$ values, high %N (and consequently high protein concentrations). The protein concentrations calculated in the present study may suffer from some limitations since they do not result from direct measurement, but were calculated on the basis of an inferred conversion factor. Since no accurate N-to-protein conversion factor is available for *P. oceanica*, using the inferred value was the most cautious solution, as an accurate but complex determination of amino acid concentrations by chromatographic methods was beyond the scope of the present study (*e.g* Augier *et al.,* 1982; Diniz *et al.,* 2011; Lourenço *et al.,* 2002). This value was lower than the 6.25 Atwater coefficient, consistently with results obtained on several macroalgal species and with the currently accepted view (Lourenço *et al.,* 1998; Diniz *et al.,* 2011). This stresses the need for dedicated analyses of the nitrogen and protein content in *P. oceanica* and for an accurate determination of N-to-protein conversion factors for Magnoliophyta. Rhizome is clearly identified as a N-storage organ and a source of amino-acids for juvenile leaves (Touchette & Burkholder, 2000b; Alcoverro *et al.,* 2001; Invers *et al.,* 2002; Romero, 2004). One study only compared isotopic composition in the different parts of *P. oceanica* and records higher $\delta^{15}N$ value in rhizomes (Vizzini *et al.,* 2003). This high value could be caused by the storage of nitrogen in rhizome as asparagine, arginine or glutamine (Pirc, 1985; Touchette & Burkholder, 2000b; Invers, Pérez & Romero, 2002; Invers *et al.,* 2004). This hypothesis is further supported by the strong correlation between leaf or rhizome $\delta^{15}N$ and asparagine content (Scartazza *et al.,* 2017). Regarding fast-growing juvenile leaves, their high $\delta^{15}N$ values can be explained by their high photosynthetic activity which increases the nutrient demand and decreases the isotopic discrimina-

tion (meaning that more ^{15}N is integrated), therefore contributing to an increase in the $\delta^{15}N$ value (Alcoverro *et al.,* 1998). In addition, the input of ^{15}N-rich amino acids such as asparagine from the rhizome would also increase the $\delta^{15}N$ value. Unfortunately, the isotopic composition of juvenile leaves has never been documented to date, and comparison is not possible. The seasonal trend observed here (maximum $\delta^{15}N$ value in spring and summer, minimum values in winter) would nevertheless be consistent with this hypothesis.

It is interesting to note that an opposite pattern is detected when considering adult and intermediate leaves separately, or when all leaves of the shoot are considered pooled (Table 2 in the present study; Vizzini *et al.,* 2003; Fourqurean *et al.,* 2007). The predominance of adult and intermediate leaves explains why their variation drives the variation observed when all leaves are pooled. This discrepancy was attributed to an excess of nutrients to support seagrass growth (Fourqurean *et al.,* 2007), and could be linked with the decline of the photosynthetic activity of the leaves with increasing age (Alcoverro *et al.,* 1998), which is also denoted by their lower %N. The lower $\delta^{13}C$ values measured in adult leaves would also be consistent with a decline in photosynthetic activity, and thus an increase in the discrimination against ^{13}C. This discrepancy between juvenile and adult leaves could confirm recent results demonstrating that juvenile leaves are the best proxy to assess the current productivity of seagrasses (Kim *et al.,* 2014).

Finally, the third group comprising senescent and drifting dead leaves was characterized by the predominance of insoluble carbohydrate and inorganic matter, low %N values and low protein concentrations. The decrease of %N with increasing age is consistent with previous studies (Pirc 1985; Lepoint *et al.,* 2002) and with the internal nutrient recycling system of *P. oceanica*, another legacy of its terrestrial origin. Before the fall of the old leaves, their nutrient content is transferred to rhizomes to support the high nutrient demand of growing tissues (Lepoint *et al.,* 2000, 2002; Romero 2004; Boudouresque *et al.,* 2006). As a result, falling leaves mostly comprised structural compounds, the amount of which is fixed throughout the leaf's life cycle, and inorganic matter. The gradual degradation of these plant parts can also explain the change in their isotopic composition. Nevertheless, explaining the seasonal changes of these plant parts appeared more complex since degradation and alteration of biochemical and isotopic content is driven by mechanisms at play for a longer period than the seasonal variation of primary production. It is also a matter of some complexity to estimate the actual age and degradation stage of drifting leaves. The seasonal variation of %C observed only for senescent leaves might thus be more an artifact of sampling than a real pattern.

Leaf epibiotic community

Using *P. oceanica* leaves as a substrate, leaf epibionts form a specific and heterogeneous community with its own functioning and under the influence of several drivers, such as depth, environmental conditions, grazing pressure, position along the leaf blade (e.g. basal vs. apical) and leaf age (Romero, 1988; Alcoverro, Duarte & Romero, 1997; Lepoint *et al.,* 1999; Bedini, Canali & Bertuccelli, 2003; Prado *et al.,* 2007; Balata *et al.,* 2008, 2010; Nesti, Piazzi & Balata 2009; Michel *et al.,* 2015). Assessing the actual species composition of this heterogeneous community is complex and requires time-consuming microscopic observations (Panayotidis & Boudouresque, 1981; Bedini *et al.,* 2003; Prado *et al.,* 2007; Balata *et al.,* 2008; Nesti *et al.,* 2009). Even if such analyses are required to fully describe the epibiotic community, its isotopic and biochemical features could provide a simple tool to roughly describe its composition and monitor changes over time. Biochemical concentrations measured in the present study were lower than values measured for *P. oceanica*. This low organic matter content is consistent with previous results, which showed that inorganic matter represented 82 to 88 % of the total epibiotic biomass (Terrados & Medina Pons, 2008). Even if the mass of ash was not determined in the present study, the strong effect of acidification on %C and $\delta^{13}C$ values similarly demonstrated the predominance of inorganic carbon in the epibiotic community. Amongst the epibiotic community, bryozoans and red algal members of the order Corallinales (Rhodophyta) are the two main calcified taxa (Van der Ben, 1971; Romero, 1988; Prado *et al.,* 2007; Nesti, *et al.,* 2009). In such a deep meadow, the epibiotic community might have been mostly composed of bryozoans, since previous results demonstrated their increased predominance with increasing depth and decreasing luminosity (Van der Ben, 1971; Lepoint *et al.,* 1999; Nesti, Piazzi & Balata, 2009). The protein content measured is higher than values available in the literature for the epiphytic community, *i.e.* a community dominated by marine primary producers, which would be consistent with the predominance of epibiotic consumers. This conclusion has nevertheless to be confirmed since the composition of the epiphytic community is generally not specified (*e.g.* Lawrence *et al.,* 1989). Seasonal variations of the isotopic and biochemical features were also consistent with previous knowledge of the biological successions regarding the epibiotic community. The results obtained for leaf epibionts in spring were markedly different than in other seasons, as denoted in particular by the distance of the spring sample from the other epibiotic samples in the PCA plot (Fig. 6). The predominance of Phaeophyta (brown algae) as epiphytes in spring, e.g *Cladosiphon* Kütz, *Giraudya sphacelarioides* Derbès et Solier, *Myriactula gracilis* van der Ben, *Myrionema orbiculare* J. Agardh, and *Sphacelaria cirrosa* (Roth) C.Agardh, previously observed by several authors (Van der Ben 1971; Panayotidis 1979; Thélin & Bedhomme 1983; Romero 1988), would be consistent with the increase in biochemical concentrations, the lowest effect of acidification and the lower $\delta^{15}N$ values recorded in this season. Nevertheless, the development of this algal community might have been limited at the studied depth (~25 m), explaining why calcified organisms remain predom-

inant.

Even if *P. oceanica* is a key species for the functioning of Mediterranean marine coastal ecosystems, its isotopic and biochemical features have never been investigated using a combined approach. In addition, few works have considered plant parts separately despite their different metabolisms. Results obtained in this study provided some useful information to fill this gap. The differences observed between plant part types were consistent with the complex photosynthetic metabolism previously described, and appeared to be a legacy of *P. oceanica*'s terrestrial origin. It gave rise to higher $\delta^{13}C$ values than those of other marine primary producers, and also the presence of several structural compounds of complex chemical structure, with an effect of seasonality and plant part-specific metabolism. Correlations were observed between isotopic and biochemical descriptors, notably between N-linked descriptors (proteins and $\delta^{15}N$). Even if not specifically investigated in the present work, high photosynthetic intensity could be considered a key driver of the isotopic and biochemical features of juvenile leaves, whereas lower values measured for older leaves were consistent with reduced metabolic activity. These results confirmed the suitability of stable isotope and biochemical analyses to serve as efficient tracers of physiological mechanisms.

Data accessibility

Raw data used for this paper are freely available online in the Seanoe digital repository at https://doi.org/10.17882/58034

Acknowledgements

This paper is part of P. Cresson's PhD work, funded by grants from the Ville de Marseille and the Agence de l'Eau Rhone Méditerranée Corse. Thanks are due to Adrien Goujard (GIS Posidonie) for drawing the map Figure 1, to Michele Perret-Boudouresque for the bibliographical review of biochemical concentrations in *P. oceanica* , and to Christos Panagiotopoulos (Mediterranean Institute of Oceanography) for fruitful discussions on the chemical structure of carbohydrates and measurement methods. Special thanks are due to Marie France Fontaine, now retired, who performed the great majority of the biochemical analyses. The English of this manuscript was revised by Michael Paul, a native English speaker. We would also like to thank two anonymous reviewers for their pertinent comments and suggestions on a previous version of the ms.

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