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Isotopic and biochemical composition of Western Mediterranean macroalgae

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Abstract :

Among marine primary producers, macroalgae support complex and productive coastal food webs, but coastal primary production relies on terrigenous inputs and remineralized organic matter which both vary seasonally. An approach combining stable isotope and biochemical analyses enables a better characterization of macroalgae specificities and highlights environmental influences on their chemical signature. This study compared the isotopic signature and biochemical composition of 22 Mediterranean macroalgae belonging to Rhodophyta (red algae), Phaeophyceae (brown algae) and Chlorophyta (green algae) between March and November 2010 to capture the differences in species chemical signatures potentially driven by metabolic traits or environmental drivers. Carbon stable isotope values were evidenced as a good proxy of specific carbon metabolism: low values observed in red algae could be related to the reported absence of carbon concentrating mechanisms (CCMs) in this group while higher values were driven by strong CCM activity in green algae. Biochemical patterns also differed between groups: soluble carbohydrates were a major component for red algae, while lipids and proteins dominated in brown algae, and insoluble carbohydrate concentrations were high in green algae. Variation within species across two collection times could be related to environmental changes and algal metabolism. δ15N values confirm the efficiency of this parameter as a proxy of the impact of human influence in the Bay of Marseille.

Highlights

► Isotopic and biochemical content was measured in 22 Mediterranean macroalgae. ► Carbon descriptors are related to the metabolism of the specific macroalgal group. ► Seasonal variation of descriptors correlates with environmental variability.

Keywords : Biochemical profile, environmental variability, macroalgae, metabolism, seaweed, stable isotopes

Introduction

Vegetated coastal habitats that encompass seagrass and seaweed ecosystems fix annually ~10% of the carbon used by marine ecosystems (Duarte, 2017). They are important contributors to the organic matter pool that supports highly productive and diversified coastal ecosystems (Leclerc et al., 2013; Udy et al., 2019). Among benthic primary producers, macroalgae are recognized for their fast growth rate compared to seagrasses, and their capacity to quickly store carbon in tissue and transfer it to higher trophic levels, sometimes as detritus (Cresson et al., 2016; Krumhansl & Scheibling, 2012; Le Bourg et al., 2022; Leclerc et al., 2013; Remy et al., 2021). Macroalgae enter coastal food webs via three pathways: through direct grazing by herbivores, as particulate organic matter (POM) when the thallus is eroded or senescent, and as dissolved organic matter (DOM) released from the thallus due to microbial activity and leaching (Ramshaw et al., 2017).

In the Western Mediterranean, the transfer of macroalgal primary production through grazing is of minor importance. Few invertebrate species such as the urchin *Paracentrotus lividus* graze directly on benthic macrophytes. The salema *Sarpa salpa* is the only native herbivorous fish species in the Western Mediterranean (Verlaque, 1990) and other species (*e.g.* such as the sparids *Diplodus* spp. or *Spondyliosoma cantharus*) may also consume algae but these species consume macroalgae in limited quantities, or only at some life history stages (Box et al., 2009; Cresson, Ruitton, Ourgaud, et al., 2014; Ruitton et al., 2000; Sala & Boudouresque, 1997; Verlaque, 1990). Therefore, organic matter fluxes entering the food web mostly derive from the detrital pathway (Palacín et al., 1998; Bulleri et al., 1999, 2000; Benedetti-Cecchi et al., 2000; Hereu, 2006). Filter feeders (Cresson et al., 2016) and detritivorous invertebrates such as holothurians are indeed able to use macroalgal detritus (Boncagni et al., 2019). Nonetheless, the increased importance of grazing is one of the expected effects of the arrival of new invasive species such as the rabbit fish *Siganus luridus* (Bariche, 2006).

Understanding the nature, intensity and drivers of the interactions between herbivores, detritivores and benthic primary producers is thus crucial to accurately depict the functioning of food webs, and has consequently been investigated for decades (Clements et al., 2009; Crossman et al., 2001; Paine & Vadas, 1969; Poore et al., 2012; Prado

& Heck Jr., 2011). The use of C and N stable isotopes has proved very useful to track the integration of benthic primary production in marine food webs. Isotopic ratios of macroalgae differ from those of phytoplanktonic and terrestrial production entering marine food webs. The biochemical composition of primary producers is a powerful tool to understand herbivore feedings choices as macroalgal composition drives herbivory selectivity (Boyer et al., 2004; Crossman et al., 2001; Dromard et al., 2015; Verlaque, 1990). Protein content is, for instance, recognized as a major factor driving food selection (Barile et al., 1999 and references therein; Jacquin et al., 2006; Schaal et al., 2010), as the diet of detritivores and herbivores is usually based on nitrogen-poor items. Increased inputs of nitrogen strongly affect growth rate and reproductive success (Bowen et al., 1995; Bracken et al., 2012; Crossman et al., 2001; Grémare et al., 1997; Prado et al., 2010). Other compounds, such as carbohydrates or lipids, are also useful proxies to understand the feeding choices of consumers. Carbohydrates can occur within two groups: soluble carbohydrates can be easily assimilated by consumers (Harmelin-Vivien et al., 1992), while the complex chemical structure of insoluble carbohydrates makes them refractory and of low nutritional interest for the vast majority of consumers (Crossman et al., 2001; Panagiotopoulos & Semperé, 2005). The high amount of energy carried by lipids also explains why lipid-rich dietary items are of high interest for consumers (Sargent et al., 2002).

The isotopic ratios and biochemical composition of benthic primary producers are influenced by differences in metabolism (mainly carbon) as well as environmental conditions under the influence of seasonality and climate change (Ito & Hori, 1989). The variability of carbon isotopes is tightly linked to the carbon source used for primary production (CO_2 or HCO_3^-), the nature of the RubisCO and seasonal variations of temperature or light intensity affecting physiological processes (Boller et al., 2015; Grice et al., 1996; Iniguez et al., 2020). Nitrogen isotopes are good indicators of nitrogen enrichment and source (Cole et al., 2004; Viana & Bode, 2013). Similarly, the composition of pigments and fatty acids in macroalgae varies considerably between seasons and taxonomic groups (Schmid et al., 2017). Proteins and carbohydrates variations are often correlated with changes in temperature, salinity or pH (Mohy El-Din, 2019). This strong link between isotopic or biochemical indicators and environmental fluctuations can sometimes preclude the understanding of trophic relationships, especially in dynamic systems. Thus, having a species-specific description of primary producer isotopic and biochemical signatures, including some seasonal variability is

a priority required to better assess ecosystem functioning and organic matter transfer in food webs, notably in a context of major ecosystem changes in the Western Mediterranean (Lejeusne et al., 2010).

In the Bay of Marseille, 400 artificial reefs were deployed between 2007 and 2008 at depths ranging 15 to 30 m (Charbonnel et al., 2011). A major monitoring and research program followed this deployment. All aspects of artificial reef functioning have been monitored since including potential alteration of the integrity of the nearby *Posidonia oceanica* meadow (Astruch et al., 2015), the settlement and trophic functioning of the benthic invertebrate community (Cresson et al., 2016; Rouanet et al., 2015) and the fish assemblages (Cresson, Ruitton, & Harmelin-Vivien, 2014; Cresson, Le Direach, et al., 2019; Le Diréach et al., 2013, 2015). As macroalgal species quickly colonized the artificial reefs, this deployment was a perfect opportunity to investigate the isotopic and biochemical diversity of Mediterranean benthic primary producers. The aim of this study was thus to characterize the isotopic and biochemical fingerprints of various macroalgal groups, and study the relative roles of taxonomy, metabolism and environment as drivers of isotopic and biochemical variability. We hypothesized that physiology and metabolism are major drivers of carbon-related isotopic and biochemical descriptors, while seasonal variability of the environmental parameters would cause the variations of nitrogen-derived descriptors. We also consider this work as a potential baseline for future studies where stable isotopes in invertebrates or fish consumers will be used to determine the future of algal primary production

Material and methods

Sampling was performed in March, June, September and November 2010 to assess intra-annual variability in the isotopic ratios and biochemical content of macroalgae. Each month was considered as representative of a season (March: Spring, June: Summer, September: Autumn and November: Winter). All observed macroalgal species on artificial reefs where hand-picked by divers following an identical protocol for each sampling. Sampling occurred on two 'metal basket' type large artificial reefs (187 m^3, 6 m high) considered in other studies (e. g. Cresson, Ruitton, Ourgaud, et al., 2014) and deployed at similar depths (~30 m). In the laboratory, samples were sorted, cleaned of epibionts, and

identified to genus or species. Samples were then stored frozen (-20°C), freeze-dried and ground with a mechanical grinder before isotopic and biochemical analyses.

Isotopic analyses were based on small aliquots (~ 1 mg) of powder placed in tin capsules. Isotopic ratios were measured with a continuous flow mass spectrometer (Delta V Advantage, Thermo Scientific) coupled with an elemental analyser (Flash EA1112, Thermo Scientific). Ratios are expressed following the classical notation, δX where:

$\delta X = \left(\frac{R_{sample}}{R_{standard}} - 1\right) \times 10^3$, where X is ^{13}C or ^{15}N respectively, and R the isotopic ratio $^{13}C/^{12}C$ or $^{15}N/^{14}N$ respectively in the sample and the standard (Vienna Pee-Dee Belemnite for C and atmospheric N_2 for N). Measurement accuracy was checked by repeated measurement of an acetanilide standard. Overall deviation was lower than 0.1‰ for both C and N. Carbon and nitrogen percentages (%C and %N hereafter) were measured with the elemental analyser and used to calculate the C:N ratio.

Carbohydrate measurements were based on the phenol-sulfuric acid method (PSA, Dubois et al., 1956). The resulting solution absorbs at 490 nm (Panagiotopoulos & Semperé, 2005). Soluble carbohydrates were extracted from the algal powder with distilled water (100°C, 20 minutes) prior to analysis, while insoluble carbohydrate concentration was measured from the remaining powder. Lipid concentrations were determined following Bligh & Dyer (1959) and were expressed as tripalmitic acid equivalent. Protein content was determined with the Lowry et al. (1951) method, considered as the best suited for marine macroalgae (Barbarino & Lourenço, 2005). Biochemical measurements were triplicated in most cases, when the amount of ground matter was sufficient. Finally, inorganic content was determined by measuring the ash weight, after burning the powder at 500°C for 5 h.

Macroalgal species sampled varied from one month to another because of the heterogeneity in algal assemblages between months. In addition, because the sampling was restricted to one depth for the sake of homogeneity, the amount of macroalgae collected per species was not always sufficient to perform all analyses. In such cases, the amount of algal powder available was allocated to isotopic analyses, as less material is required for this analysis than for the determination of biochemical content. Similarly, the quantity of sample was not sufficient to replicate ash content characterization, which was measured once for each species each month. Heterogeneity in sampling resolution (i.e. the fact that phyla do not comprise the same species at each season) is summarized in Table 1.

The data treatment process was designed to take this heterogeneity into account. We first wanted to describe the major features of each macroalgal group (red, green and brown) per month, regardless of the species sampled , and to ensure that intragroup variability, both in isotopic and biochemical descriptors, was representative of the intrinsic group variability. To do so, mean isotopic and biochemical descriptors of each group (annual means) were compared between months. Then, species sharing similar average biochemical and isotopic features were grouped by hierarchical clustering, based on Euclidean distance and Ward criterion. Hierarchical clustering was selected, as this analysis does not require any *a priori* assumption regarding the number of clusters. Biochemical concentrations of all species were then summarized with a Principal Component Analysis followed by a hierarchical clustering on the two first principal components.

In a second step, variations between months were assessed only for the species that were collected at all months (*i.e. Caulerpa cylindracea*, *Codium bursa, C*. *vermilara*, *Flabellia petiolata*, *Dictyopteris* sp., *Dictyota dichotoma*, *Halopteris* sp. and *Sphaeroccocus coronopifolius*). Isotopic and biochemical descriptors were compared between months for each species. All comparisons were performed with ANOVAs followed by Tukey posthoc tests when significant. Homoscedasticity and normal distribution of residuals were reached with *a priori* log transformation of the data, and checked by visual examination of plots produced by the *performance* package (Lüdecke et al., 2021). As $\delta^{13}C$ values followed a bimodal distribution when considering all species together, log transformation was ineffective to reach ANOVAs assumptions. Mean comparison was thus performed with a non-parametric Kruskall Wallis test, followed by Wilcoxon posthoc test.

Correlations between biochemical and isotopic descriptors were checked with two-sided Spearman correlation tests, and considered significant if $|\rho|>0.5$ and p value < 0.001. Correlations were calculated between average biochemical concentrations and isotopic ratios calculated for replicated analyses of the same sample. This analysis was aimed at identifying whether variability in isotopic ratios can be used as a proxy of biochemical variability.

All analyses were performed using R software version 4.0.2 (R Core Team, 2020) and packages *car*, *cluster*, *FactoMineR*, *mass*, *rstatix* and *multcomp* (Hothorn et al., 2008; Kassambara, 2020; Lê et al., 2008; Venables & Ripley, 2002). Plots were produced with *ggplot2* (Wickham, 2009) and ggcorplot (Kassambara & Kassambara, 2019) packages.

Table 1. Analysis undertaken for species collected at each season. (SI: Stable isotope, B: biochemical, A: ash weight).

	Species	March	June	September	November
Chlorophyta	*Bryopsis cupressina*			SI	
	Caulerpa cylindracea	SI B	SI B	SI B A	SI B
	Codium bursa	SI B A	SI B A	SI B A	SI B A
	Codium vermilara	SI B A	SI B	SI B A	SI B A
	Flabellia petiolata	SI B A	SI B A	SI B A	SI B A
Phaeophyceae	*Cladostephus spongiosus*			SI B A	SI B A
	Ericaria zosteroides		SI		
	Dictyopteris polypodioides	SI B A	SI	SI B	SI
	Dictyota sp.	SI B A	SI B A	SI B A	SI B A
	Halopteris sp.	SI B A	SI B A	SI B A	SI B A
	Padina pavonica *		SI B A	SI B A	
	Sporochnus pedunculatus		SI B A	SI	
	Taonia atomaria		SI B A		
	Zanardinia typus		SI A		
Rhodophyta	*Asparagopsis armata*	SI B A			
	Bonnemaisonia sp.		SI A		
	Bornetia secundiflora			SI	
	Dudresnaya verticillata		SI		
	Peyssonnelia sp.				
	Vertebrata subulifera	SI		SI B A	SI B A
	Sphaerococcus coronopifolius	SI B	SI B	SI B A	SI B A
	Spyridia filamentosa			SI B A	

* calcified species

Results

Taxonomic variations of biochemical and isotopic features

For all months combined, ash (i.e. inorganic content of the tissues) always represented 50% or more of the mass in the Chlorophyta, Ochrophyta and Rhodophyta groups. Among the biochemical compounds, insoluble carbohydrates were the most strongly represented class and represented ~9 to 20% of the mass of the tissues (Fig.1). Other classes each represented generally less than 10% of the total mass, with the exception of soluble carbohydrates in Rhodophyta (13.8%).

The concentrations of biochemical compounds highlight some specificity for each group (Fig. 2). The Chlorophyta exhibited significantly higher insoluble carbohydrate values, intermediate concentrations in soluble carbohydrates, low lipid and protein content, and lower C:N ratios than the two other groups. Ash content was also slightly higher than for the two other groups. The Phaeophyceae had significantly higher lipid and protein content but lower concentrations in carbohydrates. The Rhodophyta had low lipid, intermediate protein and insoluble carbohydrate concentrations.

When species were considered separately, there were inconsistencies in each group (Fig. S1). Based on the specific biochemical profile, no cluster grouped all species from the same group. Thus, there is a no group-specific biochemical profile (Fig. 3). Three of the five clusters were either monospecific (*F. petiolata* and *S. coronopifolius*) or group two species from the same group (*Sporochnus* sp. and *T. atomaria*). For instance, higher insoluble carbohydrate, protein and lipid concentrations, lower soluble carbohydrate concentration in *Flabellia petiolata* than other species from the same phylum resulted in a monospecific cluster for this species. The *Sphaeroccocus coronopifolius* monocluster was characterized by very high soluble carbohydrate content. Similarly, *Taonia atomaria* and *Sporochnus* sp. clustered away from other Phaeophyceae, due to their higher lipid and protein content. Phaeophyceae and Rhodophyta had similar C:N ratios, mostly ranging between 15 and 17 but with some outlier values, whether low (e.g. <10 for *A. armata* and *D. verticillata)* or high (>20). Average C:N ratios measured for *P. pavonica* (35.11 ± 15.53) and *C. spongiosus* (25.02 ± 2.73) were notably the highest of the dataset.

Finally, two clusters grouped species from at least two different groups: one included one Rhodophyta (*P. subulifera*) with five Phaeophyceae. The median position on the first axis for this cluster results from intermediate lipid and protein values, although contradictory patterns appear. For instance, *P. subulifera* mainly appeared in this group due to high protein content despite having higher soluble carbohydrate concentrations than *S. filamentosa*. The other cluster grouped two Rhodophyta (*S. filamentosa* and *A. armata*) and three Chlorophyta (*Codium bursa*, *C. vermillara* and *Caulerpa cylindracea*), in particular as these species had high ash content and relatively low protein concentrations. Chlorophyta were placed in an upper position within this cluster, because of their high insoluble carbohydrate concentrations. The position of *Codium bursa* in this cluster was also explained by its lower lipid and protein content, which resulted in a high C:N ratio (23.39 ± 5.69 ‰).

Mean C and N isotopic ratios differed significantly between phyla (Kruskall Wallis χ^2=33.46, pvalue=5.4 10^{-8}; ANOVA $F_{2,343}$ = 23.6 for $\delta^{15}N$, p value <0.001; Fig. 4). The Rhodophyta exhibited lower isotopic ratios for both $\delta^{13}C$ (-26.66 ± 5.74 ‰) and $\delta^{15}N$ (3.63 ± 0.58 ‰) than the two other groups, but the difference was less significant for nitrogen than carbon. As for biochemical concentrations, a wide range of values precluded definition of group-specific values. For example, $\delta^{13}C$ values ranged between -31.80 ± 1.34 ‰ (*F. petiolata)* and -14.68 ± 1.31 ‰ (*C. bursa*) for the Chlorophyta, -30.48 ± 2.50 ‰ (*Sporochnus* sp.) and -14.63 ± 1.43‰ (*P. pavonica*) for the Ochrophyta, and -32.82 ± 1.06‰ (*D. verticillata*) and -18.36 ± 0.62‰ (*S. filamentosa*) for the Rhodophyta. In contrast, the range of values was lower for nitrogen, with differences of ~1 to 2‰ between maximum and minimum average values (Tab. S2). This different behaviour between the two isotopes was a major driver of species clustering and explained the predominant effect of carbon in separating species. The five species with relatively lower $\delta^{13}C$ values (*D. verticilata*, *S. coronopifolius*, *F. petiolata*, *Bonemaisonia* sp. and *Sporochnus* sp.) occurred in the same cluster, while *C. bursa* ($\delta^{13}C$ = -14.68 ± 1.31 ‰) and *P. pavonica* (-14.63 ± 1.43 ‰), the two species with $\delta^{13}C$ values higher than -15 ‰ were clustered in the same group.

Biochemical content and isotopic ratios differences between months

All macroalgae sampled in all months (n=8) exhibited major temporal variations, most of the time significant. Carbohydrate concentrations were lower in June and March than in September and November, while proteins and lipids exhibited an inverse pattern (Fig. 5). Isotopic ratios

were generally higher in September and lower in March for both carbon and nitrogen, but with differences among species and between compounds (Table S3). The only species with non-variable isotopic ratios all year round was the green alga *F. petiolata*.

Correlations between isotopic ratios and biochemical concentrations

Strong correlations ($|\rho| > 0.5$) between isotopic and biochemical descriptors were observed 21 times, most between $\delta^{13}C$ or C:N ratios and biochemical descriptors (Fig. 6). Lipids were always strongly correlated with $\delta^{13}C$ for all species pooled, and specifically for Chlorophyta and Rhodophyta. In contrast, correlations between $\delta^{15}N$ and biochemical descriptors were observed for proteins only, but not for Rhodophyta. C:N was positively correlated with insoluble carbohydrates (i.e. the higher the C:N ratio, the higher the insoluble carbohydrate concentration) for all phyla but Chlorophyta. Nevertheless, C:N was also negatively correlated with protein content in this group only, suggesting that protein (major N compound) may be the major driver of C:N ratios for Chlorophyta.

Discussion

$\delta^{13}C$, a proxy of different carbon acquisition mechanisms

Carbon stable isotopes are used as tracers of photosynthetic metabolism and carbon acquisition, and vary according to the RubisCo and carbon-concentrating mechanisms types (Boller et al., 2011, 2015; Iniguez et al., 2020). Results obtained during the present work, at species or group levels, appear largely consistent with those of previous studies which usually discriminate algae using bicarbonates and carbon concentration mechanisms (CCM) vs species relying on CO_2 diffusion only (Cornwall et al., 2017; Giordano et al., 2005; Kevekordes et al., 2006; Korb et al., 1996; Maberly et al., 1992; Marconi et al., 2011; Raven et al., 2002, 2008). Mean $\delta^{13}C$ values were notably higher for the Chlorophyta, intermediate for the Ochrophyta and lower for the Rhodophyta (Maberly et al., 1992; Marconi et al., 2011; Mercado et al., 2009). Despite the finding that these groups use different forms of Rubisco, leading to differences in carbon fractioning (IB for Chlorophyta, ID for Ochrophyta and Rhodophyta), isotopic discrimination against ^{13}C appears similar and cannot be used to explain differences between them (Raven & Hurd, 2012).

Low $\delta^{13}C$ values measured here for the Rhodophyta are consistent with values measured for this phylum in the Mediterranean (Bricout et al., 1990; Dauby, 1989; Lepoint et al., 2000; Pinnegar & Polunin, 2000; Raven et al., 1995) and in other regions (Schaal et al. 2010, Marconi et al. 2011). For these species living in deep environments, with limiting low luminosity, available carbon is not considered as a limiting factor (Hepburn et al., 2011). Deep species are known to rely more on CO_2 diffusion than on carbon concentrating mechanisms (CCM)(Giordano et al., 2005). Under fixed environmental conditions (temperature, salinity and pressure), $\delta^{13}C$ value of dissolved CO_2 is lower than the value measured for HCO_3^- (~10 ‰), likely explaining some variation in isotopic values between groups. In addition, membrane transport and biochemical reactions associated with CCM slightly discriminate against ^{13}C (Maberly et al., 1992; Raven et al., 1995, 2008; Raven & Hurd, 2012). Finally, experimental work has generally observed lower capacity for red algae to increase external pH, with some exceptions such as *Palmaria palmata* (Kübler & Raven, 1995; Maberly, 1990). In the cytoplasm, dehydration of HCO_3^- generates OH^- that is expulsed from the intracellular fluid. Intensity of external pH increase can thus be viewed as a proxy of HCO_3^- use (Maberly, 1990; Marconi et al., 2011). Lower pH increase and low $\delta^{13}C$ values can be explained by the absence of CCM that precludes the use of HCO_3^- by Rhodophyta, and by a passive entry of dissolved CO_2. Some Rhodophyta species have nevertheless been shown to have some CCM such as *Pyropia yezoensis* (Zhang et al., 2020). The diversity of mechanisms and abilities may explain the diversity of isotopic ratios observed within this phylum (Giordano & Maberly, 1989; Marconi et al., 2011).

Among the Chlorophyta, the low value observed for *F. petiolata* is also consistent with literature values, regardless of environment and sampling depth (Belloni et al., 2019; Dauby, 1989; Lepoint et al., 2000; Mercado et al., 2009; Vizzini & Mazzola, 2006; Wangensteen et al., 2011). This species, similar to others among the green algae, such as *Udotea flabellum* and *Caulerpa* spp., is able to reach such low values, justifying dedicated investigation of metabolic peculiarities (Reiskind & Bowes, 1991). Low values appear to result from a C_4-like metabolism, with a first carboxylation step catalysed by the phospho-enol-pyruvate carboxykinase (PEPCK), to increase inorganic C supplies to Rubisco, and to limit photorespiration effects (Reiskind et al., 1988). This could nonetheless appear in contradiction with the low $\delta^{13}C$ values observed. A C_4-like photosynthetic mechanism can be considered as a CCM, and should lead to less negative $\delta^{13}C$ values. Carbon fractionation associated with PEPCK activity is also dependent upon the

available substrate amount (Arnelle & O'Leary, 1992). Further studies are thus required to explain low $\delta^{13}C$ values for this species (Raven & Hurd, 2012).

In contrast, the Chlorophyta *Codium bursa* and the Ochrophyta *Padina pavonica* displayed high carbon isotopic values (> -15‰), as previously demonstrated in the literature (Azzuro et al., 2007; Bricout et al., 1990; Dauby, 1989; Lepoint et al., 2000; Mercado et al., 2009; Wangensteen et al., 2011). High values measured for *P. pavonica* seem consistent with the calcified nature of the species and the importance of inorganic carbon in the tissue of this species. For *C. bursa*, the effect of its spherical shape on nutrient acquisition and photosynthetic activity likely explains the high value measured for this species. High $\delta^{13}C$ values are classical for spherical *Codium* species: the highest $\delta^{13}C$ values (-2.7‰) recorded for a macroalga was measured for *C. pomoides*, another spherical *Codium* species (Raven et al., 2002). Two mutually non-exclusive hypotheses can explain this value. First, the rounded shape generates a thicker boundary layer around tissues, with lower hydrodynamism and lower nutrient exchanges and renewal. Consequently, the proportion of heavy carbon ^{13}C could be higher in this layer. Similarly, lower hydrodynamics may prevent the removal of surface carbonic anhydrase, the effect of which was demonstrated to be significant in other *Codium* species to integrate isotopically enriched inorganic carbon (Raven & Hurd, 2012; Reiskind et al., 1988). The second hypothesis is based on the slow growth of this species, potentially linked with carbon limitation and the storage of nutrients in the internal medium of *C. bursa* (Vidondo & Duarte, 1995). Carbon isotopic ratios tend to increase when nutrients are limited, as the remaining and only available substrate is isotopically enriched. This explanation is further supported by the low concentrations observed for all biochemical descriptors in this species.

$\delta^{15}N$, proxy of environmental influences

Relationships between $\delta^{15}N$ and nitrogen inputs are harder to establish than the relationships between $\delta^{13}C$ and inorganic carbon, especially as the isotopic fractionation associated with nitrogen uptake is less well documented (Marconi et al., 2011; Umezawa et al., 2007). The lower range of average values observed for nitrogen (~2 ‰) than for carbon (~18‰) and the similar average $\delta^{15}N$ values (3 to 4‰) for all species is classical (Marconi et al., 2011), and may result from the use of similar nitrogen acquisition mechanisms by all species. Nitrogen isotopic ratios in primary producers are used as proxy of local influences, such as upwellings or human impact (Bode et al., 2006; Costanzo et al., 2005; Cresson, Boudouresque, et al., 2019; Riera et

al., 2000; Savage & Elmgren, 2004). Values measured in Marseille fall within the range of values observed at other Mediterranean sites with moderate human influence, in Spain or Sicily for example (Azzuro et al., 2007; Jennings et al., 1997; Wangensteen et al., 2011). Samples collected in pristine areas had lower $\delta^{15}N$ values, for example in Corsica (Lepoint et al., 2000; Pinnegar & Polunin, 2000). Higher values were recorded at sites under high anthropic pressures, such as some coastal lagoons (Deudero et al., 2011; Dierking et al., 2012; Vizzini & Mazzola, 2003). In the Bay of Marseille, the Huveaune River, a small stream flowing into the sea only after heavy rain events, may be a source of isotopically enriched nitrogen, but previous results demonstrated a low influence of this river (Cresson et al., 2012, 2016). $\delta^{15}N$ values measured for macroalgae here are thus consistent with these results.

Differences in nitrogen isotopic ratios between months were detected for two Chlorophyta species, consistently with their higher nitrogen requirements and ability to uptake nutrients faster than species from other phyla. Nitrogen isotopic ratios can be used as tracers of human influence on primary producers, but may also inform on metabolism variation and nutrient limitation. When metabolic demands exceed nutrients availability, discrimination against ^{15}N is no longer possible, leading to an increase of the algal $\delta^{15}N$ ratio. For *C. bursa*, nutrients are limiting in spring and summer as the growth rate is at a maximum in this period (Vidondo & Duarte, 1995). High $\delta^{15}N$ values can thus result from the use of all of the available inorganic N pool, regardless of the isotopic discrimination against ^{15}N (Montoya, 2007). For *C. cylindracea* (considering all varieties of this species), variations of nitrogen isotopic ratios are reported in the literature, with $\delta^{15}N$ values ranging between 1 and 8‰ (Azzuro et al., 2007; Box et al., 2009; Casu et al., 2008; Lapointe, Barile, Wynne, et al., 2005). Low values were also found in a previous study in the French Mediterranean (1.32 ± 0.11 ‰ for samples collected in Port Cros National Park in December 2018; P. Cresson, unpubl. results). Three explanations can be proposed for such a wide range of variation. Firstly, since *C. cylindracea* is an invasive species in the NW Mediterranean, it may have conserved its affinity for warm summer conditions from its SW Australian origin, increasing its growth rate and nitrogen demand in summer, precluding it from major isotopic discrimination (Gennaro et al., 2015; Klein & Verlaque, 2008; Raniello et al., 2004; Ruitton et al., 2005; Verlaque et al., 2003). Secondly, this species also uses its rhizomes to uptake organic compounds from its environment, allowing this species to be partly heterotrophic (Chisholm et al., 1996; Larned, 1998). High $\delta^{15}N$ values may thus result from an increased use of detritic nitrogen, particularly ammonium, from local sediment (average $\delta^{15}N$ =

5.13 ± 0.90 ‰; Cresson et al., 2012) to supplement needs for growth. Finally, high $\delta^{15}N$ can result from the ability of this species to better use human-derived N inputs (Lapointe et al. 2005a, b).

Biochemical concentrations, species-specific indicators of macroalgal metabolism and environmental inputs

Biochemical analyses of macroalgae have rarely been applied at assemblage level, but rather focused on some species of nutritional interest, for both animal and human diet, notably due to their high protein or lipid content (Biancacci et al., 2022; Fleurence et al., 1999; Herbreteau et al., 1997; McDermid & Stuercke, 2003; Westermeier et al., 2012). Nevertheless, from an ecological point of view, the taxonomical and temporal characterization of biochemical composition is a powerful tool to understand the relative importance of metabolic and environmental effects. The biochemical content of macroalgae can also be used as a predictor of diet selection and of the effects of their consumption on the life-history traits of consumers (Dromard et al., 2017; Frantzis & Grémare, 1992; Jacquin et al., 2006; Murakami et al., 2011; Schaal et al., 2010).

Biochemical compounds were dominated (more than 50%) by carbohydrates representing 12-34 % of the mass of the tissues, with a predominance of insoluble carbohydrates. These results are consistent with other studies which showed higher carbohydrate concentrations in green and red algae (McDermid & Stuercke, 2003). Insoluble carbohydrates, in spite of the high heterogeneity of this class of compounds, share a high chemical complexity, and are represented by molecules such as cellulose polymers. Actual measurements of the different groups of carbohydrates are rare (Dromard et al., 2017; Jacquin et al., 2006; McDermid & Stuercke, 2003; Shams El Din & El-Sherif, 2012), as most studies infer carbohydrate content from the quantification of dietary fibres, *i.e.* all indigestible compounds in vegetal cell walls (DeVries et al., 1999; Hipsley, 1953; Thebaudin et al., 1997). Fibre content is usually determined as the unreactive part remaining after enzymatic degradation steps involving amylase, proteases and amyloglucosidases, leading to the inclusion of all carbohydrates in this group (Dawczynski et al., 2007; Wong & Cheung, 2001). Regardless of the nature of the compounds actually included or not in the insoluble carbohydrate group, its

importance in algal tissue and its relatively low variability throughout the year is consistent with its structural role in cell walls.

Proteins are of major interest, notably as most benthic consumers are living in N-limited environments. In contrast to our results, protein content is usually considered high in Rhodophyta, despite seasonal variability (Dawczynski et al., 2007 and references therein). The higher protein content measured in brown algae may result from the method used to determine protein content. Most studies carried out on red and brown algae (52%, Angell et al., 2016) indirectly estimate protein content by multiplying N content by a factor 6.25, considered as the 'universal' ratio between total amino acids and nitrogen content (Angell et al., 2016). However, the universality of this factor has been questioned by direct measurements of non-proteic nitrogen in different phyla, and by arguing that the use of this 'universal' factor can induce an underestimation of the proteic content by 70% (Angell et al., 2016; Lourenço et al., 2002; Shuuluka et al., 2013). Hence, the higher protein content observed in red algae could result from a higher non-proteic N content, and thus an overestimation of the protein content when estimated from the N to protein conversion (Lourenço et al., 1998, 2002, 2004). This assumption is also supported by the results of consumers' feeding choices. Brown algae support most of the grazing pressure globally (Poore et al., 2012) and are also the principal algae consumed by the urchin *Paracentrotus lividus*, one of the main benthic herbivores in the Mediterranean (Boudouresque & Verlaque, 2007).

Spatiotemporal patterns provide evidence of an inverse relationship between concentrations of lipids and proteins (high concentrations in spring and summer, low in autumn and winter) and those of carbohydrates, that may explain the biochemical functioning of macroalgae and the decoupled synthesis of these two groups of compounds.

Carbohydrate synthesis is classically observed in late spring and summer, simultaneously with high photosynthetic activity, driven by high irradiance and higher algal biomass (Marinho-Soriano et al., 2006; Perfeto, 1998; Rosenberg & Ramus, 1982; Westermeier et al., 2012). Higher concentrations in autumn can also result from the storage of carbon fixed in excess during high photosynthetic activity in summer (Chapman & Craigie, 1978). Protein content is, however, mainly driven by the concentration of inorganic nitrogen in seawater (Durako & Dawes, 1980; Marinho-Soriano et al., 2006; Mouradi-Givernaud et al., 1993; Perfeto, 1998; Rosenberg & Ramus, 1982). High nutrient concentrations are usually recorded in spring in the

bay of Marseille and may underpin the pattern observed for algae (SOMLIT data, http://somlit-db.epoc.u-bordeaux1.fr/bdd.php).

Strong correlations observed between $\delta^{13}C$ and most of the biochemical descriptors are consistent with a comparable functioning of both types of descriptors. While the correlation between isotopic and biochemical descriptors has received less interet with reference to primary producers, it has been commonly investigated for heterotrophs, as high lipid content can bias $\delta^{13}C$ measurement (Kiljunen et al., 2006; Sardenne et al., 2015). Studies on terrestrial and freshwater producers have confirmed the low $\delta^{13}C$ value for lipids, and thus explained the negative relationship between these two descriptors (Post et al., 2007; van Dongen et al., 2002). Negative relationships between N isotopes and protein content is also consistent with previously observed low isotopic ratios for proteins (Kelly & Martínez del Rio, 2010; Perga & Grey, 2010; Podlesak & McWilliams, 2006), as a result of the low $\delta^{15}N$ values in most amino acids (Näsholm, 1994; Werner & Schmidt, 2002).

Finally, the carbohydrate group presents considerable heterogeneity. Carbohydrates as a whole are usually considered ^{13}C-enriched (Duranceau et al., 1999; Teece & Fogel, 2007; van Dongen et al., 2002), somewhat consistently with the pattern observed for Chlorophyta. However, variations may appear between groups of this phylum, driven by the cellulose content in species (Marshall et al., 2007). A negative correlation between insoluble carbohydrate and $\delta^{13}C$ might be consistent with a lower amount of cellulose.

Even if some species diverged from the main pattern of their group, some general trends underpinned by physiological processes and environmental influence were supported in our study. This study confirmed the utility of combining stable isotopes and biochemical content of macroalgae to infer major metabolic mechanisms. Carbon isotopic ratios differ between phyla because of specific carbon acquisition physiologies (RubiscCO type or Carbon Concentration Mechanisms), while N-linked descriptors are likely more controlled by temperature and nutrient availability. The higher lipid and protein content observed for the Phaeophyceae is also consistent with the higher integration of brown algae in the diet of grazers and detritivores, both at regional and global scales, and of their potential use in aquaculture. In a context of major changes for the Mediterranean marine environment, this study also provides a baseline of biological and isotopic data that could be used as a reference in the future to address the effect of global change on food web structure.

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No potential conflict of interest was reported by the authors.

Author contributions

Field and lab work: P. Cresson, S. Ruitton and M Harmelin-Vivien; Data treatment and conceptualization: P. Cresson and M. Harmelin Vivien. Writing; Data curation, and visualization: P. Cresson; Original Draft: P. Cresson, F. Noisette and M. Harmelin-Vivien. Writing - Review & Editing: P Cresson, S Ruitton, F Noisette and M. Harmelin Vivien.

Data accessibility

Raw data (isotopic ratios and biochemical content) can be freely accessed on SEANOE repository at https://doi.org/10.17882/89461

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Figures

Fig.1: Average contribution of each biochemical class (SC: soluble carbohydrates, IC: insoluble carbohydrates) and of ash mass to the content in each group. The unexplained part (Not expl.) was calculated as the difference between 1 g and the sum of the mass of biochemical compound and ash content. Actual concentrations are provided in Table S1

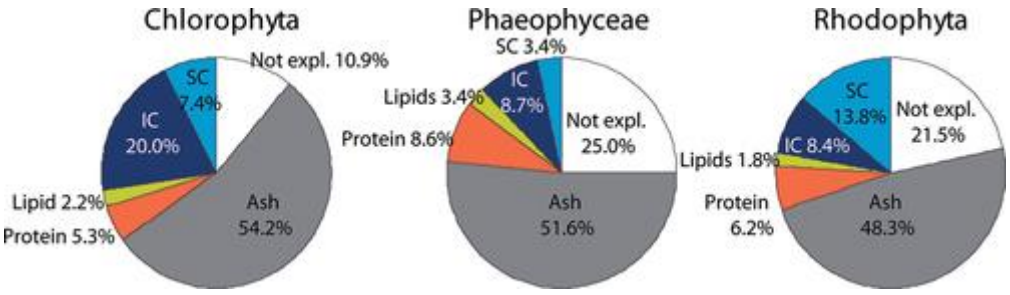


Fig. 2: Box plot of biochemical concentrations and C:N ratios for each group of algae, all months combined. Concentrations and C:N ratios were log-tranformed to comply with ANOVA assumptions. The horizontal line represents median values; boxes limit the first and third quartiles, and whiskers represent values higher or lower than mean ± 1.5 × interquartile ranges. Different letters represent significant differences between groups (p-value < 0.05).

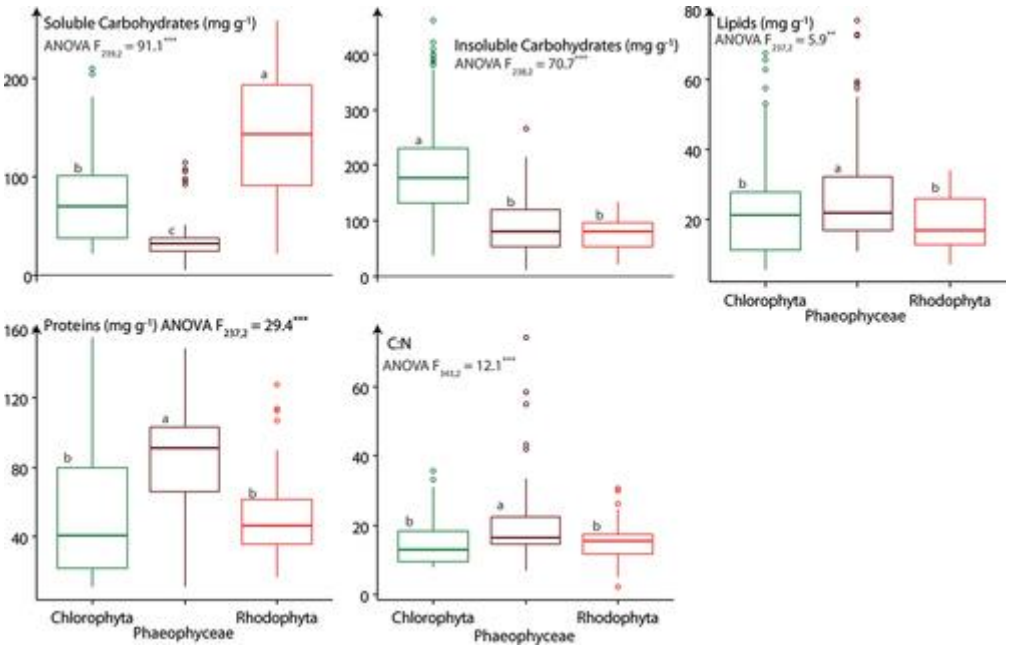


Fig. 3. First plan of the PCA analysis on average biochemical concentrations of macroalgae. Circles represent species clustered in the same group by cluster analysis on principal components.

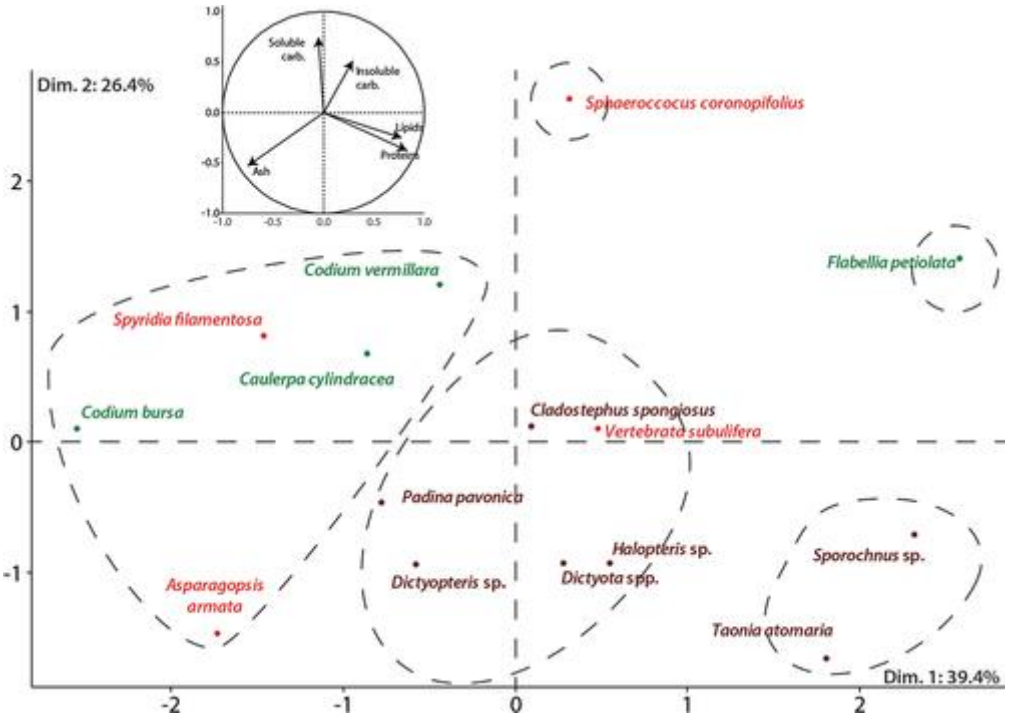


Fig. 4. isotopic ratios measured for the three macroalgal groups. The horizontal line represents median values; boxes limit the first and third quartiles, and whiskers extreme values, excluding outlier values (higher or lower than mean ± 1.5 × interquartile range).

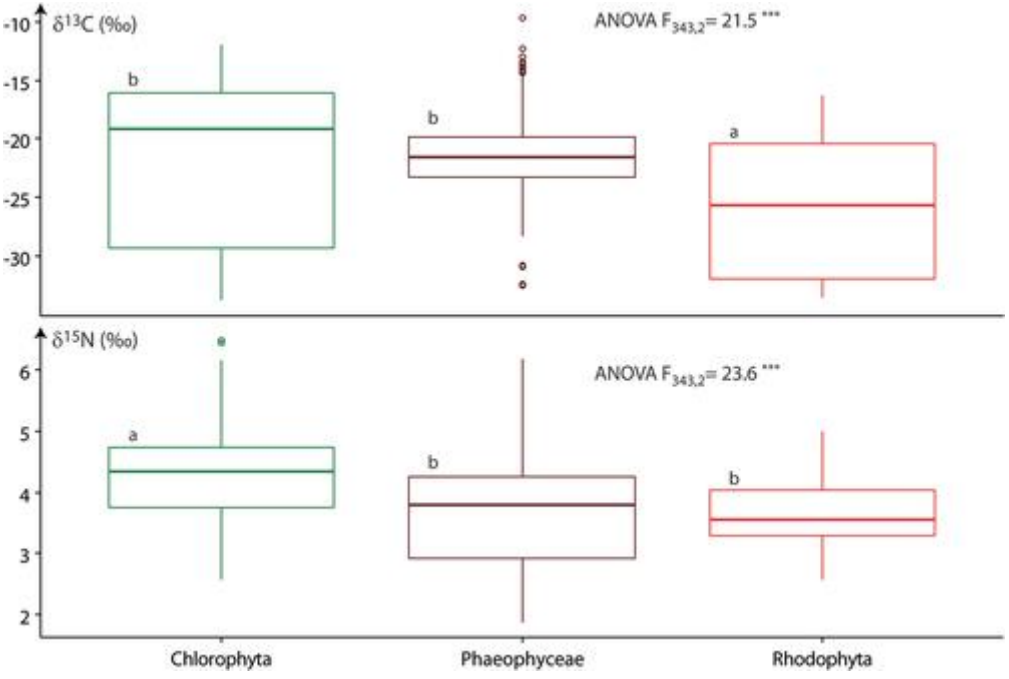


Fig. 5. Intraspecific seasonal variations of biochemical content of the species. Significant differences between seasons for each species are illustrated with different letters, or by ns when non-significant. Order of the difference is alphabetical, with a representing the lower value. The different colours of the boxplot illustrate months (green: March; red: June; brown: September; blue: November). Raw data and test statistics are provided in Tab. S3.

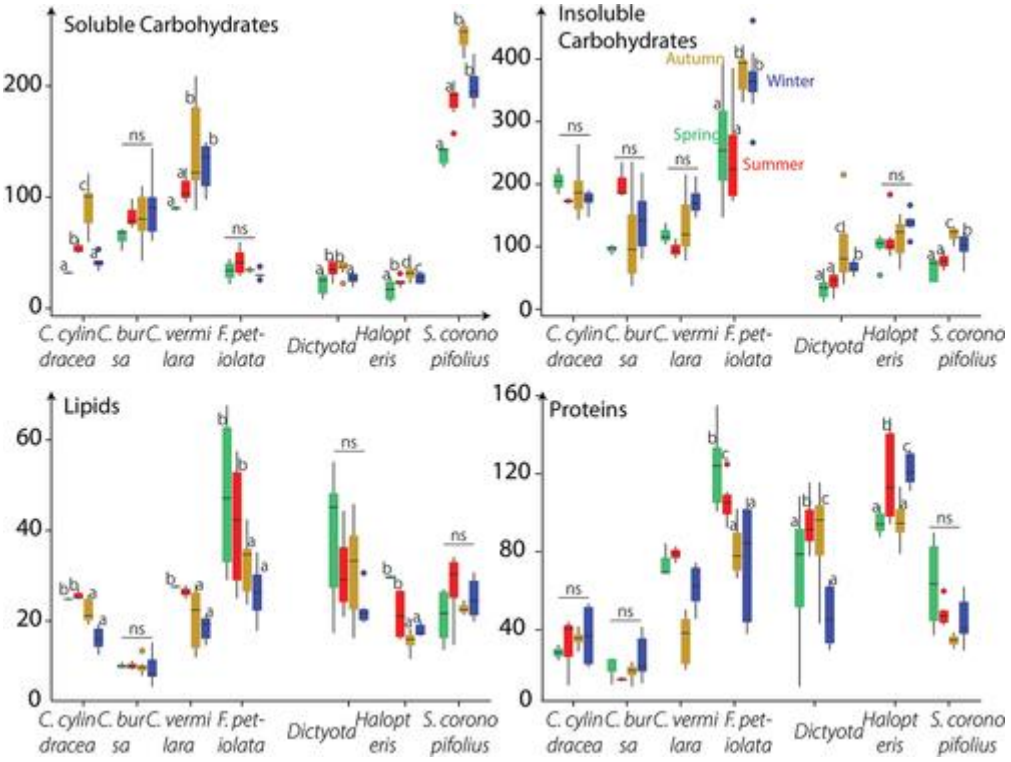
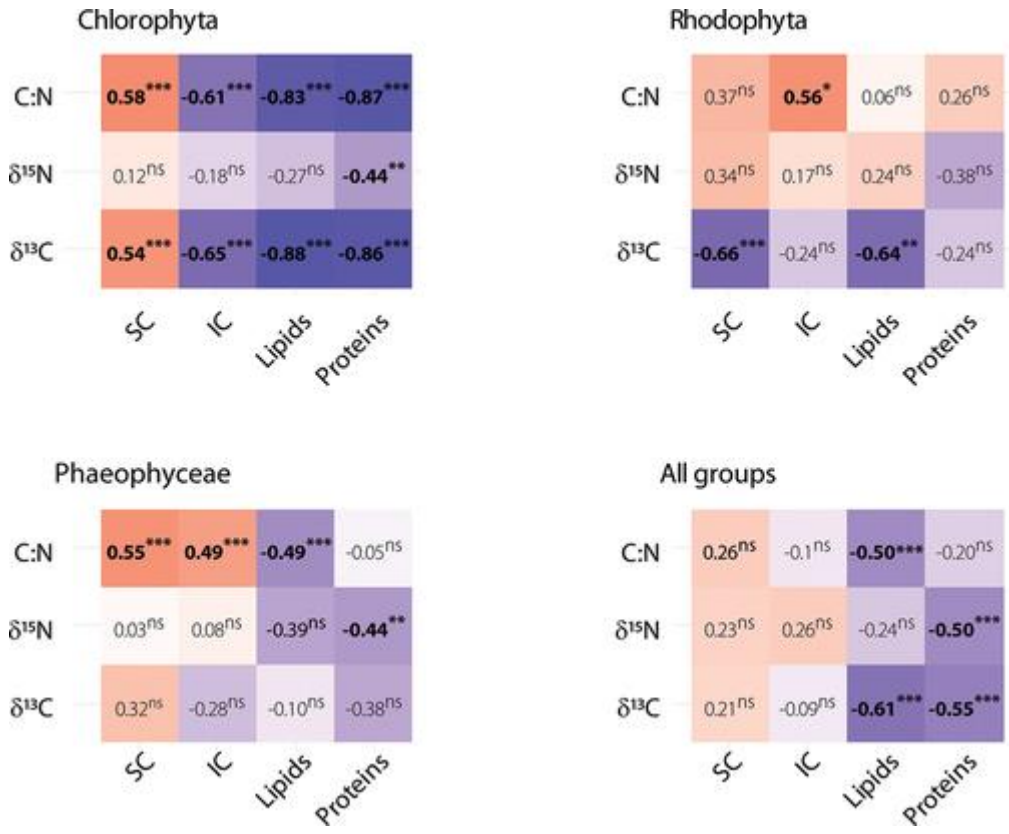


Fig. 6: Correlations between isotopic (vertical axis) and biochemical descriptors (horizontal axis). SC: Soluble carbohydrates; IC: Insolubles carbohydrates. Values are Spearman's ρ and p-value of the correlation test. Colour of the squares illustrate the sign of the correlation (red: positive; blue: negative) and the stronger the colour the stronger the relationship. Significant correlations (p value <0.005) are highlighted with bold colour fonts.



Electronic supplementary material

Tab. S1: Average concentrations (mean ± SD, mg g^{-1}) in biochemical compounds measured in the three groups.

Group	Soluble carbohydrates	Insoluble carbohydrates	Lipids	Proteins	Ash	Not Explained
Chlorophyta	74.53 ± 40.4	195.13 ± 98.24	22.33 ± 13.5	52.78 ± 35.75	556.27 ± 148.23	96.54 ± 74.04
Phaeophycea	34.25 ± 19.64	86.23 ± 47.11	27.13 ± 14.52	85.79 ± 28.15	520.44 ± 46.9	237.16 ± 56.47
Rhodophyta	136.36 ± 69.84	75.06 ± 31.9	19.14 ± 8.02	55.74 ± 33.01	495.89 ± 101.85	219.05 ± 91.69

Tab. S2: Mean isotopic ratios, for Ochrophyta, Phaeophyceae, Rhodophyta and species levels (mean and standard deviations). HCA: clusters calculated by the hierarchical clustering analysis based on isotopic ratios. n: number of replicated analyses performed for each group/species

	HCA	$\delta^{13}C$	sd	$\delta^{15}N$	sd	n
Chlorophyta		**-21.20**	**6.82**	**4.30**	**0.80**	**114**
Bryopsis cupresina	3	-17.62	0.73	3.69	0.22	3
Caulerpa cylindracea	3	-19.14	1.62	4.61	0.71	24
Codium bursa	4	-14.68	1.31	4.44	1.13	33
Codium vermillara	3	-19.43	2.05	4.28	0.38	24
Flabellia petiolata	1	-31.80	1.34	3.99	0.57	30
Phaeophyceae		**-21.47**	**3.71**	**3.66**	**0.91**	**169**
Cladostephus spongiosus	3	-21.74	1.25	4.12	0.29	12
Cystoseira zosteroides	2	-23.58	1.47	3.75	1.01	6
Dictyopteris sp.	3	-20.91	3.11	3.58	0.91	33
Dictyota spp.	3	-21.17	1.40	3.60	0.90	57
Halopteris sp.	2	-24.41	1.09	3.38	0.47	24
Padina pavonica	4	-14.63	1.43	4.76	0.56	18
Sporochnus pedunculatus	1	-30.48	2.50	2.82	1.37	8
Taonia atomaria	3	-21.69	1.58	3.25	0.44	9
Zanardinia typus	3	-20.67	0.81	2.94	0.23	3
Rhodophyta		**-26.66**	**5.74**	**3.63**	**0.58**	**60**
Asparagopsis sp.	2	-25.61	0.05	2.68	0.09	3
Bonnemaisonia sp.	1	-31.30	0.08	3.46	0.11	3
Bornetia secundiflora	3	-21.63	0.03	3.90	0.09	3
Dudresnaya verticillata	1	-32.82	1.06	4.04	0.83	3
Polysiphonia subulifera	3	-21.00	2.13	3.31	0.48	21
Sphaeroccocus coronopifolius	1	-32.05	0.97	3.95	0.49	24
Spyridia filamentosa	3	-18.36	0.62	3.70	0.21	3

Table S3. Seasonal variation of isotopic ratios for the species sampled at all seasons, and results of ANOVA and Tukey post-hoc tests

		March	June	September	November	ANOVA F	P value	Post hoc
Caulerpa cylindracea	$\delta^{13}C$ (‰)	-21.17 ± 0.84	-19.94 ± 1.96	-18.57 ± 0.88	-18.78 ± 1.81	$F_{20,3} = 2.96$	0.057	-
Codium bursa		-14.38 ± 0.43	-15.59 ± 1.10	-13.71 ± 1.20	-15.34 ± 1.06	$F_{29,3} = 5.74$	0.003	Jun=Nov<Mar<Sep[a]
Codium vermillara		-22.39 ± 0.28	-22.00 ± 0.23	-17.52 ± 1.30	-19.49 ± 0.74	$F_{20,3} = 28.93$	$<10^{-3}$	Mar=Jun<Nov<Sep
Flabellia petiolata		-32.22 ± 2.13	-31.49 ± 1.23	-31.14 ± 0.65	-32.02 ± 0.25	$F_{26,3} = 0.97$	0.42	-
Dictyopteris polyploides		-23.44 ±0.86	-18.06 ± 4.23	-20.65 ± 1.22	-22.90 ± 0.36	$F_{29,3} = 8.37$	$<10^{-3}$	Mar=Nov=Sep <Jun
Dictyota spp.		-22.63 ± 1.06	-20.12 ± 1.22	-21.13 ± 1.66	-21.40 ± 0.68	$F_{53,3} = 12.63$	$<10^{-3}$	Mar=Nov<Sep<Jun
Halopteris sp.		-24.80 ± 0.58	-23.60 ± 0.40	-23.59 ± 0.56	-25.63 ± 1.09	$F_{20,3} = 12.22$	$<10^{-3}$	Nov=Mar<Jun=Sep
Sphaeroccocus coronopifolius		-32.98 ± 0.56	-31.64 ± 0.20	-32.21 ± 0.66	-31.36 ± 1.40	$F_{20,3} = 4.97$	0.009	Mar[a] Sep[ab] Jun[b] Nov[b]
Caulerpa cylindracea	$\delta^{15}N$ (‰)	4.01± 0.05	5.22 ± 0.06	5.09 ± 0.53	4.13 ± 0.62	$F_{20,3} = 7.92$	0.001	Mar=Nov<Sep<Jun
Codium bursa		4.85 ± 0.29	6.36 ± 0.18	4.10 ± 0.73	4.25 ± 1.22	$F_{29,3} = 4.83$	0.008	Sep=Nov=Mar<Jun
Codium vermillara		4.41 ± 0.07	3.48 ± 0.09	4.52 ± 0.25	4.26 ± 0.20	$F_{20,3} = 28.93$	$<10^{-3}$	Jun<Nov=Mar=Sep
Flabellia petiolata		3.98 ± 0.51	4.06 ± 0.74	3.77 ± 0.21	4.09 ± 0.70	$F_{26,3} = 0.40$	0.75	-
Dictyopteris polyploides		3.42 ± 0.75	3.48 ± 1.10	4.13 ± 0.43	2.13 ± 0.17	$F_{29,3} = 6.07$	0.002	Nov<Mar=Jun<Sep
Dictyota spp.		4.15 ± 1.23	2.77 ± 0.36	3.81 ± 0.33	4.13 ± 0.83	$F_{53,3} = 13.05$	$<10^{-3}$	Jun<Sep=Nov=Mar
Halopteris sp.		2.86 ± 0.32	3.17 ± 0.24	3.84 ± 0.20	3.63 ± 0.34	$F_{20,3} = 14.99$	$<10^{-3}$	Mar=Jun<Nov=Sep
Sphaeroccocus coronopifolius		3.40 ± 0.19	4.50 ± 0.23	3.67 ± 0.19	4.21 ± 0.28	$F_{20,3} = 28.89$	$<10^{-3}$	Mar=Sep<Nov=Jun

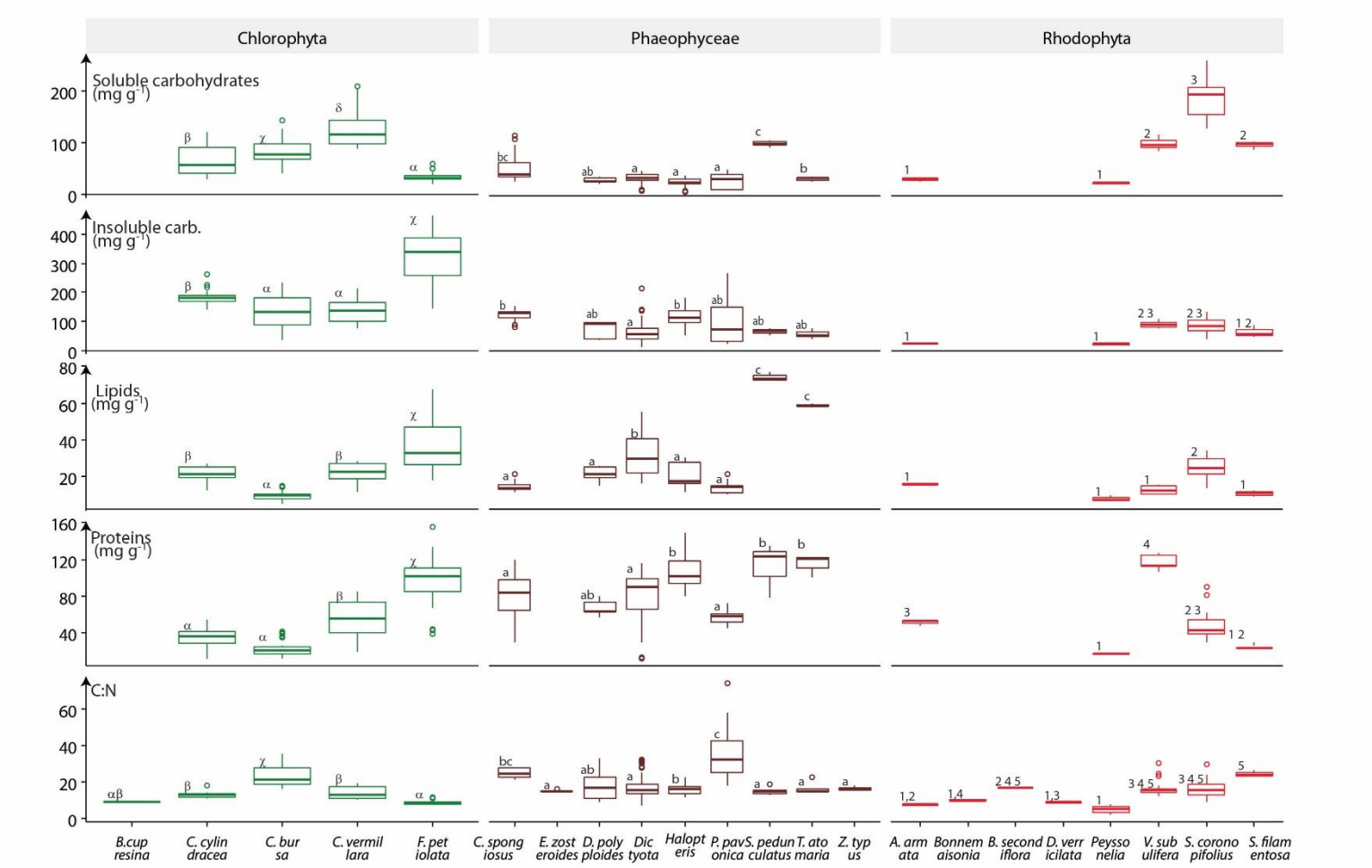


Fig. S1: Specific variations of biochemical composition and C:N ratios. Horizontal line represents median values; boxes limit the 1st and 3rd quartiles, and whiskers extreme values, excluding outlier values (higher or lower than mean ± 1.5b × interquartile range). Significant differences between species within phyla are represented by different letters or number, with letter/number presented by increasing order of concentrations or of C:N values.