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Photo-protective compounds in red macroalgae from Brittany: Considerable diversity in mycosporine-like amino acids (MAAs)

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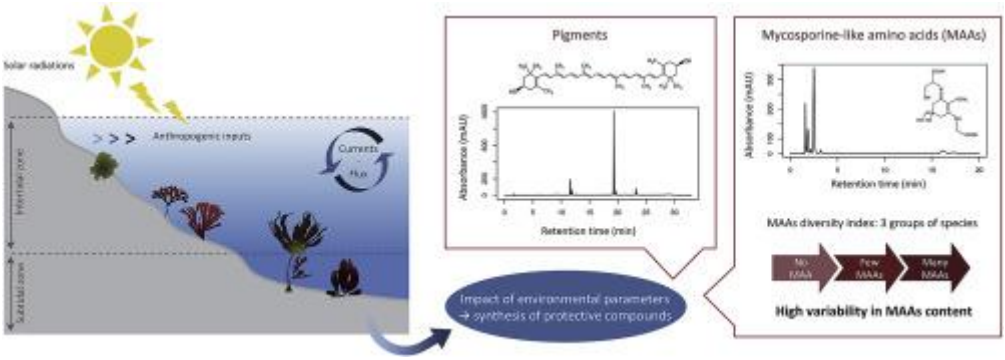
Abstract :

To cope with the biotic and abiotic stresses experienced within their environment, marine macroalgae have developed certain defence mechanisms including the synthesis of photo-protective molecules against light and particularly harmful UV radiation. The aim of this study was to screen selected red algae, a highly diverse phylogenetic group, for the production of photo-protective molecules. The pigment content and composition (i.e. chlorophyll-a, phycobiliproteins and carotenoids) and the composition of mycosporine-like amino acids (MAAs) were studied in 40 species of red macroalgae collected in Brittany (France), at two distinct periods (i.e. February and July 2017). A high inter-specific variability was demonstrated in terms of pigment content and MAA composition. Twenty-three potential MAAs were detected by HPLC, and six were identified by LC-MS (i.e. shinorine, palythine, asterina-330, porphyra-334, usurijene and palythene). This is the first study to report on the composition of pigments and MAAs in a diverse group of red seaweeds from Brittany, including some species for which the MAA composition has never been studied before. Nevertheless, the results suggested that some species of red algae are more likely to cope with high levels of light radiation since those species such as Bostrychia scorpioides, Porphyra dioica, Gracilaria vermiculophylla and Vertebrata lanosa are living in environments exposed to higher levels of irradiation, and had various MAAs in addition to their photo-protective pigments.

Graphical abstract



Highlights

► Detection of 23 potential MAAs in 40 Rhodophyta species, including 6 already identified (shinorine, palythine, asterina-330, porphyra-334, usurijene, palythene). ► First report on the MAAs composition for some red seaweeds. ► High variability in MAAs content and composition between the different species, with no link with phylogeny, morphology, position on the shore or sampling site. ► A MAAs extraction method using 70% ethanol being less toxic than conventional methanol, and giving potential valorisable extracts.

Keywords : Algae, Diversity, HPLC, MAAs, Metabolites, Photo-protection, Pigments, Rhodophyta, Screening

1. Introduction

Macroalgae are known to play a key role in coastal ecosystems as being at the basis of the food web and providing refugium for many animal species (Lüning, 1990). In particular, seaweeds produce diverse compounds under natural conditions, which could potentially be used in health, cosmetic, or food sectors (Holdt and Kraan, 2011; Stengel et al., 2011; Stiger-Pouvreau and Guérard, 2018; Surget et al., 2017). However, both the concentration and composition of these bioactive compounds can fluctuate within individuals, with their habitat and with season. In the context of climate change, the environmental parameters impacting particularly intertidal seaweeds are in a state of flux. To cope with changing environmental conditions, seaweeds have developed particular adaptive, metabolic responses and are thus able to resist and adapt to different abiotic stresses (Davison and Pearson, 1996; Gantt, 1990).

Irradiance is one of the factors which can change over the intertidal range in coastal areas, in term of quantity and quality because it varies during the day and with the tide, the turbidity of the water, the climate, the depth or seasons (Sagert et al., 1997). Light is also very important for seaweeds as algae are photosynthetic organisms (Gantt, 1990; Lobban and Harrison, 1994) with some living in the intertidal zone and thus exposed directly to the sunlight during emersion phases. One of the consequences of climate change is the amount of ultraviolet (UV) radiation reaching the Earth's surface (Bischof et al., 2006; Thomas et al., 2012). UV radiations are notably at the origin of DNA alterations, the synthesis of reactive oxygen species (ROS) and, in seaweeds, the photo-inhibition mechanism or the degradation of photosynthetic pigments (Karentz, 2001; Karsten, 2008; Van de Poll et al., 2001). However, due to human activities and

ozone depletion, the protective filter that constitutes the atmosphere gradually loses its effectiveness, which means that the amount of UV to which the organisms are exposed tends to increase (Carreto et al., 2018; McKenzie et al., 2007). Although some authors agree that the ozone layer will probably not fully recover before several decades, the hole in this layer would be resorbing at mid-latitudes due to the implementation of some policies tending to reduce greenhouse gas emissions, but this would not be the case at high latitudes and the evolution of the UV level remains complex (McKenzie et al., 2011; Wilmouth et al., 2018).

Different types of macroalgae have developed various strategies to protect themselves from harmful UV radiation. These include the synthesis of photo-protective molecules (Bhatia et al., 2011; Le Lann et al. 2016; Rastogi et al., 2010; Sinha et al., 2007). Among the algal photo-protective compounds, this study focuses on pigments and mycosporine-like amino acids (MAAs), synthesized by red algae (Rhodophyta). MAAs were discovered in 1965 in the terrestrial fungus *Ascochyta pisi* (Leach, 1965). Since then, the presence of MAAs has been demonstrated in a wide range of marine organisms from bacteria to fish (Bandaranayake, 1998; La Barre et al., 2014; Rosic et al., 2015), including different species of algae (Bedoux et al., 2014; Sinha et al., 2007). MAAs are low molecular weight molecules (< 400 Da), soluble in water, with a high molar extinction coefficient (between 28,000 - 50,000 $M^{-1}.cm^{-1}$) and a maximum of absorption (λ_{max}) between 310 - 362 nm (Rastogi et al., 2010). In addition, it has been demonstrated that red algae, which constitute a well-marked phylogenetic group, have the greatest diversity and the highest proportion of MAAs (Carreto and Carignan, 2011; Karentz, 2001; Sinha et al., 2007). Until now, about 20 MAAs have been identified in

various red macroalgae (Sinha et al., 2007). However, as some recent studies have shown (Briani et al., 2018; Hartmann et al., 2016), many MAAs have yet to be identified. The major role of MAAs in photo-protection against UV radiation has already been widely demonstrated (Bedoux et al., 2014; Huovinen et al., 2004; Singh et al., 2008), and functionality could be linked to their individual structures (Wolley et al., 2018). It appears that some MAAs may also have various functions such as anti-oxidants (De la Coba et al., 2009; Torres et al., 2018; Wada et al., 2015), osmolytes, nitrogen storage, or protective agents against desiccation or temperature variations (Bhatia et al., 2011; Oren and Gunde-Cimerman, 2007).

Pigments are part of a second significant group of photo-protective compounds in macroalgae. There are three main families of pigments: chlorophylls, carotenoids and phycobiliproteins (reviewed in Stengel et al., 2011). They do not all have a role in photo-protection, such as chlorophylls and phycobiliproteins which harvest light for photosynthesis. Among the chlorophylls, there is only chlorophyll-*a* in red algae (Stengel et al., 2011). One characteristic of red algae is the presence of phycobiliproteins (reviewed by Dumay et al., 2014). These are “secondary pigments” which also capture solar energy and transfer it to chlorophyll *a* for the photosynthesis; they are particularly effective under low light conditions (Gantt, 1990; Lobban and Harrison, 1994). There are 3 main groups of phycobiliproteins in the Rhodophyta: phycoerythrin (PE, λ_{max} = 540-570 nm) which provides their red colour, phycocyanin (PC, λ_{max} = 610-620 nm) and allophycocyanin (APC, λ_{max} = 650-655 nm) which both have a blueish hue (Dumay et al., 2014). Finally, there are two groups of carotenoids: carotenes (α-carotene, β-carotene in red algae) and xanthophylls (i.e. zeaxanthin,

97 antheraxanthin, violaxanthin and lutein in red algae) which convey a yellowish
98 colouration. These last pigments have several physiological roles (Karsten, 2008;
99 Young and Frank, 1996): they take part in photosynthesis through light harvesting in
100 thylakoids (Hashimoto et al., 2016) but they also have an important role in photo-
101 protection (Mimuro and Akimoto, 2003). Indeed, they also may participate in the
102 dissipation of solar energy (Young et al., 1997) and to the de-activation of reactive
103 oxygen species (ROS) (Rastogi et al., 2010).

104 According to the nature, quality and quantity of photo-protective compounds,
105 and also their morphology (blade, filamentous, crustose) and life cycle, different species
106 of macroalgae are then more or less sensitive to UV radiation (Davison and Pearson,
107 1996; Roleda et al., 2004). In this context, the aim of the present study was to
108 investigate the inter-specific variations in red algal photo-protective compounds within
109 a diverse, representative group of temperate and local red seaweeds. Thus, a screening
110 was carried out on a large number of red seaweeds sampled from the coast of Brittany
111 (France). In order to study the maximum number of species, seaweeds were sampled
112 during two distinctly different seasons, i.e. winter and summer, as some species occur
113 only during a part of the year. Candidate species for analysis were also collected from
114 the upper subtidal zone (i.e. emersed only at spring tides for maximum 1 h; Ar Gall and
115 Le Duff, 2014) and along the intertidal zone (i.e. with different maximum emersion
116 times from 1 h in the lower intertidal zone to 12 h in the upper intertidal zone during
117 spring tides; Ar Gall and Le Duff, 2014). A MAA diversity index was determined for
118 each species, in order to compare and provide hierarchical comparisons within the red
119 macroalgae collected in this study. Moreover, in order to test the hypotheses of a
120 relationship between the diversity of MAAs and individual red algal taxonomy, various

orders/families of red macroalgae were specifically targeted for collection.

2. Materials and methods

2.1 Sampling sites

Macroalgae were collected at low tides on four different sites in Brittany (France): Porspoder (48°28’58’’N – 4°46’5’’W), Portsall (48°33’53’’N – 4°42’5’’W), Saint-Pabu (48°34’34’’N – 4°38’45’’W) and Le Faou (48°17’44’’N – 4°10’56’’W). Portsall, exposed North-West, is a sheltered and mainly rocky site, surrounded by sand and intertidal pools. Despite its western exposure, Porspoder remains sheltered due to the presence of two large rocky over-hangs. The site chosen at Saint-Pabu, was exposed to the North; it is characterized by sandy dunes. Finally, Le Faou differs from the three previous sites because a river flows there into the Bay of Brest, forming a sheltered but muddy estuary at low tide. Two samplings periods were carried out: in February (winter) and July (summer) 2017.

2.2 Macroalgal samples

Forty species of red macroalgae were collected across the four different sites: 21 species were found both in winter and summer and 19 species were found on the shore only for one season. All species belong to the class of Florideophyceae, except *Porphyra dioica* (Bangiophyceae). These included species from different morpho-anatomical groups (i.e. MAG, according to Steneck and Dethier, 1994): filamentous algae (MAG 2); corticated or polysiphonous filaments algae (MAG 2.5); foliose algae (MAG 3); corticated foliose algae (MAG 3.5); corticated algae (MAG 4); and articulated calcareous algae (MAG 6) (Appendix 1).

After collection, algal samples were brought back to the laboratory into sampling bags. Samples were stored in a fridge and washed within 4 hours of sampling with filtered seawater to remove residual sediments and salt; visible epiphytes were removed by hand. Seaweeds were then stored in the freezer before being freeze-dried (β1-8 LD plus Christ) and the entire thallus was milled into a fine powder (MM400 Retsch). The dried powder was kept in darkness at room temperature until analyses.

*2.3 Extraction and assay of chlorophyll-*a *and carotenoids*

Pigments were extracted from 75 mg dry weight (DW) of algal powder in 750 µL of 90% acetone, according to Schmid and Stengel (2015). Two successive extractions of 30 min and 4 h were carried out at 4 °C, under magnetic agitation. Samples were then centrifuged at 10,000 rpm for 5 min (miniSpin plus, Eppendorf), and the supernatants combined and filtered for HPLC (High Pressure Liquid Chromatography) analysis (Dionex Ultimate 3000, ThermoScientific). Pigments were separated using an ACE C_{18} column (150 x 4.6 mm, 3 µm) with a guard-column, an injection volume of 6 µL and a run-time of 33 min per sample. Before injection, each sample was automatically diluted ¾ with ammonium acetate buffer (0.5 M, pH 7.2). Separation was achieved using a solvent gradient described in Table 1, delivered at a flow rate of 1.0 $mL.min^{-1}$. A photo-diode array detector (DAD3000, ThermoScientific) was used for the detection of pigments at 435, 470 and 650 nm. The identification and quantification of each pigment was based on spectral comparisons and calibration using commercial standards: chlorophyll *a* (Sigma, USA), and α-carotene, β-carotene, lutein, zeaxanthin, antheraxanthin, violaxanthin (DHI, Denmark). Only peaks with an area larger than 0.4 mAU.min for α- and β-carotenes and 1 mAU.min for the other pigments

were considered in the analysis. HPLC data were collected using Chromeleon 0.7 software (Thermo Scientific Dionex, France).

2.4 Extraction and assay of phycobiliproteins

Tissue concentrations of phycoerythrin (PE) and phycocyanin (PC) were determined using a method adapted from Sun et al. (2009). Two successive extractions of 15 min at 4 °C were performed from 75 mg DW of algal powder, with 1.5 mL of phosphate buffer (0.1 M, pH 6.8). Subsequently, samples were centrifuged for 20 min at 10,000 rpm (miniSpin plus, Eppendorf) and supernatants were used to measure absorbances at 455, 565, 592, 618 and 645 nm using a spectrophotometer (POLARStar Omega, BMG LABTECH). The concentrations of PE and PC ($mg.g^{-1}$ DW) were estimated using the following equations from Beer and Eshel (1985):

$[PE] = [(A_{565} - A_{592}) - (A_{455} - A_{592}) \times 0.20] \times 0.12$

$[PC] = [(A_{618} - A_{645}) - (A_{592} - A_{645}) \times 0.51] \times 0.15$

2.5 Mycosporine-like Amino Acids (MAAs) extraction and assay

20 mg DW of finely ground algae were extracted for 2 h with 2 mL of 70% aqueous ethanol (v/v) at 45 °C with magnetic stirring. After centrifugation (centrifuge 5810, Eppendorf) at 1,500 rpm for 10 min, the supernatant was recovered. The pellet was re-extracted twice following the same procedure and the combined supernatants were evaporated to dryness under vacuum (miVac, Genevac, France) at 45 °C. The evaporated extracts were then stored at -20 °C before analysis.

Prior to HPLC analysis, extracts were dissolved in 500 µL of 2.5% aqueous methanol with 0.1% acetic acid, centrifuged for 5 min at 10,000 rpm (miniSpin plus,

192 Eppendorf), and filtered through 0.2 µm-pore syringe filters. MAAs analyses were
193 performed using a Dionex Ultimate 3000 HPLC (Thermo Scientific, Germany)
194 equipped with a diode-array detector (DAD). MAA separation was performed using a
195 Zorbax Eclipse XDB C_{18} column, 5 µm, 4.6 × 250 mm (Agilent, USA) equipped with a
196 guard-column. For one analysis, 20 µL of extract were injected and the operating
197 parameters were as follow: 0.1% acetic acid in Milli-Q water as the mobile phase; a
198 flow rate of 1 $mL.min^{-1}$; a column temperature of 25 ± 1 °C; an injector temperature of
199 5 ± 1 °C; a run time of 20 min. MAAs were detected at 320, 330, 334 and 360 nm.
200 HPLC data were collected using Chromeleon 0.7 software (Thermo Scientific Dionex,
201 France). Individual peaks were identified by online absorption spectra and retention
202 time (Rt).
203
204 For identification, MAAs extracts were also analyzed by LC-MS using a LC-
205 ESI-Q-TOF-MS (Dionex, Ultimate 3000, Bruker, micrOTOF-QII) system (Bruker
206 Daltonik GmbH, Bremen, Germany). The same LC method was used, only the flow was
207 reduced to 0.5 $mL.min^{-1}$. Source parameters were: positive mode; source temperature,
208 200 °C; capillary voltage, 4.5 kV; nebulizer gas (N_2) at 2.8 bars and dry gas (N_2) at 12
209 $L.min^{-1}$. Mass spectra acquisition was set at 0.5 Hz from m/Z 50 to 1000. MS/MS
210 analyses were performed with a collision energy of 30 eV and an isolation width of 2
211 m/Z. All raw data were collected with Compass dataAnalysis Version 4.1. The
212 quantification of the identified MAAs was accomplished using their molar extinction
213 coefficients ε at the wavelengths of maximum absorption, according to Pelillo et al.
214 (2004), and the molar extinction coefficient of Karsten et al. (1998b). For MAAs whose

molecular structure has not yet been elucidated, their quantification was not established. Each MAA was then numbered in the order in which it appeared during the run.

Finally, the diversity of MAAs within each seaweed species tested was estimated by the development of a new index. The presence and absence of each MAA for each species was coded in a table, as 0 or 1 respectively. A distance matrix was then calculated by comparing species two by two using the following index:

$$Index = \frac{M}{M + N}$$

were 'M' is the number of matches between the two species (presence and absence) and 'N' the total number of MAAs which were present in only one of the two species. The closer the index was to 1, the more similar the two species were in terms of the composition of their MAAs. A species with many weak indices thus represented a specific composition. A dendrogram was made from this matrix with the R program (R development core team, 2008) to represent species that had similar MAA compositions (hclust with single method, hclust package). In order to highlight any links between the composition of MAAs by algal species with their phylogeny, a phylogenetic analysis was performed using *rbc*L and *cox1* genes sequences, as retrieved from GenBank (Accession numbers for GenBank sequences are listed in Appendix 1 and are available on https://www.ncbi.nlm.nih.gov). Sequences were aligned using the ClustalW programme in the BioEdit alignment editor (Hall, 1999). A phylogenetic tree was built using MEGA version 7 (Kumar et al., 2016), based on the Neighbor-Joining clustering method. Evolutionary distances were computed using the Maximum Composite Likelihood. The tree was subjected to a bootstrap test (5000 replicates) to estimate robustness at each branch (pvclust, pvclust package).

238

239 *2.6 Statistical analyses*

240 All data were statistically analyzed with the R program (R development core team,
241 2008). All extractions were performed in triplicate, and results expressed as average ±
242 standard deviation (SD). Comparisons were carried out using a Student's t-test (2
243 samples) or an ANOVA (more than 2 samples). Beforehand, the conditions of
244 application were controlled using the Shapiro test for compliance with a normal
245 distribution, and the F-test or Bartlett's test for the homogeneity of variance (Dytham,
246 2011). The non-parametric Wilcoxon test (2 samples) or Scheirer-Ray-Hare test (SHR,
247 more than 2 samples) were used when at least one of the conditions of application was
248 not met (to replace the t-test or the ANOVA, respectively). If possible, when significant
249 results were highlighted, *post-hoc* tests (i.e. multiple comparisons) were performed: a
250 Tukey *post-hoc* test after an ANOVA (TukeyHSD), or a non-parametric *post-hoc* test
251 after a Sheirer-Ray test (kruskalmc, pirgmess package) (Dytham, 2011). Principal
252 Component Analysis (PCA) was carried out (package FactoMineR) to highlight species
253 which stood out.

254

255 **3. Results**

256 *3.1 Pigments: chlorophyll* a, *carotenoids and phycobiliproteins*

257 The chlorophyll *a* content (Fig. 1) varied significantly among the analyzed red algal
258 species (SHR test, p-value < 0.0001). In some species, such as *Mastocarpus stellatus* or
259 *Furcellaria lumbricalis*, chlorophyll *a* concentrations did not exceed 1 $mg.g^{-1}$ DW -
260 whatever the season. However, *Callithamnion tetragonum* and *Plumaria plumosa* for
261 example contained in winter 8.60 ± 0.26 and 8.37 ± 0.21 $mg.g^{-1}$ DW of chlorophylle *a*,

respectively. Moreover, a seasonal variability was demonstrated with chlorophyll *a* concentrations decreasing in summer (SHR test, p-value = 0.0040). For example, *Palmaria palmata* contained 7.02 ± 0.31 $mg.g^{-1}$ DW of chlorophyll *a* in February, and 1.50 ± 0.27 $mg.g^{-1}$ DW in July. Conversely, *Bostrychia scorpioides* was the only species with a significantly increased concentration in summer (i.e. 5.44 ± 0.43, as compared to 4.32 ± 0.09 $mg.g^{-1}$ DW in winter).

Similarly, total concentration of carotenoids changed with species (SHR test, p-value < 0.0001) (Fig. 2). For example, *Chondrus crispus* and *F. lumbricalis* contained less than 0.2 $mg.g^{-1}$ DW of carotenoids, whereas *Vertebrata lanosa, Porphyra dioica* or *B. scorpioides* contained up to 0.78, 0.84 and 0.65 $mg.g^{-1}$ DW of total carotenoids, respectively. A difference in term of composition was also observed (Fig. 2): overall, lutein was the most common carotenoid in 31 species out of 40. However, the amount of lutein remained low as compared to chlorophyll *a*, which constituted 90.1% on average of the total liposoluble pigment content. Moreover, there was no seasonal variation in the carotenoid composition as a whole, but the concentration was seen to decrease in summer (SHR test, p-value = 0.0012) (Fig. 2), except for *V. lanosa* in which the concentration increased (e.g. 0.78 ± 0.01 and 0.66 ± 0.04 $mg.g^{-1}$ DW in summer and winter, respectively). *Gigartina pistillata*, *Metacallophyllis laciniata* and *Membranoptera alata* did not contain any detectable levels of carotenoids in summer. However, although there were seasonal variations in chlorophyll *a* and overall carotenoid amounts, the ratio of carotenoids / chlorophyll *a* appeared to be constant between both seasons (SHR test, p-value = 0.4919) (data not shown).

The different studied red algae contained variable levels of phycobiliproteins similarly to their chlorophyll *a* or carotenoid amounts (SHR test, p-value < 0.0001)

(Fig. 3). For example, *Bornetia secundiflora* and *Membranoptera alata* contained 27.39 ± 3.13 and 19.67 ± 0.15 $mg.g^{-1}$ DW of PE in winter, respectively, whereas *C. crispus*, *Mastocarpus stellatus* or *V. lanosa* contained less than 5.0 $mg.g^{-1}$ DW (Fig. 3). *P. plumosa* was more differentiated from the other species with high levels of PE in both seasons (23.18 ± 0.49 $mg.g^{-1}$ DW in winter and 13.58 ± 1.36 $mg.g^{-1}$ DW in summer). Moreover, it would appear that, on the whole, the PE content decreased in summer (SHR test, p-value < 0.0001). *Bostrychia scorpioides* was the only species for which the PE concentration really increased in summer (6.62 ± 0.22 $mg.g^{-1}$ DW in summer, compared to 5.32 ± 0.50 $mg.g^{-1}$ DW in winter). The results were similar for phycocyanin (data not shown).

A Principal Component Analysis (PCA) of all pigments (Fig. 4a, 4b) pointed out six species: *B. scorpioides*, *Porphyra dioica*, *V. lanosa*, *Gracilaria vermiculophylla*, *Gracilaria gracilis* and *Plumaria plumosa*. This last species contained the highest concentration of chlorophyll *a* and phycoerythrin. The five other species were the only ones containing zeaxanthin with a concentration of 0.5 $\mu g.g^{-1}$ DW or greater. *G. gracilis* also contained antheraxanthin. Finally, the species belonging to similar MAG showed different pigment content (chlorophyll *a*, carotenoids and phycoerythrin) and composition. Moreover, no link between pigment composition of the red seaweeds studied here and their phylogeny, morphology, position on the shore or sampling site could be demonstrated.

3.2 Mycosporine-like Amino Acids

309 HPLC analyses showed 23 different peaks that may correspond to MAAs (Fig. 5
310 and Appendix 2), and six (i.e. shinorine, palythine, asterina-330, porphyra-334,
311 usujirene and palythene) were elucidated by LC-MS. The composition and
312 concentration of various MAAs showed a difference between the various species of red
313 macroalgae sampled in this study. These observations were independent of the sampling
314 season. Amongst the 21 species of algae commonly occurring in both seasons
315 considered, six did not contain any MAA (these included: *F. lumbricalis*,
316 *Metacallophyllis laciniata, Polyides rotunda, Osmundea pinnatifida, Membranoptera*
317 *alata* and *Plumaria plumosa*). For the eight species which could be collected only in
318 summer, no MAAs were detected in four and for those harvested only in winter, five did
319 not contain any detectable MAAs. At the same time, *Mastocarpus stellatus* contained a
320 high concentration of only shinorine (i.e. 3.12 ± 0.26 and 1.62 ± 0.07 $mg.g^{-1}$ DW,
321 respectively in winter and in summer). One should note that three species, i.e. *Palmaria*
322 *palmata*, *B. scorpioides* and *G. vermiculophylla*, contained eight different MAAs,
323 visible as separate peaks on the chromatograms (data not shown). A chromatogram of
324 the MAAs analysed from *P. palmata* (Fig. 6) showed the presence of six identified
325 MAAs (i.e. shinorine, palythine, asterina-330, porphyra-334, usujirene and palythene)
326 and two other molecular structures that may correspond to MAAs. Moreover, some
327 unidentified MAAs were present in only one algal species, such as *B. scorpioides*,
328 which contained four unidentified (new) MAAs (i.e. MAA_4, MAA_13, MAA_18 and
329 MAA_23), *G. pistillata* which had MAA_14 and MAA_22, and *V. lanosa* MAA_1
330 (Fig. 5).
331

Based on HPLC chromatograms, shinorine was the predominant MAA identified among all sampled red macroalgae in this study. Indeed, with the exception of *V. lanosa*, shinorine was the only MAA common to all of those species which appeared able to produce MAAs. Shinorine was found to be in sizeable quantities in *M. stellatus*, *G. gracilis*, and *Ceramium nodulosum* with 3.12 ± 0.26, 4.00 ± 0.06 and 1.92 ± 0.11 $mg.g^{-1}$ DW, respectively for species harvested in winter (February). These amounts were approximately halved in July with 1.62 ± 0.07, 1.47 ± 0.10 and 0.86 ± 0.06 $mg.g^{-1}$ DW, respectively for the same algae (t test, p-value = 0.0004). The next most common MAA was palythine which was found in 19 different species.

Moreover, no large modifications in the level of MAAs (Wilcoxon test, p-value = 0.7804) and their composition was observed between both sampling periods in the different species (Fig. 5). However, some changes were noticed such as in *O. pinnatifida* with the presence of asterina-330, usujirene and palythene in those thalli collected in winter (February), but not in those collected in summer (July). The same was observed for *G. pistillata* with the presence of two new MAAs (i.e. MAA_14 and MAA_22) in the winter samples.

In winter and summer, the analysis of the diversity indices for the MAAs highlighted three groups of species (Fig. 7A and Appendix 3A): those with no MAA (Group 1), those with few MAAs (between 1 and 5; Group 2) and those with many MAAs (6 or more; Group 3). Only *B. scorpioides* seemed to stand out and did not correspond to any group, even though it contained eight different MAAs. Shinorine was present in all species of Groups 2 and 3, except in *V. lanosa* which was also the only species having MAA_1. Moreover, asterina-330 and palythine were also two MAAs

found in some species from Group 2, and in all species from Group 3 (with the exception of *V. lanosa* and *B. scorpioides*). Finally, there were some MAAs which were only found in Group 3, notably usujirene and palythene which were present in six of the nine species of the Group 3 in winter. The high diversity in MAAs did not seem to be related to phylogeny (Fig. 7B and Appendix 3B), anatomy and morphology, the height on the shore nor the sampling site (Appendix 1). Hence, species which are close phylogenetically (i.e. *G. vermiculophylla* and *G. gracilis*, or *Callithamnion tetricum* and *C. tetragonum* in our study), morphologically (*M. stellatus* and *C. crispus*), or the only two species harvested from a muddy habitat (i.e. *B. scorpioides* and *G. vermiculophylla*) did not have the same MAAs profile (see Appendix 1 and Fig. 5). No link was also found between the MAAs and the fact that a species was introduced or indigenous to Brittany (data not shown).

4. Discussion

As intertidal macroalgae are organisms directly exposed to solar radiation, they have developed some protective mechanisms in order to survive, such as the synthesis of photo-protective compounds. In this sense, the objective of this study was to collect many different red algal species from a temperate shore in order to have a first overview of the specific distribution of these compounds, and more particularly of mycosporine-like amino acids for which data are not actually available for some species.

Firstly, a high inter-specific variability has been demonstrated in terms of pigment composition and concentration (Figs 1-3). Some species had a high content of chlorophyll-*a* (*P. plumosa* or *Callithamnion* sp.) while others had more carotenoids

(*P. dioica*, *B. scorpioides*, *V. lanosa*), or others had low concentrations of both pigments (*C. crispus* or *G. pistillata*). Three groups were observed from the carotenoids composition, as suggested by Schubert et al. (2006): species rich in lutein, species rich in zeaxanthin, and those with violaxanthin or antheraxanthin (Fig. 2). Lutein was the major carotenoid, as already illustrated (Esteban et al., 2009; Marquardt and Hanelt, 2004).

Furthermore, the quantity of photosynthetic pigment (i.e. chlorophyll-*a* and phycobiliproteins) decreased in the summer samples (Figs. 1, 3) due to an increase in irradiance. Indeed, the increase in light energy availability leads species to reduce their pigment complement to harvest the same quantity of light (Ak and Yücesan, 2012; Ramus et al., 1976). Another explanation would be linked to the nitrogenous nature of chlorophyll-*a* and phycobiliproteins (Huovinen et al., 2006; Lüning, 1990; Parjikolaie et al., 2013). They are used for nitrogen storage in winter and as a nitrogen source when limitations occur in summer (Barufi et al., 2011; Lapointe and Duke, 1984; Surget et al., 2017). Stack et al. (2017) demonstrated that the protein content of *Porphyra dioica* doubled in winter months, perhaps because of phycobiliproteins acting as a nitrogen storage. In general, the impact of N concentration on pigments has already been widely demonstrated in many species such as in *Palmaria palmata* (Corey et al., 2013; Parjikolaei et al., 2013).

The aim of the second part of this study was to elucidate the MAAs composition of 40 red macroalgae. The extraction method in 70% ethanol, coupled with the extract analysis by HPLC, provided reliable data, and had the advantage of being non-toxic,

404 unlike using methanol which is commonly used in the literature (e.g. Briani et al., 2018;
405 Gröniger et al., 2000; Guihéneuf et al., 2018; Pandey et al., 2017; Yuan et al., 2009).
406 The use of a less-toxic solvent such as ethanol allowed for the valorization of extracted
407 MAAs as they have a significant potential for biotechnology applications in for example
408 human health and cosmetics (Chrapusta et al., 2017; Lawrence et al., 2018; Pangestuti
409 et al., 2018). For example, Helioguard 365 (Mibelle group, Switzerland) is a
410 commercial formulation used to protect the skin or hair from UV radiation and based on
411 the MAAs shinorine and porphyra-334 extracted from the red macroalga *Porphyra*
412 *umbilicalis* (La Barre et al., 2014; Schmid et al., 2006).

413 The results of the screening revealed a high diversity in MAA composition in
414 the 40 studied species, including some species for which the MAA composition had not
415 been reported before, e.g. *Plumaria plumosa*, *Bornetia secundiflora*, *Dilsea carnosa*,
416 *Gastroclonium ovatum*, *Hypoglossum hypoglossoides*, *Champia parvula* and
417 *Chylocladia verticillata*. It was already demonstrated that some species of the
418 Rhodophyta have the capacity to accumulate a high degree of diversity and
419 concentration of MAAs (e.g. Rastogi et al., 2010; Sinha et al., 2007), but this present
420 study is the first to report on MAA profiles from as many species of red alga from a
421 temperate region, here from Brittany. Based on their absorption maxima and retention
422 time, 23 potential MAAs were identified by HPLC analyses in the 40 red seaweeds
423 harvested along the Brittany coasts. Shinorine and palythine were found to be the most
424 common MAAs in this study (Fig. 5), which is in agreement with Karentz (2001). The
425 composition in MAAs of each species coincided with the literature, even for poorly
426 studied species such as *Asparagopsis armata* (Figueroa et al., 2008). For example, in
427 accordance with the bibliography, shinorine and porphyra-334 are found in *P. dioica*

(Guihéneuf et al., 2018); shinorine, palythine and porphyra-334 in *C. nodulosum*; or species contained no MAA such as *Phycodrys rubens* and *F. lumbricalis* (Karsten et al., 1998b).

The development of a diversity index for MAAs allowed for the identification of three groups of species (Fig. 7A): i.e. species without MAA (e.g. *Plumaria plumosa*, *H. plumosa*, *M. alata*, *Polyides rotunda* or *F. lumbricalis*), species with a few MAAs (e.g. *Porphyra dioica*, *Gracilaria gracilis*, *C. tetricum*), and some with more than six MAAs (e.g. *Palmaria palmata*, *B. scorpioides*, *Gracilaria vermiculophylla*, *V. lanosa*). These observations suggested a huge diversity of mycosporine-like amino acids among different members of Rhodophyta, with no apparent link with phylogeny, or morphology, position on the shore or sampling site. Similarly to our study, the distribution of various MAAs in other publications is not related to a phylogenetic pattern (Karentz et al., 1991; McClintock and Karentz, 1997), but more to an acclimatization to environmental variations (Briani et al., 2018).

Furthermore, 23 MAAs of which some potentially new and as yet unidentified were observed in the 40 species knowing that approximately 20 MAAs have been reported so far in red algae, suggesting that there may be in this study some MAAs that have never been yet identified. *B. scorpioides* was the species that stood out the most because it had four, unique, as yet un-identified MAAs (i.e. MAA_4, MAA_13, MAA_18 and MAA_23). This species also did not fit into any of the three groups based on the diversity index (Fig. 7A). Only six MAAs were identified by LC-MS in the studied species (i.e. shinorine, palythine, porphyra-334, asterina-330, usurijene, palythene). In addition, we propose that MAA_11 corresponded to palythinol by comparing the results with the literature: indeed, palythinol is a MAA often observed in

452 many red macroalgae in the literature (Sinha et al., 2007) and MAAs_11 is the fifth
453 most common MAA in the red algae studied here. MAA_11 is present for example in
454 *P. palmata*, which should contain palythinol according to the litterature (Gröniger et al.,
455 2000; Karsten and Wiencke, 1999; Yuan et al., 2009). It is difficult to identify MAAs
456 based only on the absorbance spectra and retention times of known MAAs in the
457 bibliography, especially since some MAAs are present in very small quantities which
458 makes it impossible to detect them in LC-MS. The lack of commercial standards makes
459 this study particularly complex, in addition to making impossible the quantification of
460 the different MAAs. In addition, some peaks have similar absorption maxima and
461 retention times. For example, usujirene and palythene only differ by their cis- or trans-
462 conformation and less than one minute separated them (Carreto and Carignan, 2011). In
463 order to identify other MAAs, more biomass for the extractions (if the available
464 biomass allowed it), or the development of a purification protocol to concentrate each
465 MAA would therefore be necessary.

466 Moreover, there was no large seasonal difference in terms of the composition
467 and concentration of MAAs (Fig. 5). This is an unexpected result as the role of photo-
468 protection by MAAs has been widely demonstrated (e.g. Conde et al., 2000; Singh et
469 al., 2008). We expected that the concentration of total MAAs would increase in relation
470 to the intensity of radiation in the marine ecosystem (Karsten et al., 1998a; Torres et al.,
471 2016). However, it is possible that a possible increase in MAAs content occurred
472 between the two sampling periods, February and July, indicating that two sampling
473 periods are not enough to study seasonal variation in MAAs content. This is suggested
474 by Guihéneuf et al. (2018), who found that the concentration of MAAs increased
475 between February and May in *P. palmata* and *C. crispus* collected on Irish shores and

476 not during summer as expected. Another explanation is that, as MAAs are nitrogenous
477 compounds, they are used in summer as a source of nitrogen (Karentz, 2001). In this
478 sense, a number of studies have already demonstrated the positive effect of nitrogen on
479 concentrations of MAAs (Figueroa et al., 2014; Korbee et al., 2005; Peinado et al.,
480 2004).

481 Thus, the variability observed in pigments and MAAs composition suggests that
482 all species do not seem to have the same protective capabilities against solar radiation.
483 In an attempt to obtain a first explanation of this diversity, we tried to relate this
484 composition to phylogeny, or morphology, position on the shore or sampling site, which
485 was not successful. To date, few studies have examined the link between pigment
486 composition and phylogeny, and no clear relationship has been found (Vandervelde,
487 1973; Wang et al., 2018). The MAAs composition would also not be related to a
488 phylogenetic pattern (Karentz et al., 1991; McClintock and Karentz, 1997). Thus there
489 is currently no explanation for the composition (presence/absence) in MAAs between
490 the different species that seems to be random. However, it has been experimentally
491 demonstrated that an increase in UV radiation could have an impact on the levels of
492 MAAs, for example in *Palmaria palmata* or *Chondrus crispus* (Hoyer et al., 2002;
493 Kräbs et al., 2004); or that the MAAs concentration depended on the season (Guihéneuf
494 et al., 2018), nutrient concentrations and pH (Briani et al., 2018), latitude (Karsten et al.
495 1998b) or depth (Franklin et al., 1999; Karsten and Wiencke, 1999). For example, the
496 total MAA concentration in *P. palmata* and *Devalerea ramentaceae* collected at
497 different depths decrease with sampling depth, although the composition remains the
498 same (Hoyer et al., 2001; Karsten et al., 1999).
499

In our study, four species stand out due to their diversified compositions of MAAs: *B. scorpioides*, *G. vermiculophylla*, *V. lanosa* and *P. dioica*, which are the sampled species the most exposed to solar radiation due to their relatively high position on the shore. Indeed, *B. scorpioides* lives at the top of muddy estuaries (Sanchez de Pedro et al., 2014); *G. vermiculophylla* lives at the surface of the intertidal muddy estuaries (Roleda et al., 2012; Surget et al., 2017); *P. dioica* is located on the upper intertidal, and *V. lanosa* is an obligate epiphyte on the brown macroalga *Ascophyllum nodosum* (Garbary et al., 2014) which has a wide range of coverage on intertidal rockyshores at mid-tide level. These four red algal species were characterized by the presence of several MAAs and a high concentration of carotenoids, in particular zeaxanthin, a pigment synthetized under higher light conditions (Rmiki et al., 1996; Schubert and Garcia-Mendoza, 2008). Thus, they stood out from the others on the Principal Component Analysis of pigments (Fig. 4). Another species, *G. gracilis*, also stood out the PCA since it contained antheraxanthin, in accordance with Rmiki et al. (1996) and Schubert et al. (2006). Conversely, *P. plumosa*, with no observed MAA, was the single species with the highest concentrations of chlorophyll *a* and phycoerythrin. This alga lives in shaded areas (Yakovleva et al., 1998), which may explain its necessity to have high concentrations of pigments which absorb light energy, rather than act as photoprotectants.

5. Conclusions

This study highlights the occurrence of numerous MAAs in red macroalgae from Brittany, with variations in the number and level of MAAs per species. Thus, this research contributes to reinforce the few existing databases on the MAAs composition

of red algae as described by Gröniger et al. (2000) and Sinha et al. (2007), knowing that the identification of these molecules is difficult due to the lack of commercial standards. Here we attempted to identify the factors controlling this MAAs diversity: MAAs composition was no related to a phylogenetic pattern; however, the algal species most exposed to light radiation were those with a high level and diversity of photoprotective compounds which could protect their photosynthetic mechanisms against UV-radiation. Nevertheless, it remains complex to provide conclusions about the actual impact of the different biotic and abiotic factors on the synthesis of each MAA. Although MAAs seem to be photo-protective, they may play other and as yet unknown roles in macroalgal metabolism. Subsequently, it is necessary to select few species and carry out macroalgal cultures under controlled conditions in order to understand factors responsible for the production of each MAAs. Thus, the impact of temperature, UV radiation, nutrients or pH could be tested one by one or in combination, allowing to highlight a change in the ratio of MAAs for example. This would result in a better understanding of the synthesis and function of these compounds.

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Authors' contributions

F.L. performed the analysis of the samples collected in winter, drafted the manuscript and conducted the statistical analyses. S.L. performed the analysis of the samples collected in summer and contributed to the writing of the manuscript. G.B. and L.T. performed the LC-MS analyses. V.S-P designed the diversity index, helped in carrying out the phylogenetic analyses and contributed to the interpretation of the results. S.C. designed and supervised the project. All authors reviewed and approved the final manuscript.

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Table 1. HPLC solvent gradient. (A) methanol:ammonium acetate buffer 0.5 M (80:20), (B) acetonitrile:milliQ water (87.5:13.5), (C) ethylacetate 100%.

Time (min)	% A	% B	% C
0	90	10	0
1	0	100	0
11	0	78	22
24	0	25	75
26	0	25	75
27	0	100	0
28	90	10	0
33	90	10	0

Appendix 1. List of the 40 red macroalgal species collected in February and/or July 2017, with their associated GenBank accession numbers (for the genes *rbc*L and cox1), morpho-anatomical group (i.e. MAG; according to Steneck and Dethier, 1994), localisation on the shore, substrate and sampling site. The different MAGs are: (2) filamentous algae; (2.5) corticated or polysiphonous filaments algae; (3) foliose algae; (3.5) corticated foliose algae; (4) corticated algae and (6) articulated calcareous algae.

	Accession numbers					
Species	*rbc*L	cox1	MAG	Localisation	Substrate	Sampling sites
Ahnfeltiopsis devoniensis	KU640212.1	KF641876.1	4	Lower intertidal	Rocky	Portsall
Asparagopsis armata	GQ337068.1	KJ960343.1	2.5	Lower intertidal	Rocky	Portsall
Bonnemaisonia hamifera	FJ195604.1	KJ960353.1	2.5	Lower intertidal	Rocky	Portsall
Bornetia secundiflora	No data		2.5	Upper subtidal	Rocky	Portsall
Bostrychia scorpioides	AY920825.1	MF094019.1	2.5	Upper intertidal	Muddy	Le Faou
Calliblepharis jubata	KC121138.1	KJ960410.1	4	Lower intertidal	Rocky	Portsall
Callithamnion tetragonum	AF439301.1	MF447481.1	2	Lower intertidal	Rocky	Portsall
Callithamnion tetricum	AF439300.1	KJ960434.1	2	Lower intertidal	Rocky	Portsall
Ceramium echionotum	AF439313.1	KJ960509.1	2.5	Lower intertidal	Rocky	Portsall
Ceramium nodulosum	No data		2.5	Lower intertidal	Rocky	Portsall

Ceramium secundatum	KT250273.1	KT250269.1	2.5	Lower intertidal	Rocky	Portsall
Champia parvula	EF613312.1	HQ422864.1	2.5	Lower intertidal	Rocky	Portsall
Chondracanthus acicularis	KJ202090.1	KJ202081.1	4	Lower intertidal	Rocky	Portsall
Chondrus crispus	KF026483.1	GU645233.1	4	Lower intertidal	Rocky	Portsall
Chylocladia verticillata	No data		2.5	Intertidal	Rocky	Portsall
Corallina sp.	No data		6	Lower intertidal	Rocky	Porspoder[a]; St-Pabu[b]
Dilsea carnosa	KT310705.1	KY572820.1	3.5	Upper subtidal	Rocky	Portsall
Furcellaria lumbricalis	No data		4	Intertidal	Rocky	Portsall
Gastroclonium ovatum	KU726714.1	KJ960700.1	2.5	Lower intertidal	Rocky	Portsall
Gelidium corneum	HM629821.1	KJ960706.1	4	Lower intertidal	Rocky	Porspoder
Gigartina pistillata	AY294375.1	KJ960717.1	4	Lower intertidal	Rocky	Porspoder[a]; Portsall[b]
Gracilaria gracilis	AY049400.1	KF714853.1	4	Lower intertidal	Rocky	Portsall
Gracilaria vermiculophylla	JQ768774.1	JQ794759.1	4	Upper intertidal	Muddy	Le Faou
Griffithsia corallinoides	No data		2.5	Lower intertidal	Rocky	Portsall
Heterosiphonia plumosa	AF083379.1	KJ960780.1	2.5	Upper subtidal	Rocky	Portsall[a]; St-Pabu[b]
Hypoglossum hypoglossoides	AF257368.1	KJ179930.1	3.5	Lower intertidal	Rocky	Portsall

Lomentaria articulata	KU726701.1	KU707860.1	2.5	Lower intertidal	Rocky	Portsall
Mastocarpus stellatus	U02992.1	KY572683.1	4	Lower intertidal	Rocky	Portsall
Membranoptera alata	JQ864359.1	KJ960846.1	3.5	Lower intertidal	Rocky	Portsall
Metacallophyllis laciniata	No data		3.5	Upper subtidal	Rocky	Portsall
Osmundea hybrida	AF281878.1	KX258831.1	4	Lower intertidal	Rocky	Portsall
Osmundea pinnatifida	JX828140.1	KU566536.1	4	Lower intertidal	Rocky	Porspoder[a]; Portsall[b]
Palmaria palmata	LN999410.1	KY572816.1	3.5	Lower intertidal	Rocky	Porspoder[a]; Portsall[b]
Phycodrys rubens	JX110932.1	KY572841.1	3.5	Upper subtidal	Rocky	Porspoder
Plocamium cartilagineum	HQ224543.1	JF271583.1	4	Lower intertidal	Rocky	Portsall
Plumaria plumosa	KU381993.1	HQ412551.1	2.5	Lower intertidal	Rocky	Portsall
Polyides rotunda	No data		4	Lower intertidal	Rocky	Portsall
Porphyra dioica	JN703282.1	JN847313.1	3	Upper intertidal	Rocky	Portsall[a]; St-Pabu[b]
Sphaerococcus coronopifolius	AY294376.1	KJ961109.1	4	Upper subtidal	Rocky	St-Pabu
Vertebrata lanosa	MF120849.1	KX344122.1	2.5	Intertidal	Rocky	Portsall

[a]Samples collected in winter; [b]Samples collected in summer

Figure legends

Figure 1. Chlorophyll *a* concentrations (mg.g^{-1} DW) (mean ± standard deviation, n=3) of 40 red macroalgal species, collected from Brittany in February (winter) and July (summer) 2017. Blank spaces indicate that chlorophyll-*a* level was below the limit of detection or quantification.

Figure 2. Carotenoid concentrations (mg.g^{-1} DW) (mean, n=3) in 40 red macroalgal species, collected from Brittany in February (winter) and July (summer) 2017. Blank spaces indicate that carotenoids level was lower than the limit of detection or quantification.

Figure 3. Phycoerythrin concentrations (mg.g^{-1} DW) (mean ± standard deviation, n=3) in 40 red macroalgal species, collected from Brittany in February (winter) and July (summer) 2017. Blank spaces indicate that phycoerythrin level was lower than the limit of quantification.

Figure 4. Principal Component Analysis (PCA) of pigment contents, for 21 species of red macroalgae collected in winter and summer (A: variables; B: samples). Ellipses were drawn around species that had a confidence level of 95% or more. Dimension 1 was principally characterised by chlorophyll *a* level and dimension 2 by zeaxanthin presence and level (correlation coefficient = 0.93 and 0.88, respectively).

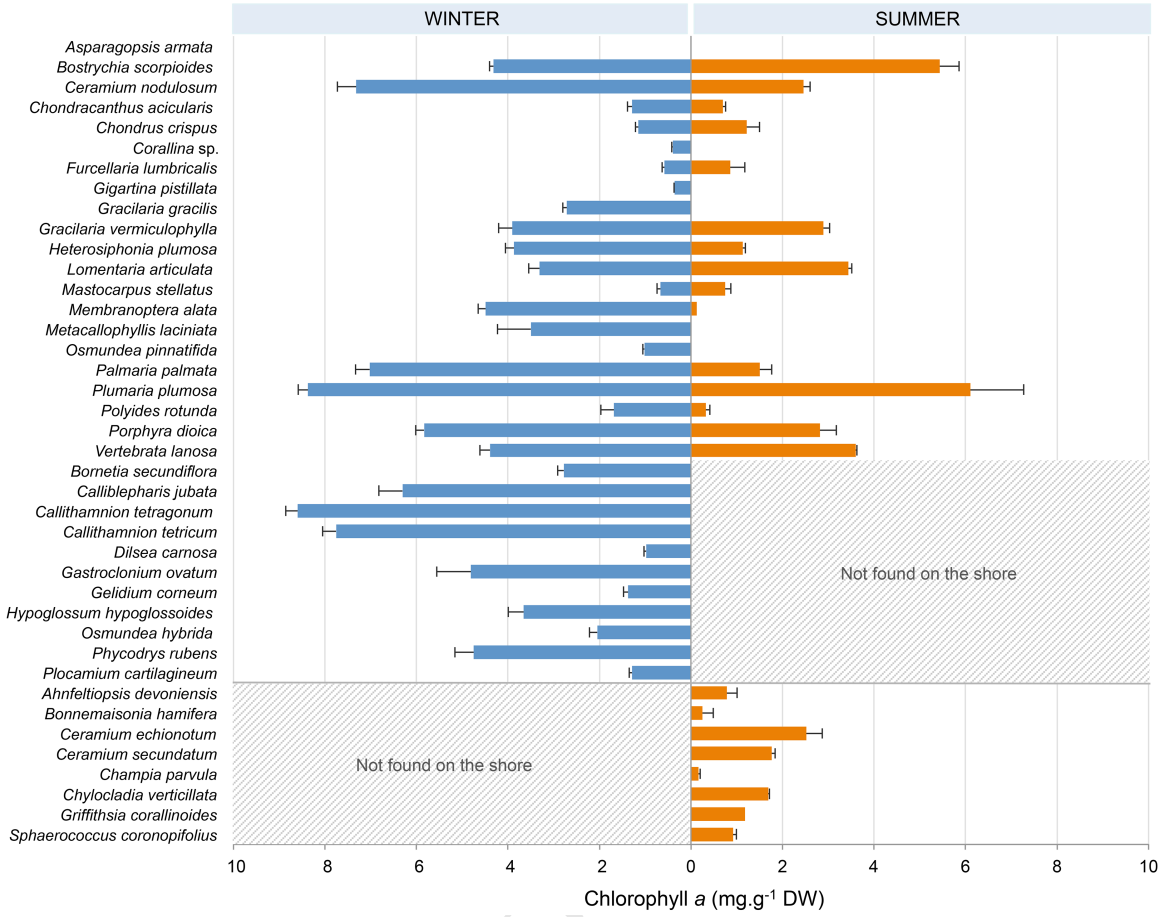
Figure 5. Mycosporine-like amino acids composition (mAU.min) (mean, n=3) in 40 red macroalgal species, collected from Brittany in February (winter) and July (summer) 2017. Blank spaces indicate that MAAs level was lower than the limit of detection or quantification.

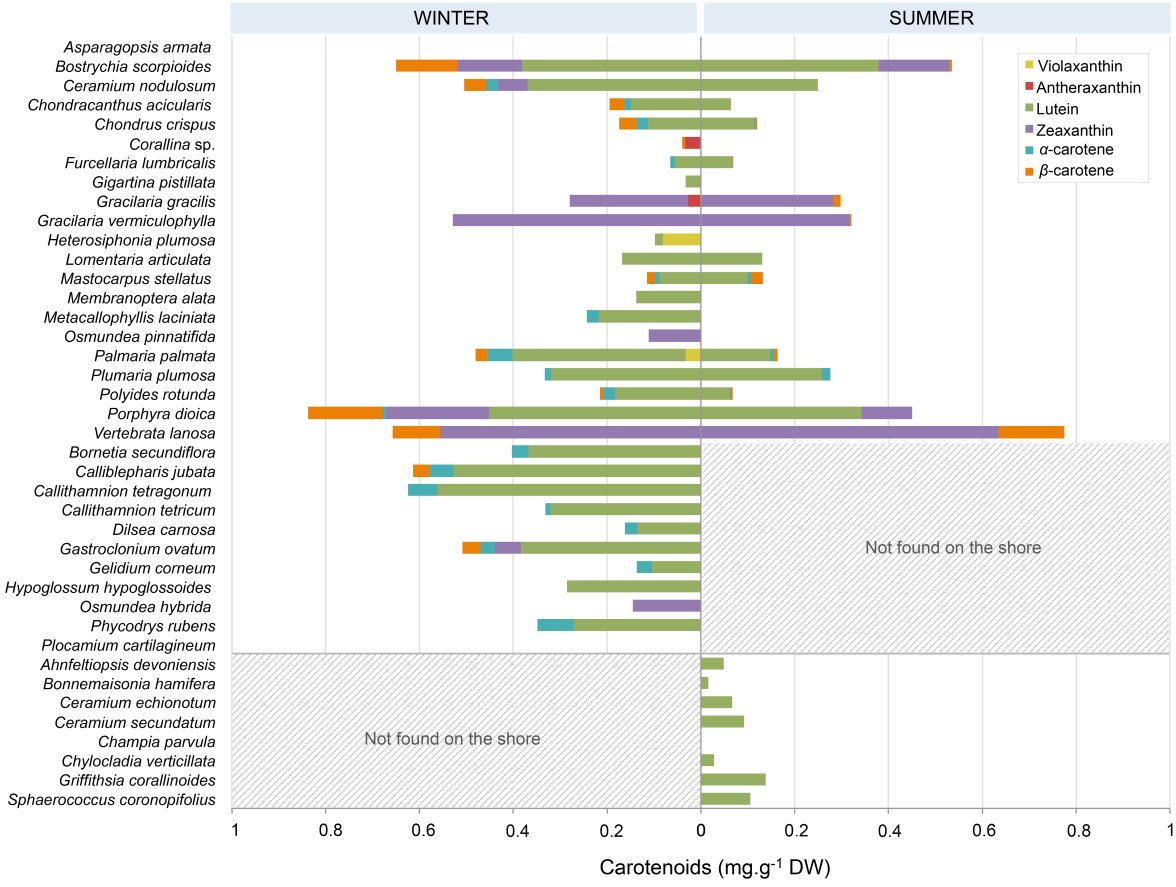
Figure 6. Chromatogram at 330 nm of MAAs extracted from the red macroalga *Palmaria palmata* collected in February 2017 (winter) from Porspoder (Brittany, France).

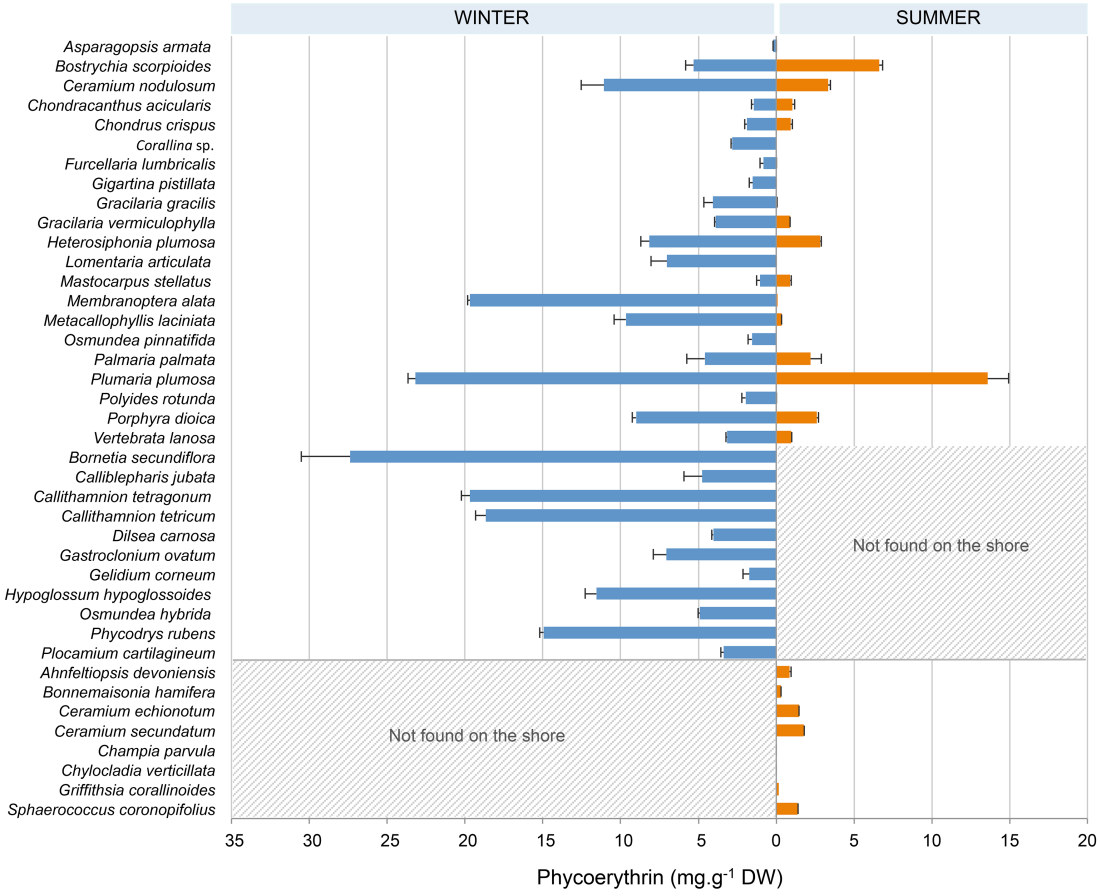
Figure 7. (A) Dendrogram based on the diversity indices of MAAs, obtained from hierarchical cluster analysis (single linkage method); (B) Neighbor-joining phylogenetic tree based on *rbc*L and cox1 genes sequences, for the 32 macroalgal species collected from Brittany during winter. The most relevant boot-strap values are shown next to the branches. Some species are not represented on the phylogenetic tree due to missing sequences on GenBank (see Appendix 1).

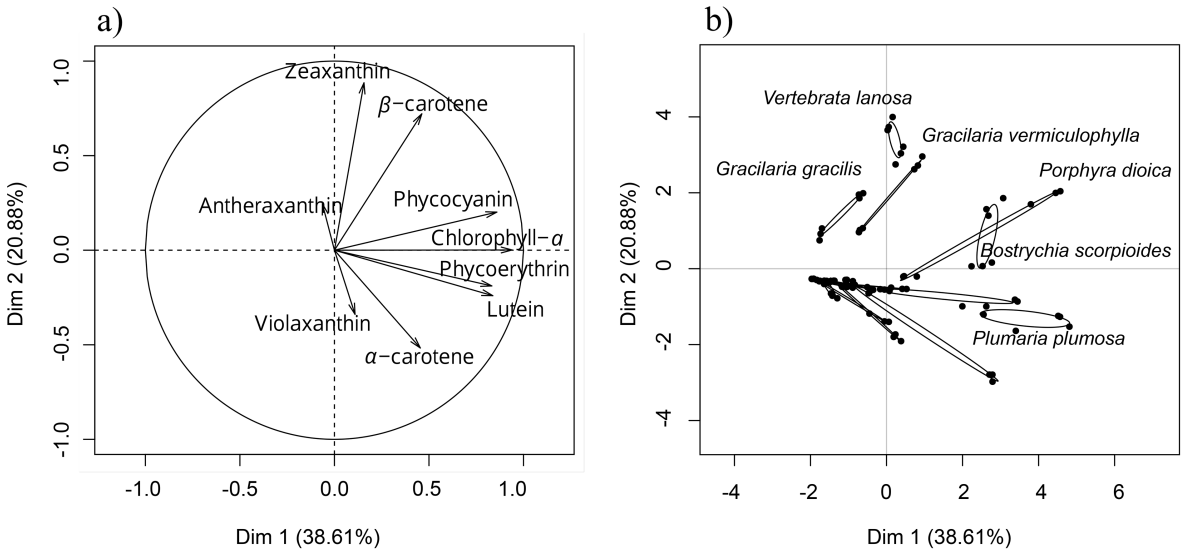
Appendix 2. List of the 23 different peaks analysed in 40 red macroalgae species, that may correspond to MAAs, with their absorption spectra, λ_{max} and their retention time (Rt).

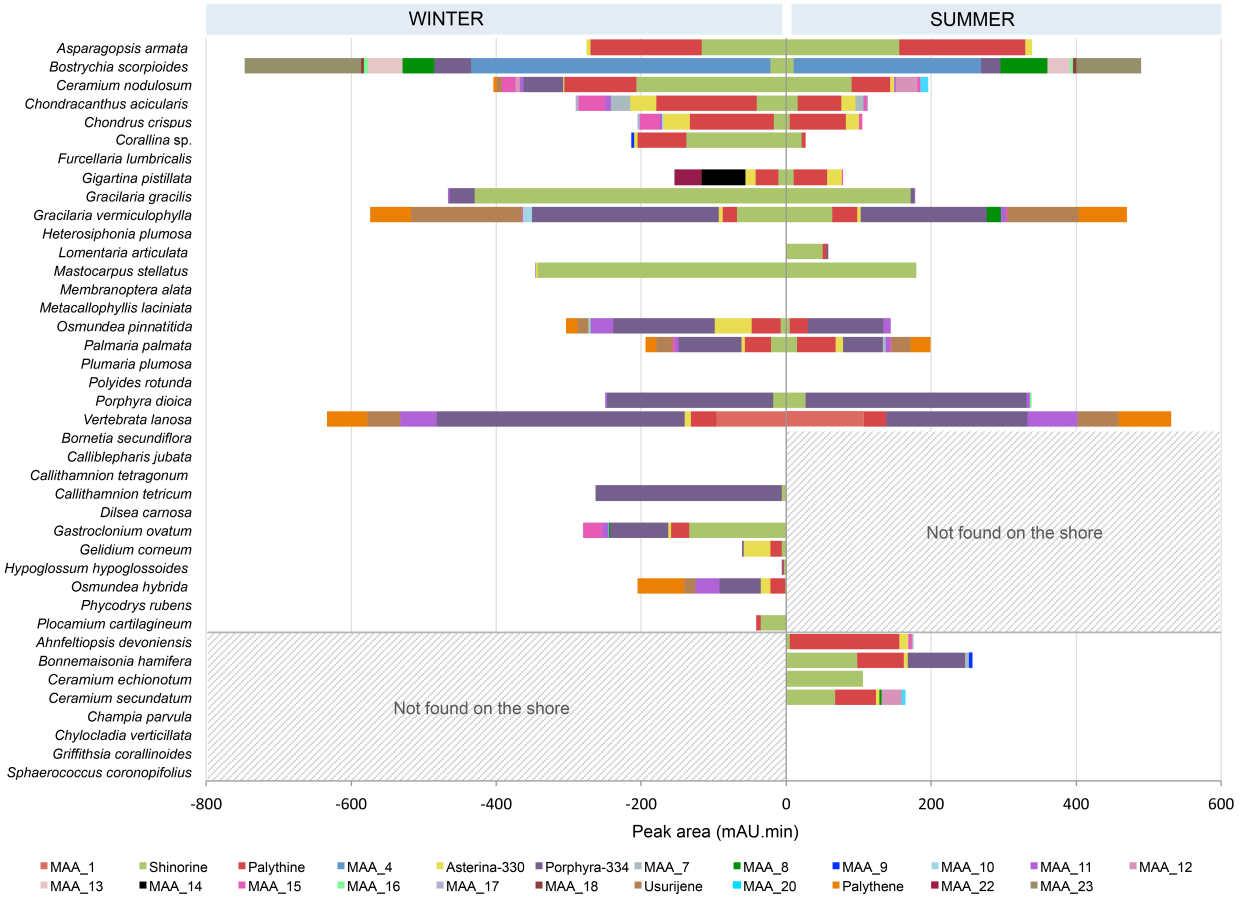
Appendix 3. (A) Dendrogram based on the diversity indices of MAAs, obtained from hierarchical cluster analysis (single linkage method); (B) Neighbor-joining phylogenetic tree based on *rbc*L and cox1 genes sequences, for the 29 macroalgal species collected in summer. The most relevant boot-strap values are shown next to the branches. Some species are not represented on the phylogenetic tree due to missing sequences on GenBank (see Appendix 1).

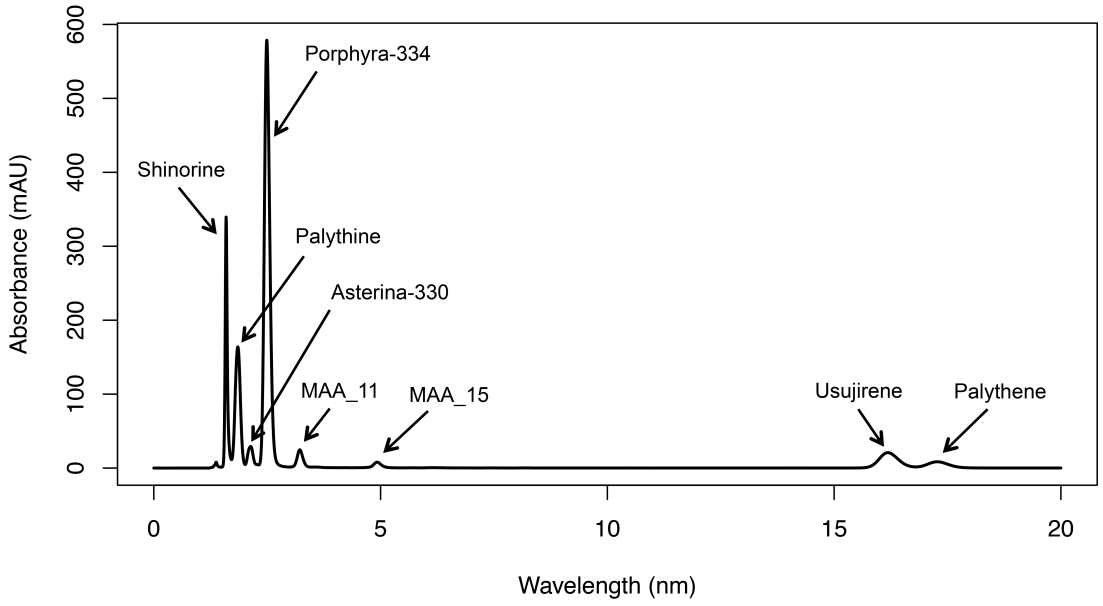




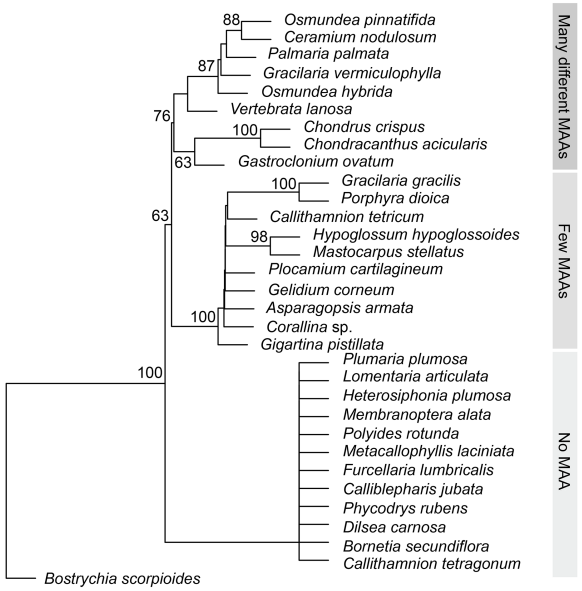








a)



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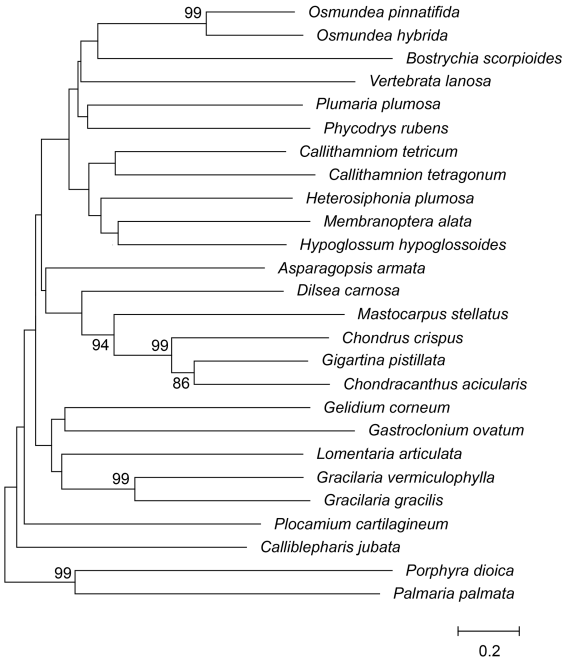

Highlights

- Detection of 23 potential MAAs in 40 Rhodophyta species, including 6 already identified (shinorine, palythine, asterina-330, porphyra-334, usurijene, palythene)
- First report on the MAAs composition for some red seaweeds
- High variability in MAAs content and composition between the different species, with no link with phylogeny, morphology, position on the shore or sampling site
- A MAAs extraction method using 70% ethanol being less toxic than conventional methanol, and giving potential valorisable extracts