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Seasonal plasticity of the polar lipidome of *Ulva rigida* cultivated in a sustainable integrated multi-trophic aquaculture

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ABSTRACT

Green macroalgae of the genus *Ulva*, commonly known as sea lettuces, are currently traded worldwide for multiple applications, including human consumption. In this work, we evaluated the seasonal variation of the total content of lipids, esterified fatty acids (FAs) and polar lipids (the major carriers of FAs) at the level of the classes and molecular species of *Ulva rigida* cultivated in Portugal in a land-based integrated multitrophic aquaculture (IMTA) system. Comparing winter, spring, summer and autumn samples, the lipid content and abundance of unsaturated FAs, namely *n*-3 polyunsaturated FAs, were highest in winter and lowest in summer. However, regardless of seasonal variations, *U. rigida* had a low *n*-6/*n*-3 ratio in all seasons, which is associated with health benefits. Among the polar lipids, those that varied the most were betaines, glycolipids and phospholipids, including a high number of lysolipid species that increased during autumn and spring. Multivariate analysis using principal component analysis (PCA) showed better discrimination of the four seasons when the dataset on molecular lipid species was used. Knowledge of the seasonal variability of lipid composition in biomass of *Ulva rigida* can be used to better explore these green macroalgae as a source of bioactive polar lipids with high market value.

1. Introduction

Algae lipids are of increasing interest, not only for their nutritional value, but also as reservoir of bioactive compounds with multiple applications, notably in the pharmaceutical, cosmetic and functional food industries [1–3]. However, the study of these bioactive molecules is a challenge, not only because of their very large chemovariety, but also due to their quantitative plasticity in response to development processes and, in particular, to abiotic factors [2–4].

Seasonal variations translate into changes in environmental factors (*e.g.* temperature, light and salinity) which are known to modulate the plasticity of lipidomes, including for macroalgae [5–8]. The main lipid components of macroalgae are polar lipids, including glycolipids, betaine lipids, and phospholipids [9,10]. They have important functions as membrane building blocks and signalling molecules [10–12]. Plastid (chloroplast) membranes are mainly composed of glycolipids, and extraplastidial cell membranes are mainly composed of phospholipids and betaine lipids [9]. There is an exception for phosphatidylglycerol (PG), a phospholipid with a crucial role in photosynthesis, which is present in large quantities in the plastidial thylakoid membranes [9]. Polar lipids are natural carriers of FAs, including polyunsaturated (PUFAs), with high nutritional value [2].

Nowadays, knowledge about the polar lipidomes of algae goes beyond the simple screening of their FA composition. Mass spectrometry (MS)-based lipidomics is the most modern and promising methodology to unravel polar lipid profiles and describing the quantitative and qualitative changes occurring in response to environmental variations [2]. In particular, liquid chromatography (LC) coupled with MS has already been used to successfully reveal the polar lipidome of several macroalgae [13–15]. However, to our knowledge, only one study on

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brown macroalgae *Fucus vesiculosus* has dealt with the seasonal plasticity of the polar lipidome of macroalgae at the level of molecular species [16].

The possibility of predicting the plasticity of polar algae lipidomes in response to environmental variations is essential to fully exploit the nutritional value of algal polar lipids and their biotechnological applications, in particular those which improve human and animal health [17]. Polar lipids have been associated with several biological activities with potential health benefits, namely antimicrobial, anti-inflammatory and anti-tumour effects [18–25]. In addition, the bioactivity of polar lipids has been shown to depend on both the fatty acyl composition and the polar head [25], which confirms the importance of their structural characterization at the molecular level.

The green macroalgae of the genus *Ulva*, commonly known as sea lettuces, are a group of edible algae widely distributed along the coasts of the world oceans. The seasonal variation of the total lipid content, of the lipid classes and of the fatty acid composition, either in total extract or in lipid classes obtained by thin layer chromatography, was studied for *Ulva* spp. samples collected from the wild [7,26–30]. Regardless of taxonomic and geographic differences, a common trend for higher and lower lipid content and unsaturated fatty acids (UFAs) levels has been reported for seasonal variations at low and high temperature, respectively [7,26–28]. In agreement, a laboratory study has showed that *Ulva pertusa* increased its total lipid and PUFAs content in response to the low temperature. However, seasonal variations in its total lipid and PUFA content may not be explained solely by variations in temperature. High salinity also led to an increase in the total lipid content of *U. pertusa* [31], while high light increased its saturated fatty acid (SFA) 16:0 [31], as has also been observed for *U. fenestrata* [32]. Moreover, the lipid composition may be modulated by changes in nutrients [33,34], namely a decrease in the proportion of the major PUFAs 16:4*n*-3 and 18:4*n*-3 was observed in *U. pertusa* by increasing nitrogen level [34].

Despite the evidence of temporal variability in *Ulva* spp. lipid composition, in particular associated with temperature variations, no study to date has simultaneously compared all seasons and analysed in detail the molecular species present in each class of lipids. Such a study would provide a more complete understanding, both qualitative and quantitative, of the seasonal variability of the lipid classes and of the species displayed by *Ulva* and would therefore be of major interest for biotechnological lipid-based applications targeting these macroalgae.

In this study, the polar lipidome of *Ulva rigida*, cultivated in Portugal in a land-based integrated multitrophic aquaculture (IMTA), was characterized using two complementary approaches based on MS: gas chromatography–mass spectrometry (GC–MS) to study the profile of total esterified FAs, and high-resolution hydrophilic interaction liquid chromatography–mass spectrometry and tandem mass spectrometry (HILIC–MS and MS/MS) to study the profile of polar lipids, both at the level of lipid classes and molecular species. Thalli of *U. rigida* were collected during winter, spring, summer and autumn and analysed, allowing to fully characterise the response of the polar lipidome to seasonal variations. The evaluation of seasonal variations was first carried out by a multivariate analysis using principal component analysis (PCA) to visualize the general clustering trends of the samples. A univariate analysis (Kruskal–Wallis followed by a Dunn post-hoc analysis) was used to test for significant differences between the seasons.

2. Materials and methods

2.1. Biomass

Dry samples of *Ulva rigida* were supplied by ALGAplus (production site in Ria de Aveiro coastal lagoon, mainland Portugal, 40° 36′ 43″ N, 8° 40′ 43″ W). ALGAplus operates an IMTA system which integrates the semi-intensive aquaculture of seabream (1/3) and seabass (2/3) on earthen ponds with seaweed production on a land-based outdoor tank system. Seaweeds are cultivated exclusively using nutrient rich effluent water that flows from the fish production ponds to the seaweed tanks. After the seaweed tanks, the water is discharged into a settling basin connected to the coastal lagoon, with a significantly lower load of nutrients.

Using the same cultivation conditions of stocking density and flow rate, *U. rigida* was grown by clonal propagation methods in summer (August 2016), autumn (November 2016), winter (March 2017), and spring (May 2017). During the culture period in the seaweed tanks (two weeks), average water temperature (°C) and salinity (PSU) was 21.9 ± 0.8 and 40.5 ± 0.3 in the summer, 15.1 ± 2.3 and 36.5 ± 0.9 in autumn, 12.5 ± 0.2 and 30.1 ± 0.4 in the winter, and 17.5 ± 1.5 and 31.1 ± 1.8 in spring. According to ALGAplus standard harvesting and processing procedures, *U. rigida* samples were collected, cleaned to remove epiphytes, and dried at 25 °C to obtain a moisture content below 12% (w/w). Before lipid extraction, dried samples were milled, and residual moisture was determined by drying milled samples at 105 °C for 15 h [13].

2.2. Lipid extraction

Lipids were extracted using a modified Bligh and Dyer protocol [13,35]. Samples of 250 mg (five replicates per season) were mixed with MeOH (2.5 mL) and $CHCl_3$ (1.25 mL) in a glass tube and then homogenized by vortexing for 2 min and sonication for 1 min. After incubation in ice on a rocking platform shaker for 2.5 h, the mixture was centrifuged for 10 min at 626 ×*g*. The organic phase was collected in a new glass tube and washed with ultrapure water (2.3 mL), followed by centrifugation for 10 min at 626 ×*g*. The biomass was re-extracted twice with MeOH (2 mL) and $CHCl_3$ (1 mL). The final organic phase (lipid extract) was dried under nitrogen stream, transferred to amber vials, dried again, weighed, and stored at −20 °C. Lipid content was estimated as dry weight percentage.

2.3. Fatty acid analysis by GC–MS

Fatty acids (FAs) were analysed by gas chromatography–mass spectrometry (GC–MS) after transmethylation [36]. For that, 1 mL of internal standard used for quality control (0.75 μg mL^{-1} of methyl nonadecanoate in n-hexane) was added to 30 μg of dried lipid extract, followed by 200 μL of a methanolic solution of potassium hydroxide (2 M). After 2 min vortexing, 2 mL of an aqueous solution of sodium chloride (10 mg mL^{-1}) were added and the sample was centrifuged for 5 min at 626 ×*g*. The upper organic phase containing FA methyl esters (FAMEs) was transferred to a microtube and completely dried under nitrogen. FAMEs were then redissolved in 60 μL n-hexane and 2 μL were used for GC–MS analysis on an Agilent Technologies 6890 N Network chromatograph (Santa Clara, CA, USA) equipped with a DB-FFAP column with 30 m length, an internal diameter of 0.32 mm and a film thickness of 0.25 μm (J&W Scientific, Folsom, CA, USA). The GC equipment was connected to an Agilent 5973 Network Mass Selective Detector operating with an electron impact mode at 70 eV and scanning the mass range *m/z* 50–550 in a 1 s cycle in a full scan mode acquisition. The oven temperature was set at an initial temperature of 80 °C for 3 min, followed by successive linear increases to 160 °C at 25 °C min^{-1}, then to 210 °C at 2 °C min^{-1} and finally to 250 °C at 30 °C min^{-1}. Following these ramps, temperature was maintained at 250 °C for 10 min. The injector and detector temperatures were 220 and 280 °C, respectively. Helium was used as carrier gas at a flow rate of 1.4 mL min^{-1}. FAME identification was performed by comparing retention times and mass spectra with those of commercial FAME standards (Supelco 37 Component FAME Mix, ref. 47885-U, Sigma-Aldrich, Darmstadt, Germany) and confirmed by comparison with spectral libraries from Wiley and "The Lipid Web" [37].

The double bond index was calculated as DBI = Σ (relative percentage of each fatty acid × N) / 100, where N corresponded to the

number of double bonds [38,39]. The average chain length was calculated as ACL = Σ (relative percentage of each fatty acid × C) / 100, where C corresponded to the total number of carbon atoms [39].

2.4. Polar lipid analysis by HILIC−MS and MS/MS

Lipid extracts were analysed by hydrophilic interaction liquid chromatography−mass spectrometry (HILIC−MS) using a high-performance liquid chromatograph Ultimate 3000 Dionex (Thermo Fisher Scientific, Bremen, Germany) with an autosampler and coupled online to the Q-Exactive® hybrid quadrupole Orbitrap® mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Mobile phase A consisted of 50% acetonitrile, 25% methanol and 25% water (per volume) with 1 mM ammonium acetate. Mobile phase B consisted of 60% acetonitrile and 40% methanol (per volume) with 1 mM ammonium acetate. Initially, 40% of mobile phase A was held isocratically for 8 min, followed by a linear increase to 60% of A within 7 min and again held isocratically for 5 min, returning to the initial conditions in 5 min and equilibrating during 10 min. A volume of 5 μL of each sample containing 5 μg of lipid extract, 4 μL of a mixture of phospholipid standards (dimyristoyl phosphatidylcholine - 0.02 μg, dimyristoyl phosphatidylethanolamine - 0.02 μg, sphingomyelin (17:0) - 0.02 μg, lysophosphatidylcholine (19:0) - 0.02 μg, tetramyristoyl cardiolipin - 0.08 μg, dipalmitoyl phosphatidylinositol - 0.08 μg, dimyristoyl phosphatidylglycerol - 0.012 μg, dimyristoyl phosphatidic acid - 0.08 μg, dimyristoyl phosphatidylserine - 0.04 μg, and ceramide (17:0/d18:1) - 0.02 μg) and 91 μL of starting eluent mixture (40% eluent A and 60% eluent B, final volume 100 μL) was introduced into the Ascentis® Si column (150 mm × 1 mm, 3 μm, Sigma-Aldrich) with a flow rate of 40 μL min^{-1} and at 30 °C. The mass spectrometer was operated simultaneously in positive (3.0 kV) and negative (−2.7 kV) modes with capillary temperature 250 °C, sheath gas flow 15 U, and auxiliary gas flow 5 U (arbitrary units). Acquisition cycles consisted of one full scan mass spectrum at high resolution (resolution of 70,000 and AGC target of 1×10^6) and ten data-dependent MS/MS scans (resolution of 17,500 and AGC target of 1×10^5) that were repeated continuously throughout the experiments with the dynamic exclusion of 60 s and intensity threshold of 1×10^4. Normalized collision energy ranged between 25, 30, and 35 eV. Data acquisition was carried out using the Xcalibur data system (V3.3, Thermo Fisher Scientific, Waltham, MA, USA).

Peak integration and assignments of HILIC–MS data were performed using MZmine 2.32. The software was used for filtering and smoothing, peak detection, peak processing, and assignment against an in-house database. During the processing of full MS raw data, all the peaks with intensity lower than 1×10^4 were excluded. For all assignments, peaks within 5 ppm of the lipid exact mass were considered. Each identification was further validated by manual analysis of the MS/MS data. Analysis of the MS/MS spectra acquired in positive ion mode was performed to confirm the identity of the molecular species belonging to the monogalactosylmonoacylglyceride (MGMG), digalactosylmonoacylglyceride (DGMG), monogalactosyldiacylglyceride (MGDG) and digalactosyldiacylglyceride (DGDG) classes, occurring as $[M + NH_4]^+$ ions, and diacylglyceryl-*N*,*N*,*N*-trimethyl homoserine (DGTS), monoacylglyceryl-*N*,*N*,*N*-trimethyl homoserine (MGTS), phosphatidylcholine (PC), lysophosphatidylcholine (LPC) and phosphatidylethanolamine (PE) classes, occurring as $[M + H]^+$ ions. The MS/MS spectra acquired in negative ion mode were used to confirm the identity of the molecular species belonging to the sulfoquinovosylmonoacylglyceride (SQMG), sulfoquinovosyldiacylglyceride (SQDG), phosphatidylinositol (PI), lysophosphatidylinositol (LPI), phosphatidylglycerol (PG), PE, and lysophosphatidylethanolamine (LPE) classes, occurring as $[M–H]^-$ ions. Negative ion mode MS/MS data were also used to identify the fatty acid carboxylate anions. All the MS/MS fragmentation patterns characteristic for the lipid classes analysed in the present study, acquired in positive and negative ion modes, were previously described [13,16]. Relative quantitation was performed by exporting integrated peak areas values into a computer spreadsheet (Excel, Microsoft, Redmond, WA). For normalization of the data, the peak areas of the extracted ion chromatograms of each lipid species within each class (listed in Supplementary Tables S1–S3) were divided for the peak area of the internal standard selected for the class. The resulting data matrix with normalized areas of all species was used to study the seasonal variation in polar lipids of *U. rigida* at molecular level, as well as calculate relative abundances for each class, obtained by dividing the normalized peak areas of each molecular species by the sum of total peak areas. Also, from this matrix was constructed a new matrix used to study seasonal variation of polar lipids at class level, calculated using the sum of normalized areas of all molecular species within each class.

2.5. Statistical analysis

Multivariate and univariate analyses were performed using R version 3.5 [40] in Rstudio version 1.1.4 [41]. GC–MS data (fatty acids) and HILIC–MS data (classes and species) were glog transformed and auto scaled using the R package Metaboanalyst [42]. Principal component analysis (PCA) was conducted with the R built-in function and ellipses were drawn using the R package ellipse [43], assuming a multivariate normal distribution and a level of 0.95. Kruskal-Wallis test followed by Dunn's post-hoc comparisons were performed with the R built-in function. P-values were corrected for multiple testing using Benjamin-Hochberg method (q values) [44]. Heatmaps were created using the R package pheatmap [45] using “Euclidean” as clustering distance, and “ward.D” as the clustering method. All graphics and boxplots were created using the R packages ggplot2 [46], plyr [47], dplyr [48], tidyr [49] and ggrepel [50].

3. Results

The lipid contents (expressed as a percentage of the dry weight of the biomass, %DW) obtained from *U. rigida* cultivated in different seasons were 3.4 ± 0.3 in winter (March), 2.2 ± 0.2 in spring (May), 1.7 ± 0.2 in summer (August), and 2.7 ± 0.2 in autumn (November) (Fig. S1).

The lipid content decreased, while the temperature increased seasonally, with the biomass of *U. rigida* containing twice as much lipid in winter than in summer ($q < 0.001$). Other significant variations included the 1.5-fold decrease ($q < 0.05$) observed between winter and spring, as well as the 1.6-fold increase observed between summer and autumn ($q < 0.05$) (Fig. S1).

3.1. Seasonal variation in the fatty acid profile

In this study, a total of 15 fatty acids (FAs) were quantified (Table 1). Each FA was present in all the samples analysed, but large seasonal variations were observed. Of the 15 FAs identified, the saturated FA (SFA) 16:0 was predominant in all seasons (26.84 ± 3.18% in winter, 29.61 ± 3.56 in spring, 43.80 ± 3.85 in summer and 30.77 ± 2.37% in autumn), followed by polyunsaturated FA (PUFA) 18:4*n*-3 in winter (20.23 ± 2.29%), in spring (17.15 ± 2.04%) and in autumn (13.83 ± 1.93%), while SFA 18:0 was the second most abundant in the FA summer profile (12.85 ± 4.20%). On a first analysis of the overall seasonal variations, it should be noted that there was an average decrease of 2.3-times in ΣUFA/ΣSFA between winter and summer, leading to a decrease of 1.9 times the double bond index (DBI) and 3.3 increase of *n*-6/*n*-3 ratio. An average chain length (ACL) of 17 was maintained throughout the year.

To visualize the grouping of the samples with season, we performed a PCA using the FA dataset (Fig. 1a and Table S4). The eigenvalues of the two first principal components (PCs) represented 68.7% of the observations (PC 1 43.2% and PC 2 25.5%) and the four groups were well differentiated in the PCA score plot with samples grouped by season.

Table 1
Fatty acid (FA) profile of samples of *Ulva rigida* cultivated in winter, spring, summer and autumn, determined by GC–MS analysis of fatty acid methyl ester derivatives. The values presented are means (% total FA) ± standard deviations for five replicates. ACL, acyl chain length; DBI, double bond index; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; UFA, unsaturated fatty acid.

	Seasons			
Fatty acids	Winter	Spring	Summer	Autumn
14:0	0.87 ± 0.12	1.10 ± 0.11	0.78 ± 0.11	3.46 ± 0.61
16:0	26.84 ± 3.18	29.61 ± 3.56	43.80 ± 3.85	30.77 ± 2.37
16:1*n*-7	0.82 ± 0.13	1.98 ± 0.28	1.91 ± 0.28	3.15 ± 0.47
16:1*n*-9	2.74 ± 0.36	2.26 ± 0.22	3.22 ± 0.25	2.16 ± 0.33
16:4*n*-3	14.02 ± 1.49	12.42 ± 1.62	6.27 ± 0.72	10.56 ± 1.41
18:0	9.97 ± 4.40	8.69 ± 4.33	12.85 ± 4.20	12.15 ± 4.52
18:1	7.17 ± 0.98	9.17 ± 1.29	10.60 ± 1.97	8.45 ± 0.98
18:2*n*-6	1.55 ± 0.21	1.96 ± 0.25	2.09 ± 0.34	2.35 ± 0.80
18:3*n*-6	0.17 ± 0.04	0.18 ± 0.05	0.19 ± 0.05	0.32 ± 0.05
18:3*n*-3	10.39 ± 1.33	9.58 ± 1.26	8.29 ± 1.43	7.87 ± 0.95
18:4*n*-3	20.23 ± 2.29	17.15 ± 2.04	7.85 ± 1.34	13.83 ± 1.93
20:4*n*-3	0.81 ± 0.15	0.58 ± 0.12	0.25 ± 0.08	0.62 ± 0.09
20:5*n*-3	0.80 ± 0.16	1.49 ± 0.29	0.37 ± 0.11	2.10 ± 0.28
22:0	0.67 ± 0.20	0.92 ± 0.27	0.77 ± 0.41	0.63 ± 0.14
22:5*n*-3	2.98 ± 0.60	2.90 ± 0.57	0.76 ± 0.28	1.59 ± 0.23
Σ SFA	38.34 ± 7.22	40.32 ± 7.57	58.20 ± 6.56	47.01 ± 6.17
Σ MUFA	10.72 ± 1.47	13.41 ± 1.75	15.72 ± 2.40	13.75 ± 1.66
Σ PUFA	50.94 ± 5.75	46.27 ± 5.84	26.08 ± 4.17	39.24 ± 4.58
Σ UFA/ΣSFA	1.68	1.55	0.74	1.16
Σ *n*-6/Σ*n*-3	0.03	0.05	0.10	0.07
ACL	17.26	17.23	16.94	17.07
DBI	2.05	1.89	1.09	1.62

The grouping of the summer and spring, even if largely separated, remained close, while the winter and autumn groups were clearly differentiated along PC 2.

A univariate analysis (Kruskal-Wallis followed by a Dunn's post-hoc analysis and FDR-adjusted *P* values) was used to test for significant differences between the seasons. FAs were sorted using the *q* values from Kruskal-Wallis H test and 12 of them showed significant differences between seasons (Fig. 1b). Of these, the first 7 FAs, with $q < 0.01$, included PUFAs 20:5*n*-3, 18:4*n*-3, 22:5*n*-3, 16:4*n*-3 and 20:4*n*-3, which decreased significantly in summer compared to winter or spring. The top 7 FAs also included MUFA 16:1*n*-7 and SFA 14:0, both of which increased significantly in autumn when compared to the winter.

3.2. Seasonal variation in the polar lipid profile

The polar lipidome of *U. rigida* from each of the four seasons was evaluated in order to study its acclimation to different abiotic conditions induced by seasonality. A lipidomic approach based on high resolution LC–MS, mass accuracy and by the interpretation of LC–MS/MS data, as detailed previously [13,16], made it possible to identify 150 lipid species of distinct molecular weight that have been semi-quantified in the lipidome of samples of *Ulva rigida* from the four seasons. These species were distributed over 16 classes of glycerolipids, which were grouped by glycolipids, phospholipids, and betaine lipids (Fig. S2). For each class, a variable number of species has been identified, differing by the total number of carbon atoms and double bonds in the fatty acyl chain(s), and thus presenting a distinct molecular weight. In some cases, different combinations of fatty acyl chains have been found for species of the same molecular weight (Tables S1–S3).

The glycolipids included 59 lipid species divided into 6 classes, 4 of neutral galactolipids and 2 of acidic sulfolipids. The galactolipid classes included monogalactosyldiacylglyceride (MGDG, 9 species) and digalactosyldiacylglyceride (DGDG, 13 species), as well as their respective lyso forms, monogalactosylmonoacylglyceride (MGMG, 6 species) and digalactosylmonoacylglyceride (DGMG, 6 species). The sulfolipids included sulfoquinovosyldiacylglyceride (SQDG, 20 species) and its lyso form, sulfoquinovosylmonoacylglyceride (SQMG, 5 species) (Table S1).

The phospholipids included 43 lipid species divided into 8 classes as follows: phosphatidylcholine (PC, 9 species), phosphatidylethanolamine (PE, 3 species), phosphatidylinositol (PI, 3 species), phosphatidylglycerol (PG, 10 species), and their respective lyso forms, lyso-PC (LPC, 9 species), lyso-PE (LPE, 4 species), lyso-PI (LPI, 1 species), and lyso-PG (LPG, 4 species) (Table S2).

Betaine lipids included 48 lipid species divided into 2 classes, diacylglyceryl-*N*,*N*,*N*-trimethyl homoserine (DGTS, 33 species) and its lyso form, monoacylglyceryl-*N*,*N*,*N*-trimethyl homoserine (MGTS, 15 species) (Table S3).

The same lipid species, with the same fatty acyl composition, were identified for the four seasons, but with significant differences in relative abundance (Figs. S3–S6). The fatty acyl chains associated with each species mainly consists of 16 and 18 carbon chains with variable degree of unsaturation (up to 4 double bonds), which corresponds to the FA profiles obtained from total extracts (Table 1).

3.2.1. Seasonal variation of polar lipids at class level

To analyse the variation of lipid classes with the season, a data matrix was constructed for lipid classes (by the sum of lipid species). The PCA analysis showed that the samples were well grouped by season in a two-dimensional score plot with the eigenvalues describing 67.6% of the total variance, including PC 1 (41.6%) and PC 2 (26.0%) (Fig. 2a). Two groups were clearly separated along PC 1, the main discriminating component, and included summer and autumn located at positive values of PC 1, and winter and spring located at negative values of PC 1.

We have sorted the lipid classes by the contribution for the variability in the PC 1 (Fig. 2b). The most relevant contributors to PC 1 were the classes DGTS (12.6%), LPE (12.3%), PG (12.2%), PE (11.1%) and DGDG (8.8%) (Table S5). These classes were increased in winter and spring. In terms of univariate analysis, the *q*-values of the Kruskal-Wallis H test showed significant differences for all classes, except for the SQDG class. The most significant variations ($q = 0.003$) were observed for the betaine lipid class DGTS, the phospholipid class PE, the glycolipid class MGDG, and the lysolipid forms of different classes (MGTS, LPE and LPC).

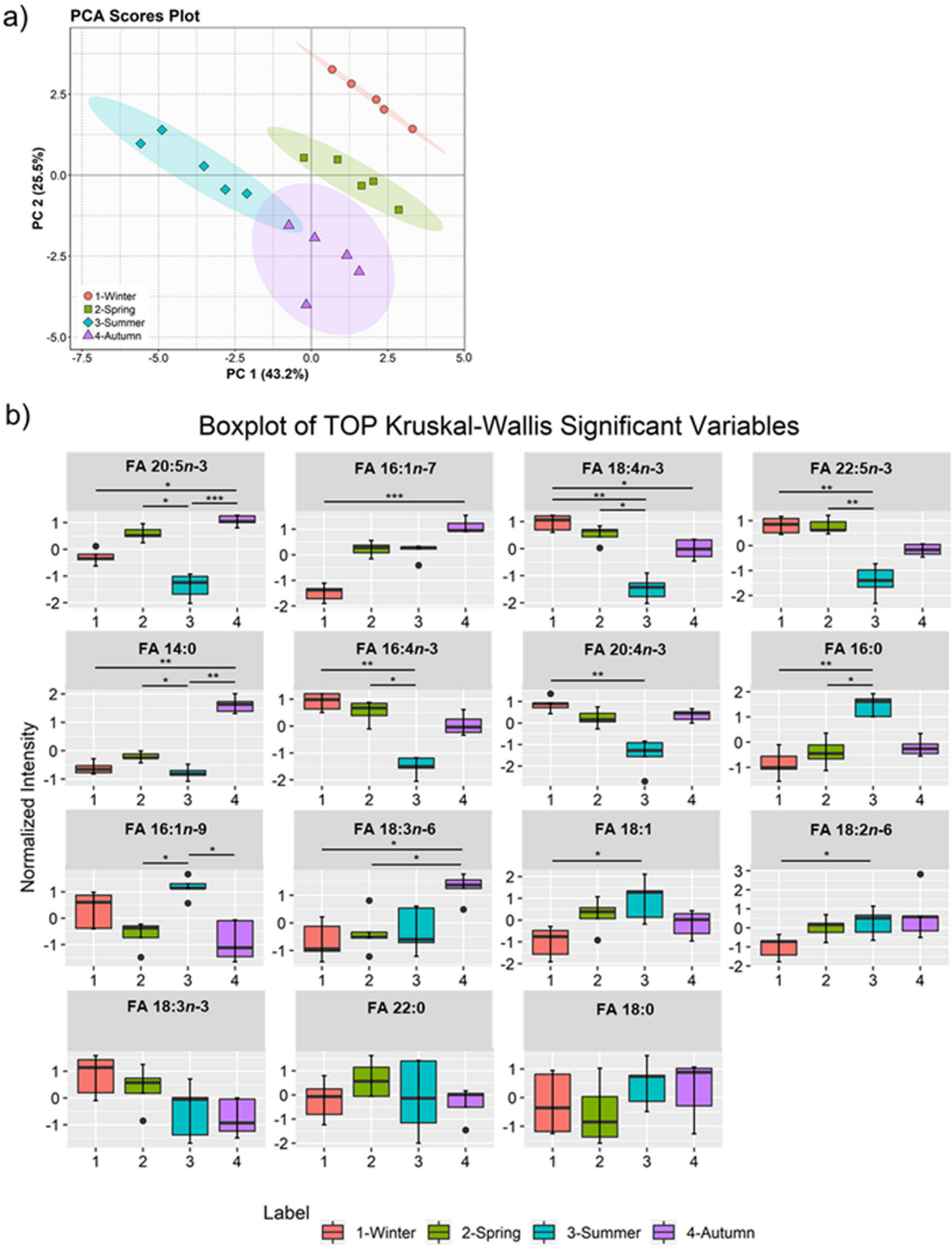

Fig. 1. PCA scores plot of two first principal components of the fatty acids (FAs) data set obtained by GC–MS for the four seasons (a) and boxplots of the FA sorted (from left to right, from top to bottom) using the Kruskal-Wallis H test *q* values (b). FAs are labelled as follows: FA xx:i (xx = number of carbon atoms in fatty acid; i = number of double bonds), followed by *n*-y for unsaturated FA (y = double-bond position related to the methyl end). Significant differences between seasons revealed by the Dunn's pairwise multiple comparison test are identified by horizontal lines and marked with * if $q < 0.05$, ** if $q < 0.01$ and *** if $q < 0.001$.

3.2.2. Seasonal variation of polar lipids at molecular level

The PCA of lipid species dataset was established and showed clear discrimination of the four different seasons (Fig. 3a). The eigenvalues of the first two principal components represented 70.5% of the total variance (PC 1 40.2% and PC 2 30.3%). The PCA plot of the determinant scores (PC 2 *vs* PC 1) shows a clear discrimination along PC1 in the summer (positive values of PC 1) and other seasons (negative values of PC1), which are discriminated along PC 2. The top 25 variables with contribution to the PC 1 (Table S6), except one (LPG 16:0), include molecular species that have PUFAs, which decrease in summer.

The lipid species dataset was also sorted using the *q*-values of the Kruskal-Wallis H test, and the results showed 146 species with

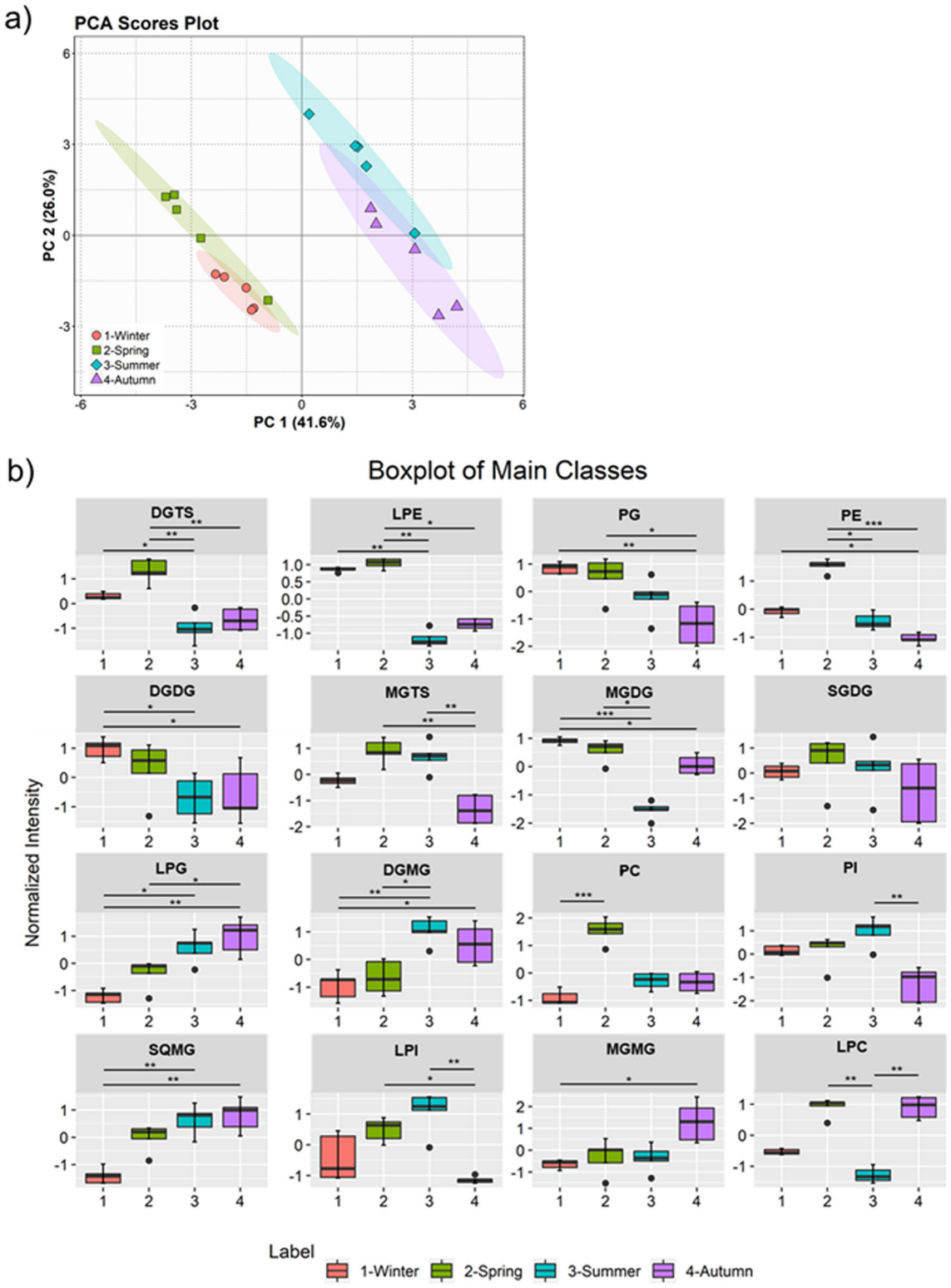

Fig. 2. PCA scores plot of the two first principal components of the data set lipid classes obtained by LC–MS for the four seasons (a) and the boxplots of the sorted classes (from left to right, top to bottom) according to the contribution to the variability of PC1 (b). Significant differences between seasons revealed by the Dunn's pairwise multiple comparison test are identified by horizontal lines and marked with * if $q < 0.05$, ** if $q < 0.01$ and *** if $q < 0.001$.

significant differences between seasons (133 species with $q < 0.01$ and 13 species with $q < 0.05$). The boxplots of the top 16 species with the lowest q values, as well as the results of Dunn's test of multiple comparisons, are shown in Fig. 3b. Among these species, the betaine lipids DGTS (34:5) and DGTS (40:6), and the sulfolipid SQDG (38:5) were more abundant in winter and spring and minor in summer. The lysophospholipid LPG (16:0), and the lysosulfolipids SQMG (14:0), SQMG (16:1) and SQMG (18:3) showed a similar trend and were most abundant in autumn and minor in summer. The phospholipids PE (32:2) and PE (34:2), and the lyso form LPE (18:1) were more abundant in spring and winter, and minor in autumn, while the betaine DGTS (30:1) was abundant in spring and summer and minor in winter and

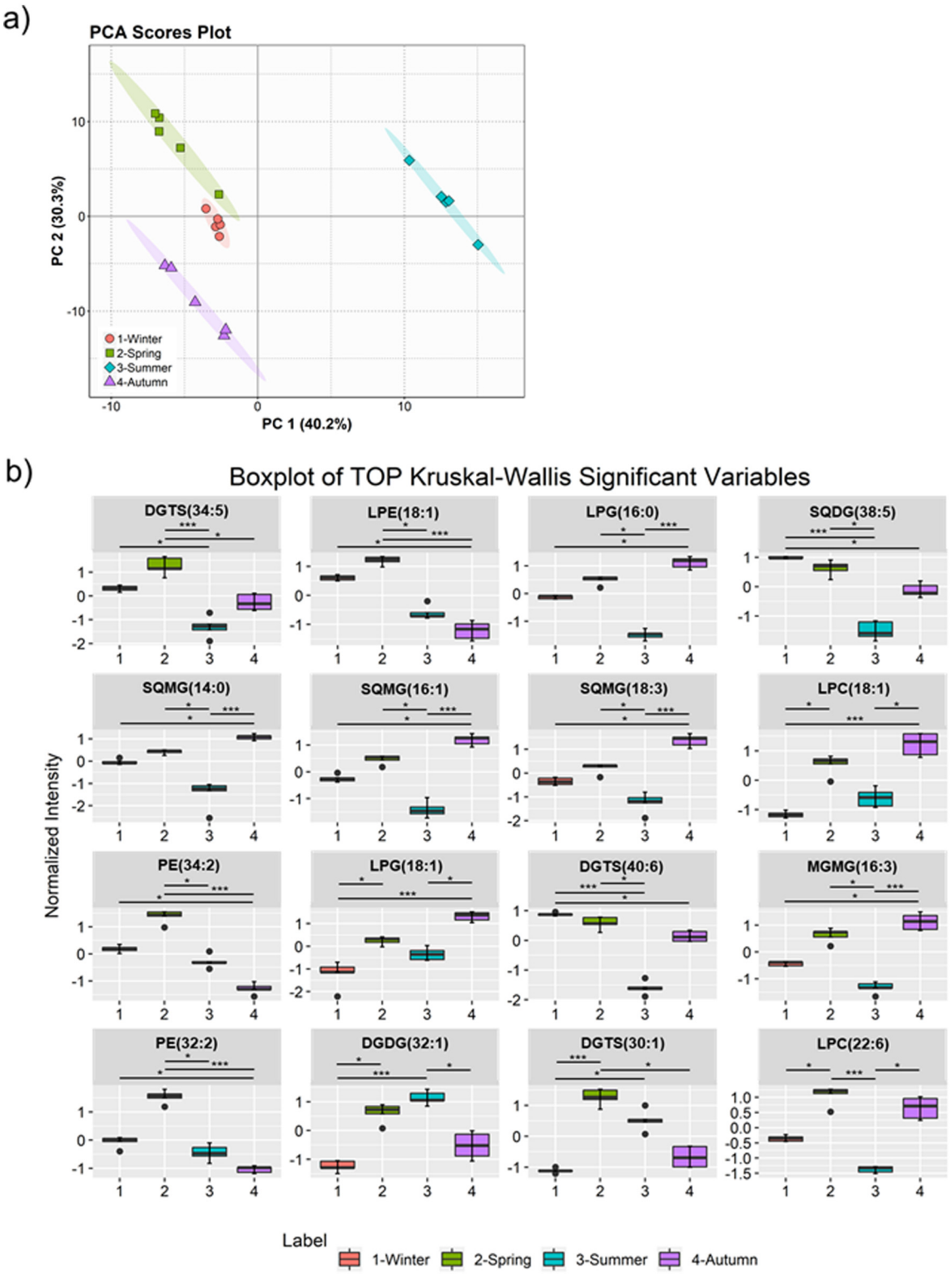

Fig. 3. PCA scores plot of first two principal components of the lipid species data set obtained by LC-MS for the four seasons (a) and the boxplots of the top 16 lipid species sorted (from left to right, top to bottom) by the Kruskal-Wallis H test lower q values (b). Lipid species are labelled as follows: AAAA(xx:i) (AAAA = lipid class; xx = number of carbon atoms in fatty acid(s); i = number of double bonds). Significant differences between seasons revealed by the Dunn's pairwise multiple comparison test are identified by horizontal lines and marked with * if $q < 0.05$, ** if $q < 0.01$ and *** if $q < 0.001$.

autumn, and the LPC (22:6) was higher in spring and autumn and minor in summer. For the lysophospholipids LPC (18:1) and LPG (18:1), high and low amounts were observed in autumn and winter, respectively. The maximum and minimum levels of MGMG (16:3) were reached in autumn and summer. The glycolipid DGDG (32:1) was abundant in spring and summer, and minor in winter.

The information from the univariate analysis was used to create a two-dimensional hierarchical clustering heatmap, using the top 25 lipid species with the lowest q-values ($q < 0.01$) (Fig. 4). The first level of the upper hierarchical dendrogram shows the samples clustered into

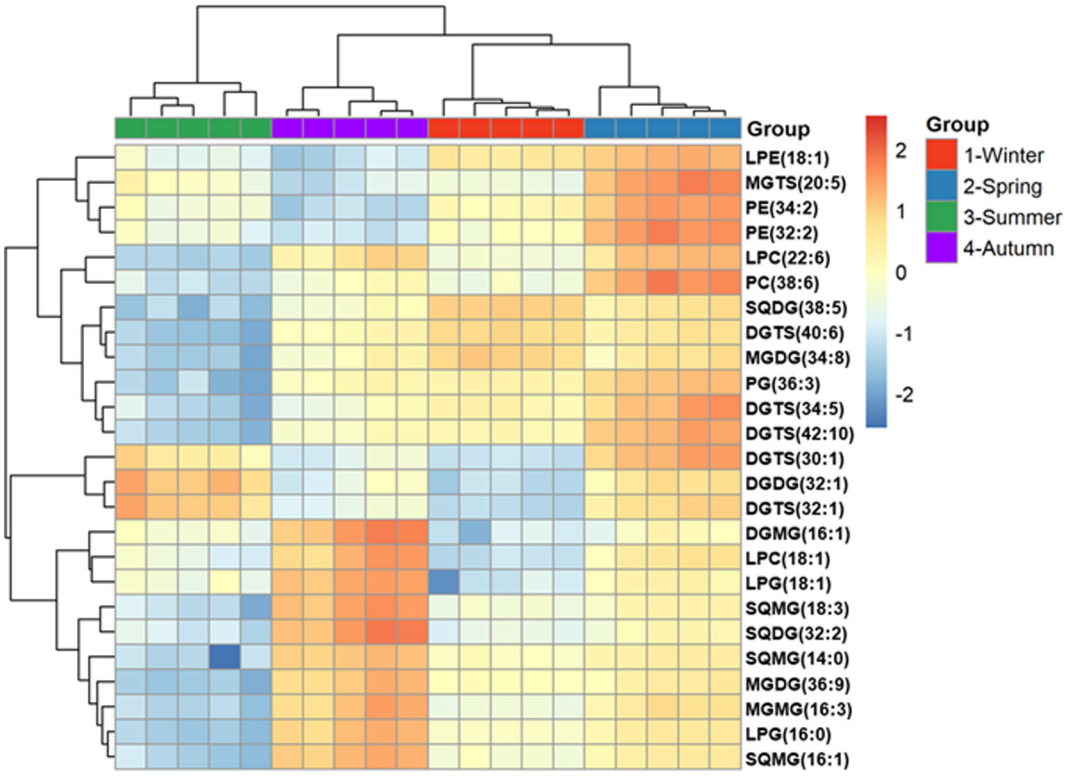

Fig. 4. Two-dimensional hierarchical cluster heatmap of the top 25 lipid species with the lowest *q* values of the Kruskal-Wallis H test. The dendrogram at the top represents the clustering of the sample groups, and the dendrogram on the left represents the clustering of the lipid species. Relative abundance levels are indicated on the colour scale and the numbers indicate the fold difference from the mean. Lipid species are labelled as follows: AAAA(xx:ii) (AAAA = lipid class; xx = number of carbon atoms in fatty acid(s); ii = number of double bonds).

two groups: a cluster for the summer group, and another for the remaining groups. This second cluster is separated in the second level into two different branches, one with autumn samples, and other with winter and spring samples. The proximity between winter and spring, as well as the separation of summer from other seasons, is consistent with what was observed in the PCA score plot (Fig. 3a).

The clustering of individual lipid species with respect to their similarity in the abundance variation from one season to another showed 4 groups at the second level of the dendrogram (on the left of the heatmap in Fig. 4). From top to bottom, the first group included 4 extraplastidial species (1 LPE, 1 MGTS and 2 PEs) and were abundant in spring and minor in autumn. The second group included 8 species (3 DGTSs, 1 LPC, 1 PC, 1 SQDG, 1 MGDG and 1 PG) with high unsaturation (up to 10 double bonds) and they were minor in summer. The third group included 3 monounsaturated species (1 DGDG and 2 DGTSs) which were abundant in summer and spring, and minor in winter and autumn. The fourth group included 10 species, including 8 lysolipids (3 SQMGs, 1 DGMG, 1 MGMG, 1 LPC and 2 LPGs), and were more abundant in autumn, and then in the spring. Regarding the composition of these top 25 species, the species with a higher level of unsaturation, bearing PUFAs (18:3, 18:4, 16:4, 22:5 and 20:5), were minor during summer. Lysolipids, with the exception of LPE (18:1) and MGTS (20:5), were most abundant in spring and autumn (transition seasons between the coldest and warmest seasons).

4. Discussion

Lipid metabolism is well regulated in living systems, including macroalgae, and polar lipids have a characteristic distribution in the different subcellular compartments of algal cells [9]. It is known that the lipidome, in particular in plants and algae, is modulated by different environmental conditions [2,12], but its plasticity is still far from being fully understood. On the other hand, there is a growing interest in polar algal lipids as valuable phytochemicals, as they are a source of omega-3 fatty acids and have a range of bioactivities which make them interesting for multiple applications [2]. Thus, detailed research is necessary to understand the natural plasticity of the polar lipidome of macroalgae, in order to provide a fundamental understanding of lipid metabolism and its regulation, as well as taking into account its biotechnological potential and market value. The knowledge of the lipidome plasticity of green macroalgae of the genus *Ulva* is of special importance, as they are traded worldwide for human consumption and other applications.

The present study, to the knowledge of the authors, is the first to evaluate the plasticity of the polar lipidome of *Ulva rigida* over four different seasons (winter, spring, summer, and autumn). The samples of *U. rigida* were produced in a land-based IMTA system, a more sustainable and environmentally friendly farming approach. In this case, *U. rigida* was grown in outdoor tanks and therefore subject to seasonal variations in environmental factors, namely temperature and salinity, as recorded during the study period (see Material and Methods). Although natural irradiance data from the production site have not been recorded, light is another important factor which has changed over the different seasons.

Comparing lipid contents obtained with those found in literature, the lipid content in autumn (2.7 ± 0.2%DW) is in agreement with a previous study [13] performed on IMTA-cultivated *U. rigida* thalli collected in the autumn (2.5 ± 0.2%DW) where the authors used chloroform:methanol for the extraction of lipids. In contrast, the lipid content in winter (3.4 ± 0.3%DW) is higher than that obtained from IMTA-cultivated *U. rigida* in winter after extraction with soxhlet with light petroleum (0.9 ± 0.2% DW) [51].

Lipid contents varied with the season, reaching minimum and maximum values in summer and in winter (1.7 ± 0.2 *vs* 3.4 ± 0.3%

DW), respectively. Consistently, the lowest lipid content was observed in summer (2.0 ± 0.09%DW) for *Ulva lobata* collected in southern California, while the highest content was observed in spring (2.9 ± 0.2%DW) and in winter (2.5 ± 0.01%DW) [27]. When the effects of temperature, light intensity, salinity and nitrogen source (nitrate and ammonia) were studied in a laboratory experiment with *Ulva pertusa*, low temperature was the most important factor for increase the total lipid content [31]. In fact, the lower and higher temperatures were recorded during growing period of *U. rigida* produced in winter and summer, respectively.

Despite the seasonal differences in lipid content, the same esterified fatty acids (FAs), as well as the same polar lipids (the main carriers of FAs), were identified from lipid extracts of macroalgae grown during all seasons. Nevertheless, significant differences were observed between seasons in the relative abundance of these biomolecules. The total number of lipid species identified in this study (150), was lower than those (202) previously identified for *U. rigida* produced by IMTA [13]. In the present study, only peaks with an area larger than 1×10^4 were considered for additional relative quantification, which explains the smaller number of species identified. This choice was motivated by the fact that the most abundant species are the most important from a perspective of valorisation for potential applications.

Regarding FA profile, overall, it is in agreement to the literature for *U. rigida* [13,52], as well as for other species belonging to the same genus [27,31,33], with the most abundant FAs including SFA 16:0, and PUFAs of 16 and 18 carbon chains. The abundance of MUFA 18:1 resulted from the sum of two peaks, probably corresponding to *n*-7 and *n*-9, as reported for other species of the genus *Ulva* [7,27,33].

According to the PCA multivariate analysis of FA data, the summer, spring and autumn groups remained closer, while the winter group more distant. Winter showed the highest level of PUFAs, the highest UFA/SFA ratio and DBI, while the *n*-6/*n*-3 ratio was the lowest, as previously observed for *Ulva fenestrata* [28]. In all seasons, *n*-6/*n*-3 ratio was < 1, which is important, with the perceived need for more *n*-3 PUFAs in human nutrition. Health benefits have been widely associated with *n*-3 PUFAs, including anti-inflammatory properties, reduction of cardiovascular disease, and prevention of breast cancer and cancer-related complications [53–55], while *n*-6 PUFAs have been linked to the promotion of inflammatory processes and tumour growth [56]. In this context, the biomass of *U. rigida* produced in winter had the highest added value, as a lower *n*-6/*n*-3 ratio was associated with beneficial health effects for consumers.

In terms of physiology of algae, it is well-established that the level of unsaturation of membrane lipids increases in response to lower temperatures in order to maintain the fluidity of cell membranes at appropriate levels [9,12]. On the other hand, the need of a particular high content of *n*-3 PUFAs in photosynthetic lipids has been associated with the high number of double bonds in *n*-3 PUFAs which can facilitate the electron-transport activity of photosystems, namely during winter [28]. Although increasing the level of unsaturation has been generally accepted as one of the acclimation mechanisms at low temperatures, other factors, namely light intensity and salinity, can also significantly influence the FA profiles [31,32,57]. In accordance with the results obtained for *U. pertusa* exposed to different levels of salinity [57], the increase of 18:2*n*-6 observed in *U. rigida* biomass between winter and summer can be due to the increase in salinity. On other hand, the higher abundance of SFAs, namely 16:0, observed in *U. rigida* produced in summer can be related to high light conditions, as observed for *U. pertusa* [31] and *U. fenestrata* [32]. Also, the length of the day, which affects daily photosynthesis rates and therefore growth rates, is a factor that varies widely between seasons and could also affect the lipidome.

Multivariate analysis of the classes data set showed greater proximity of *U. rigida* biomass between summer and autumn, and between winter and spring. The DGTS, LPE, PG, PE, and DGDG classes contributed the most to this separation into pairs of seasons, and all these classes were increased in winter and spring. An increase in the DGTS content has been stressed as crucial in the adaptation of *Nannochloropsis oceanica* to low temperatures and phosphate deficiencies [58]. In the case of *U. rigida*, further studies will be needed to understand the effect of these and other environmental factors on the content of betaine lipids. Regarding potential applications, the observed changes in lipid class levels for *U. rigida* are important to define the most appropriated season(s) to produce biomass of *U. rigida*, depending on the desired lipid profile. For example, the LPE class, with potential use in agricultural applications as a retardant of leaf and fruit senescence [59], was higher in the winter and spring biomass.

Adjustments in the content and FA composition of the glycolipid (MGDG, DGDG and SQDG) and of the phospholipid (PG) classes present in the chloroplast membranes of macroalgae have been associated with changes in temperature and radiation, as well as with different stages of life, in order to maintain and adjust photosynthetic activity [16,32,60]. Unlike that observed in this study for *U. rigida* cultivated in IMTA, an increase in the MGDG content in *U. fenestrata* harvested from the Sea of Japan in summer compared to spring has been reported [60]. The contrasting trends observed in seasonal variations in polar lipid classes may be due to different local environmental conditions, as well as to species-specific responses to environmental parameters [4,61], which merits further research.

PCA multivariate analysis of molecular species data set showed greater discrimination of the four seasons than that observed from FA and classes, emphasizing the importance of carrying out a detailed study of the polar lipidome. The top 16 species that showed the most significant variation between seasons included 3 betaine lipids (DGTS), 2 phospholipids (PE), 2 glycolipids (1 DGDG and 1 SQDG), 5 lysophospholipids (1 LPE, 2 LPG and 2 LPC) and 4 lysoglycolipids (3 SQMG and 1 MGMG). Thus, the lysolipid species were 9, more than half of the 16 species which presented a major variation. These species increased mainly during the autumn and spring seasons, suggesting that lysolipids may play an important role in the adaptations of the polar lipidome of *U. rigida* that occurred between summer and winter.

Regarding the biotechnological interest of polar lipids, it should be noted that the closest composition in terms of lipid species abundance was observed for *U. rigida* biomass produced in winter and spring, while the summer biomass was clearly distinct from the remaining seasons, as supported by PCA and by two-dimensional hierarchical clustering analysis. Also, glycolipid species with previously reported bioactivity were here identified, namely SQDG (16:0/16:0) [21] and SQMG (16:0) [20] with antimicrobial activity, SQDG (20:5/14:0) [24], MGDG (18:4/16:4) [22], MGMG (16:3) [23] and MGMG (16:2) [23] with anti-inflammatory activity, and SQDG (20:5/16:0) associated with antimicrobial [19] and anti-inflammatory [24] effects. Of these, MGMG (16:3) and MGDG (34:8) (18:4/16:4) are in the top 25 species with the most significant seasonal variations, and the two molecular species are minor during the summer. Thus, in case of specific interest in MGMG (16:3) and MGDG (18:4/16:4), the winter biomass should be favoured. These results reinforce the importance of documenting the seasonal variation of macroalgal lipids to choose the most appropriate harvest period, thus ensuring that produced biomass displays the highest levels of the compounds of interest.

5. Conclusions

The present study demonstrates a clear modulation of the lipid profile of *Ulva rigida* under contrasting environmental conditions experienced by this green macroalgae during the different seasons. The variations recorded on its lipidome were observed in terms of lipid content, as well as of fatty acid and polar lipids profile. The polar lipids that most varied include glycolipids, which are important in chloroplasts and are known to exhibit seasonal variations, but also betaine lipids and phospholipids. Lysolipids, which are signalling molecules and whose knowledge may be important for understanding the metabolism of algae, also exhibit a high level of seasonal variation. As some

of the lipid species that varied the most were previously described as bioactive, the knowledge of the lipidome of *U. rigida* is essential to select premium biomass for different applications. On the other hand, as *Ulva* is an edible seaweed, the variation in lipid composition is important when it comes to provide the biomass with constant nutritional value. Irrespective of seasonal variations, *U. rigida* had a low *n*-6/*n*-3 ratio, suitable for food and feed, probably with potential health benefits.

Future studies on the lipidome of this and other macroalgae of commercial interest are considered necessary to shed light on the environmental parameters which play the most important role in seasonal changes in lipid content and composition of these important marine resources.

CRediT authorship contribution statement

Ana S.P. Moreira: Conceptualization, Data curation, Investigation, Writing - original draft, Writing - review & editing. **Elisabete da Costa**: Data curation, Writing - review & editing. **Tânia Melo**: Data curation, Writing - review & editing. **Ronan Sulpice**: Writing - review & editing. **Susana M. Cardoso**: Writing - review & editing. **Bárbara Pitarma**: Writing - review & editing. **Rui Pereira**: Resources, Writing - review & editing. **Maria H. Abreu**: Resources, Writing - review & editing. **Pedro Domingues**: Data curation, Formal analysis, Software, Validation, Writing - review & editing. **Ricardo Calado**: Writing - review & editing. **M. Rosário Domingues**: Conceptualization, Investigation, Writing - original draft, Resources, Supervision, Project administration, Writing - review & editing.

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Declaration of competing interest

The authors declare that they have no competing interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2020.101958.

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