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Amino acids profile of 56 species of microalgae reveals that free amino acids allow to distinguish between phylogenetic groups

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ABSTRACT

Microalgae represent a phototrophic resource with a high protein content, whose nutritional value is very high as a result of its amino acid profile. Although the total amino acid profile has been repeatedly described to change little among phylogenetic groups of microalgae, some variability has occasionally been reported. Part of this uncertainty is associated with the low phylogenetic diversity encompassed in the work to date and the high methodological variability between studies. Among these studies, very few have differentiated between total and free amino acid content for common microalgae samples. Thus, in the present work, the profile of total and free amino acids has been determined in a diverse group composed of 56 species of microalgae belonging to 7 phyla. A multivariate analysis of the total amino acid profile in the studied microalgae group revealed close amino acid patterns throughout microalgae phylogeny and agreed with results obtained from similar analysis performed with published data. Conversely, the free amino acid profile strongly differentiated between phylogenetic groups. On the one hand, species of Cyanobacteria, Plantae, Cryptophyta, and Bacillariophyta showed close free amino acid patterns, characterized by the highest abundance of free glutamic acid. Ochrophyta species were particularly rich in free proline, while the free amino acid profile of Miozoa and Haptophyta species stood out from the rest of the phylogenetic groups for their outstanding levels of the two essential amino acids phenylalanine and lysine. Haptophyta species were also characterized by their much higher free tyrosine content.

1. Introduction

Among phototrophic organisms, microalgae stand out for their particularly high protein content and a nutritionally relevant amino acid (AA) profile [1]. Although production costs are still high [2], microalgae are increasingly considered as an alternative protein source both in aquaculture [3] and in human nutrition [4]. Despite the nutritional importance of proteins, studies on the nutritional quality of microalgae proteins have been much less frequent than those dedicated to other nutritionally relevant compounds, such as lipids. This difference can be attributed to the greater phylogenetic diversity for the lipid profile of microalgae [5]. Initial reports on AA content of microalgae date back similarly to reports on lipids, with pioneering multi species studies performed in the 1960s [6]. However, a look to the literature since that time reveals that fewer published resources are available regarding AA profiling in microalgae. Most of the studies involved a limited number of microalgae with only a few seminal works dealing with more diversified groups [6,7]. In these studies, gas chromatography (GC) and high-performance liquid chromatography (HPLC) coupled with mass spectrometry (MS) have been widely used for amino acid analysis [8]. GC–MS presents mayor robustness although derivatization is required. Silylated reagents and alkyl chloroformates were used to derivatize

Abbreviations: AA, amino acid; TAA, total amino acids; FAA, free amino acids; ECF, ethyl chloroformate; EI, electronic ionization; PFTBA, per-fluorotributylamine; CAP, canonical analysis of principal coordinates.

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amino acids [9]. One of the advantages of alkyl chloroformate is that the reaction is rapid, occurs in an aqueous medium and at room temperature [10]. Furthermore, different extraction methods have been tested for the extraction of high value compounds with promising results. In recent studies, the use of green solvents and supercritical fluids appeared as a low cost and ecologically safe alternative to extract high value compounds from biomass, such as amino acids or L-dopamine [11,12].

Early research pointed to a low interspecific variability in the AA profile of microalgae [6,13] and this circumstance was confirmed in some subsequent studies [7,14]. However, other studies reported some differences in the AA profile between diverse algal species [15–19]. It should be noted that most studies to date have used a limited number of species that poorly represents the very high phylogenetic diversity of phytoplankton [20]. As an example, it can be highlighted that approximately half of the AA profiles that have been published to date correspond to species of cyanobacteria and chlorophytes. This coincides with the fact that these taxonomic groups have been the most frequently cultivated but, nevertheless, they represent only a small fraction within the phytoplankton phylogenetic diversity. In addition to the taxonomic bias, the methodological variability between works carried out in different laboratories [21] makes it difficult to adequately understand the degree of AA composition constancy between phytoplankton groups.

Information on the free AA (FAA) content of microalgae is even more disperse and scarce than that for the total AA (TAA) profile. Microalgae, like most organisms, may contain, in addition to protein amino acids, non-protein bound FAA, which amount is closely related to the physiological state of the microalga and typically ranges from approximately 1 % to 20 % TAA [22,23]. Some taxa-specific FAA variability has been occasionally reported for a limited number of microalgae species [24,25]. In this regard, there is no study that includes a number of species that does not even minimally approach the phytoplankton phylogenetic diversity. FAA are known to be involved in specific metabolic processes and that variability has been useful to differentiate between taxonomic groups other than microalgae [26]. Nothing similar is known about a plausible differentiation between phytoplankton taxonomic groups on the basis of their FAA. It should be considered that, given the specific importance of FAA in the nutrition of aquatic consumers [27,28], identification of FAA variability among phytoplankton groups can shed new light for a better understanding of their nutritional value. FAA are also relevant due to their role in taste sensation [19]. Precise information on inter-taxa FAA variability is very helpful in studies involving microalgae metabolism [29], as well as for the use of FAA as biomarkers in phytoplankton ecology [30,31].

In addition to the scarce taxonomic representation already mentioned, it has been observed that only around one third of the studies on AA content in microalgae include information for an AA of high nutritional value, such as tryptophan. Therefore, there is a need to achieve complete essential AA profiles in order to make more precise nutritional value assessments of the different microalgae groups with respect to other protein sources. According to the above reasons, in this work a phylogenetically diverse set of 56 microalgae was selected in which their TAA and FAA content was determined, including tryptophan analysis. The selected set of microalgae was structured into 7 phyla and 38 genera, and 24 of the 56 species had not yet been reported for their AA composition. The present study is the most diversified to date among those involving analysis of AA in microalgae, notably expanding the number of species compared to previous larger reports that included 20 genera [6] and 12 genera [7,21]. Through multivariate analysis of AA profiles determined under uniform conditions in this diverse set of microalgae, it is intended to identify trends of variation in the AA profile within a strongly conserved composition of proteins that perform common and highly specific functions across the phytoplankton phylogeny [7]. A similar multivariate analysis is performed on the information published on AA by different authors in order to identify possible sources of variation associated with the methodology used. The study has the main goal of detecting patterns of TAA or FAA variation that can be used to distinguish phytoplankton phylogenetic groups.

2. Materials and methods

2.1. Selection of microalgae and culture conditions

The study on phylogenetic variability in the TAA and FAA profile was based on a set of 56 marine and freshwater microalgae (Table S1). Microalgae species were representative of 8 phyla, according to the AlgaBase classification [32]. To evaluate the variability in the AA profile, the microalgae were grouped based on their phylogenetic ascription to the following groups: Cyanobacteria, Plantae (included phylum Chlorophyta and phylum Rhodophyta), Ochrophyta, Haptophyta, Miozoa, Cryptophyta and Bacillariophyta. Marine microalgae were cultured in filtered and autoclaved seawater (salinity of 36) fertilized with the f/2 medium [33]. Freshwater strains were grown using the culture medium described by Fábregas et al., [34]. In the case of diatoms, 50 mg·L^{-1} of silica was added to the culture. Cultures were performed under continuous irradiance (100 μM photon·m^{-2} s^{-1}) and temperature set at 20 ± 1 °C in a 20 L tubular bioreactor. Late exponential cultures were harvested by centrifugation at 2000*g* for 15 min, washed twice and re-centrifuged in 0.9 % ammonium formate (isotonic solution with seawater), to remove the salts. Then, microalgae pellets were immediately frozen at −80 °C for subsequent lyophilization. Freeze-dried samples were kept dried and vacuum sealed until AA analysis. Subsamples were taken for elemental (C, N, H) analysis that was carried out in a Flash 112 series EA, Thermo Quest elemental analyser after oxidation at 900 °C in a CuO-electrolytic Cu column.

2.2. Sample processing and determination of amino acid profiles

For FAA determination, 0.05 g of lyophilized microalgae biomass were placed in vials with 0.5 mL of glass beads and 0.5 mL of Mili-Q water. The cells were disrupted by bead beating with the Digital Disruptor Genie® (Scientific Industries, Bohemia, NY, USA) for 3 cycles of 30 s; and the cell debris were centrifuged twice at 12000*g* for 20 min, as described in [25]. The supernatant obtained was stored at −80 °C.

For TAA determination 0.05 g of lyophilized microalgae biomass was hydrolyzed to break protein peptide bonds by acid hydrolysis. Samples were placed in vials with 1 mL of 6 M HCl, and the vials were flushed with N_2 and placed at 150 °C for 70 min under agitation, as described by [10]. Samples were then centrifuged at 3000*g* for 5 min and the supernatant was diluted 60 times with Mili-Q water and stored a −20 °C. For tryptophan, glycine, valine, threonine and tyrosine determination, 0.05 g of lyophilized biomass was hydrolyzed by alkaline hydrolysis. The biomass was placed in vials with 1 mL of 4.2 M NaOH. The vials were flushed with N_2 and placed at 110 °C for 24 h, centrifuged, and the supernatant diluted as in the acid hydrolysis. This additional hydrolysis was performed in order to determinate tryptophan, which cannot be detected on acid hydrolysis [35]. It was also helpful to obtain more precise results of the other AA, which are less damaged with this hydrolysis.

After acid and basic hydrolysis, amino acid derivatization was performed using ethyl chloroformate (ECF), as described in [36], and analysis in a Shimadzu GC–MSTQ (GCMS-TQ8030) equipped with an Agilent HP-5MS fused silica capillary column (60 m × 0.25 mm i.d., 0.25 mm film thickness). For both TAA and FAA, aliquots of 100 μL were placed in 2 mL vial and 400 μL of a water:ethanol:pyridine (60:32:8) and 40 μL of ECF solutions were added. The mix was vigorously vortexed for 60 s at room temperature and 200 μL of chloroform (containing 1 % ECF) were added. The derivatives were extracted into the organic phase by striking the tube in a vortex for 60 s. The organic phase was dried with anhydrous sodium sulphate and transferred into a new vial with a 300 μL fixed insert.

Aliquots (1 μL) of the derivatized extracts were subjected to the gas chromatography system which was operated in the split-less mode. The

column was initially kept at 120 °C for 1 min and ramped at 5 °C min^{-1} to 200 °C. Temperature was then increased to 260 °C at 30 °C min^{-1}, held for 10 min, and it was finally ramped at 10 °C min^{-1} to 280 °C and held for 3 min. Helium was used as the carrier gas with a constant flow of 1.2 mL min^{-1}.The temperatures of the injector, the transfer line and the ion source were maintained at 250, 280 and 230 °C, respectively. A solvent delay of 4 min was selected. An electron ionization (EI) at 70 eV was used in the mass spectrometer, which was operated in the selected-ion monitoring (SIM) mode. The MS was tuned to *m/z* 69, 219 and 502 for EI corresponding to per-fluorotributylamine (PFTBA). Each compound was identified using one quantifier ion and two qualifier ions, as well as the relative intensity of the qualifier ion over the quantifier (±20 %). Quantification was performed by the external standard method following the same procedure for all samples. Arginine cannot be detected with this analytical method and was estimated following the recommendation of Lourenço et al., [37], as the difference between the total protein determined as N*4.8 [21] and the sum of all analyzed AA, expressed as mg g^{-1} dry biomass weight. Asparagine and glutamine were quantified along with aspartic acid and glutamic acid, respectively. The limits of detection (LOD) and mass characteristics for each amino acid are shown in Table S2.

2.3. Statistical analysis

The data on AA content determined as weight per unit of dry microalgae biomass were converted into a percentage of the total AA in order to have a measuring unit that can be better contrasted with the existing information in the literature. Differences in individual AA content between microalgae groups were checked by one-way analysis of variance (ANOVA) followed by a multiple comparison test (Tukey) when significance ($P < 0.05$) was detected. Comparisons between TAA and FAA profiles in microalgae groups were performed by permutational multivariate analysis of variance (PERMANOVA) based on the Bray Curtis similarity matrix [38]. Each term in the analysis was tested using 999 unique permutations, with post hoc pair-wise comparisons (Tukey) using the PERMANOVA t statistic. The similarity percentage (SIMPER) analysis [39] identified those AA that contributed most to between group dissimilarity. The multivariate dispersion test PERMDISP [40] was used to evaluate AA variability between different groups of microalgae. A canonical analysis of principal coordinates (CAP) was applied to variability in the AA profile in order to find axes in a multivariate space that best separated a priori established groups [41], which in present study were those built on microalgae phylogeny. CAP is a constrained ordination method that uses the first squared canonical correlation (δ_1^2) and the leave-one-out allocation success to indicate the precision of group discrimination. Significant differences in AA profiles were determined by the trace statistic which is the sum of all squared canonical correlations. The multivariate analyses were performed using the software PRIMER v7.0.21 & PERMANOVA+ 1 statistical package (PRIMER-E Ltd., Plymouth, UK). Results from multivariate analysis of TAA and FAA profiles were contrasted with those obtained from published results in order to detect differences in data variability and confirm the hypothetical existence of any phylogenetic-specific AA pattern. Referenced information was obtained for the microalgae species listed in Table S3 (TAA) and Table S4 (FAA), for which all AA values published in the corresponding literature were recalculated, when needed, to achieve uniform expression as percent of total AA.

3. Results

3.1. Variations of protein and amino acid content of microalgae

The conversion of N to total protein resulted in values above the sum of all identified TAA and such difference was attributed to the arginine content of microalgae. Cyanobacteria and Cryptophyta presented a protein content higher than 40 % of the dry biomass, which significantly exceeded ($P < 0.05$) that of Haptophyte, Miozoa and Bacillariophyta microalgae groups (Fig. 1). The higher N content in Cyanobacteria and Cryptophyta resulted in minimum values for their respective C/N ratios that were significantly lower than those in all other microalgae groups (Fig. 1).

AA content within the TAA pool was very constant across microalgae phylogenetic groups and significance (one-way ANOVA, $P < 0.05$) was only identified for alanine, although it occurred within a narrow range of variation (Fig. 2). A similar ANOVA performed from the published information and arranging the data on TAA in the same phylogenetic groups as those used for the studied set of microalgae revealed significant ($P < 0.05$), although moderate, between-group differences for alanine, glycine, proline, tyrosine, isoleucine, lysine and histidine (Fig. S1). The higher proline content in Ochrophyta and isoleucine in Cyanobacteria marked the main difference. Results were very different when the FAA pool was analyzed, since all FAA showed significantly different content ($P < 0.05$) depending on the phylogenetic group of microalgae (Fig. 3). The largest range of variation occurred for glutamic acid, with average maximum content over 70 % in Cyanobacteria and Bacillariophyta. The minimum free glutamic acid content of around 5 % in the Ochrophyte coincided with this phylum as the richest by far in free proline (Fig. 3). Also noteworthy were the relatively higher contents in tyrosine of the Haptophyta and in the nutritionally essential AA phenylalanine and lysine in microalgae belonging to the Haptophyta and Miozoa phyla. This latter group including only dinoflagellates was

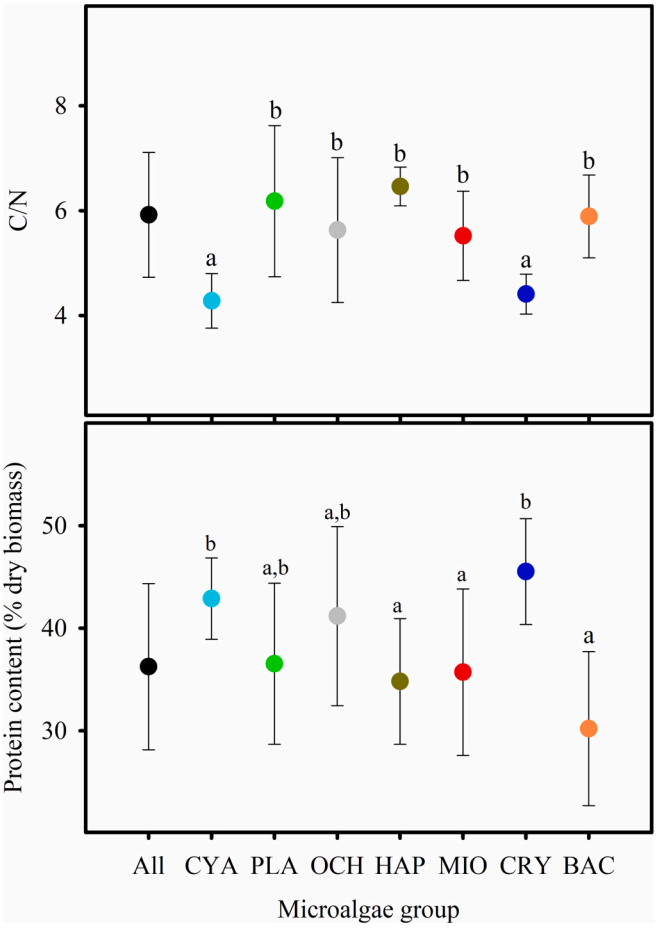

Fig. 1. Carbon to nitrogen ratio (C/N) and protein content of the total set of studied microalgae (All) and for the phylogenetic groups of Cyanobacteria (CYA), Plantae (PLA), Ochrophyta (OCH), Haptophyta (HAP), Miozoa (MIO), Cryptophyta (CRY) and Bacillariophyta (BAC). Values are expressed as mean ± standard deviation and significance (Tukey test, $P < 0.05$) for heterogenous groups is indicated by different letters.

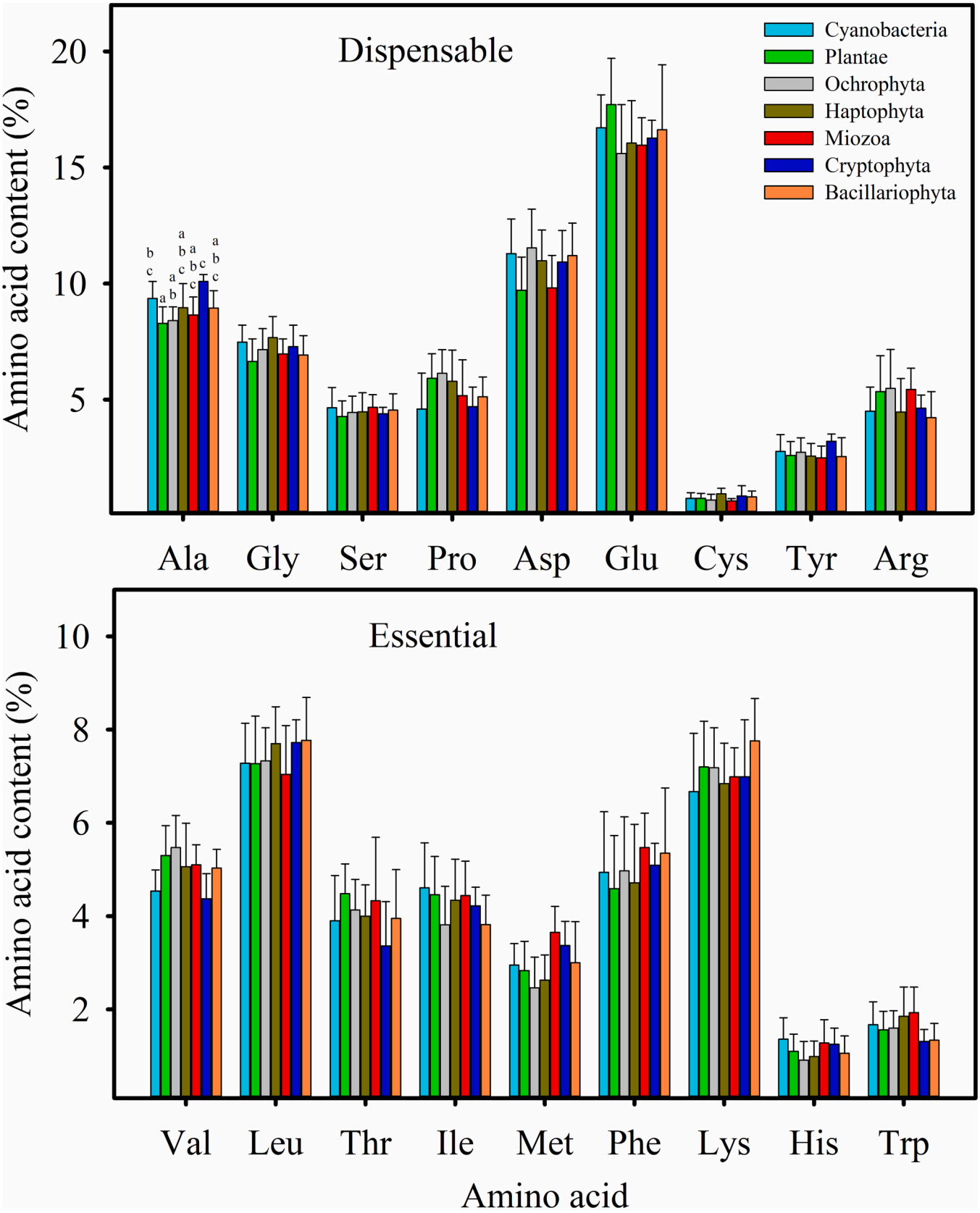

Fig. 2. Mean and standard deviation for the percentage content of amino acids with respect to the total amino acids in the studied set of microalgae. Significant differences (Tukey test, $P < 0.05$) for the content of dispensable (upper plot) and essential (lower plot) amino acids between phylogenetic groups are indicated with a different letter.

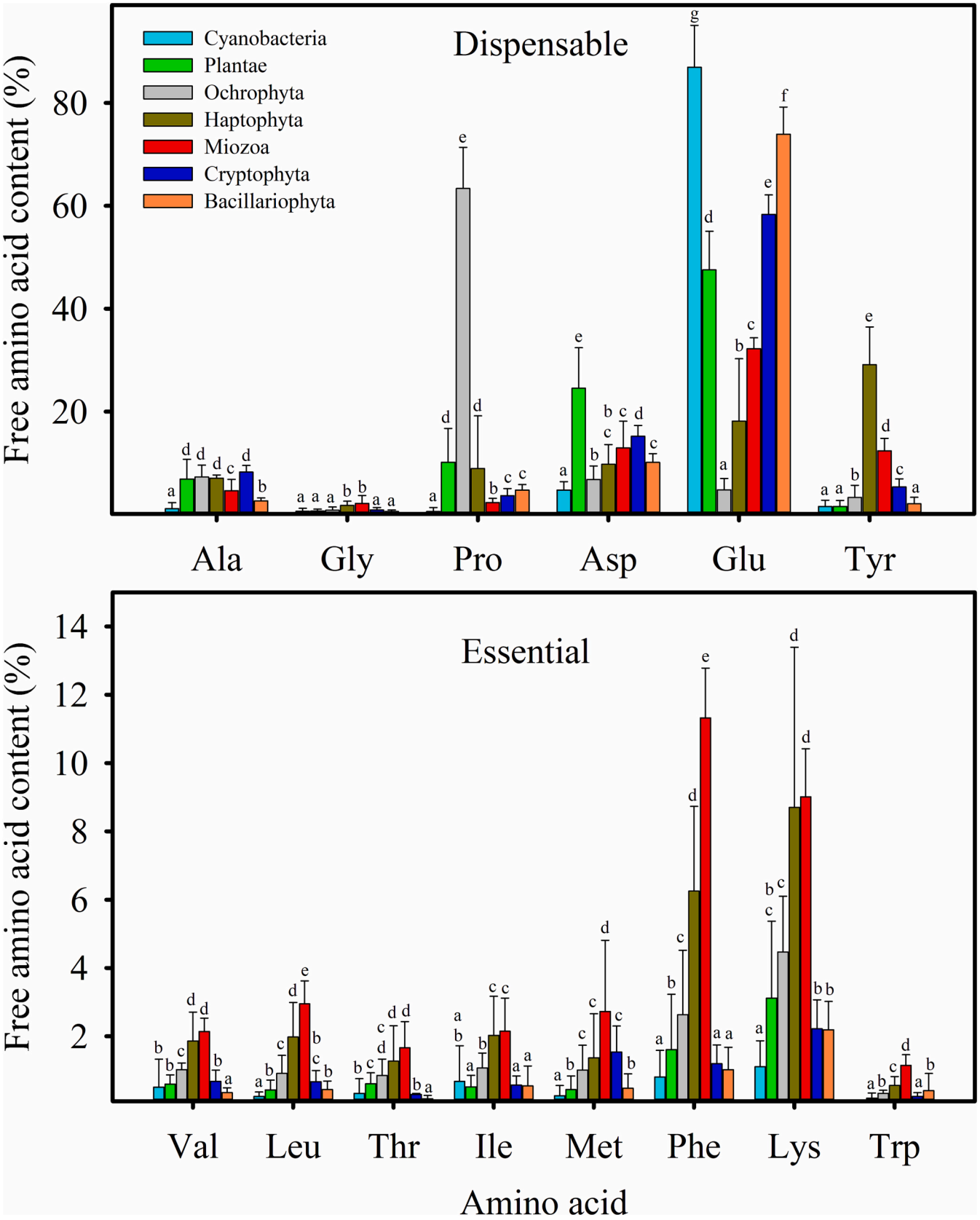

Fig. 3. Mean and standard deviation for the percentage content of amino acids with respect to the free amino acid pool in the studied set of microalgae. Significant differences (Tukey test, $P < 0.05$) for the content of dispensable (upper plot) and essential (lower plot) amino acids between phylogenetic groups are indicated with a different letter.

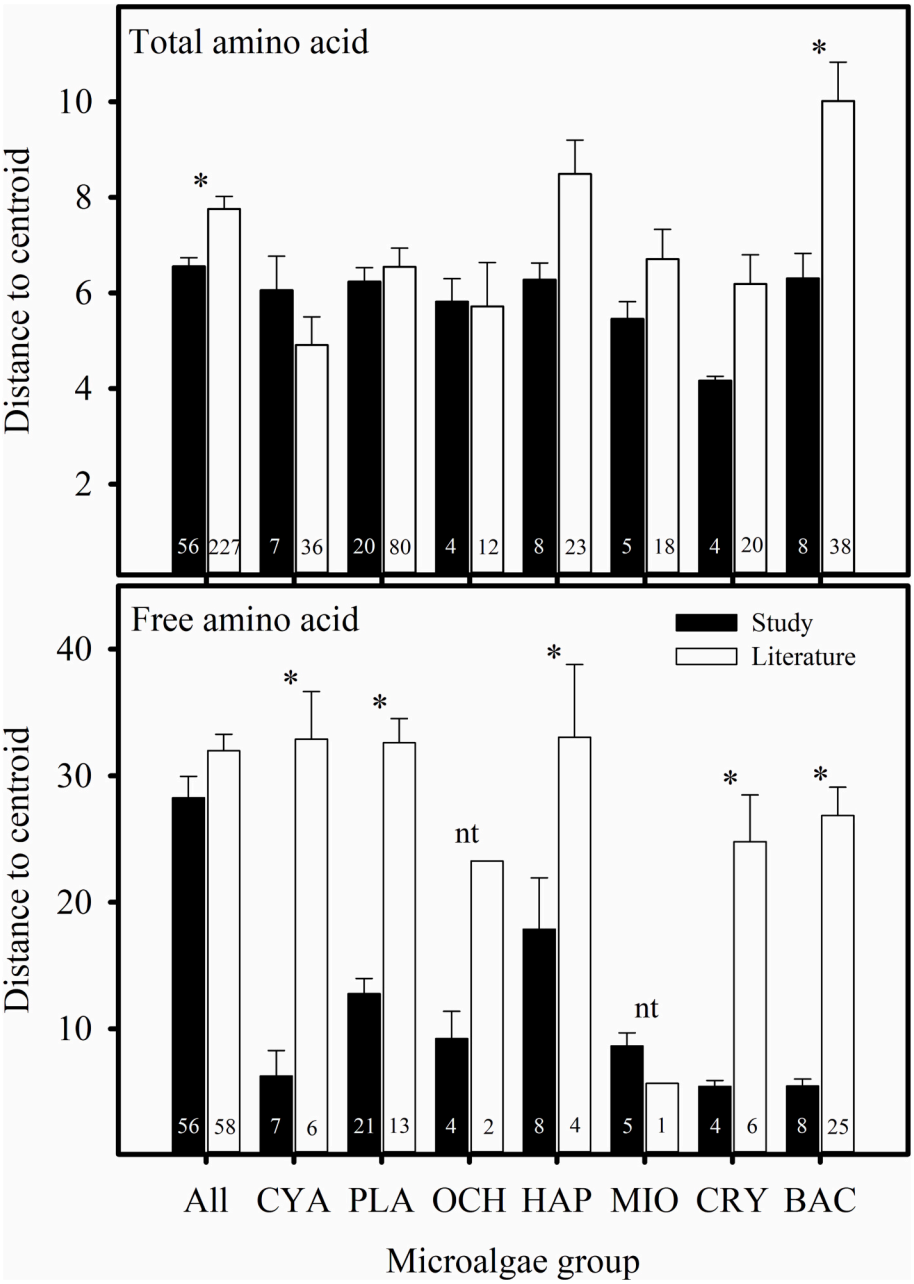

Fig. 4. Mean and standard error for the distance to centroid of the profile of total amino acids (upper plot) and free amino acids (lower plot). An asterisk denotes significant differences in data dispersion (PERMISP test, $P < 0.05$) between the studied microalgae (solid bars) and those reported in the literature (empty bars). The number at the base of the bars indicates the sample size of each microalgae group, which are labelled on the X axis as in Fig. 1. Unable to test (nt) due to insufficient sample size from the literature.

also notable for its relatively higher methionine content (Fig. 3). Free tryptophan showed one of the lowest intergroup variabilities and its percentage with respect to the total FAA was lower than that represented in the TAA pool. Regarding literature results, glutamic acid was the most abundant FAA in Cyanobacteria, Ochrophyta, Haptophyta, Miozoa and Bacillariophyta, while the Haptophyta was the group richest in tyrosine (Fig. S2). When comparing the dispersion of results for the AA profiles between the present study and the information collected from the literature, it is observed that the PERMDISP test almost always attributed higher distance to centroid and standard error for the literature data set (Fig. 4). Such difference in dispersion was particularly greater for FAA, with information for Cyanobacteria, Plantae, Haptophyta, Cryptophyta and Bacillariophyta that was much more scattered ($P < 0.05$) for literature results (Fig. 4).

3.2. Amino acid distribution patterns through phylogenetic groups of microalgae

The small differences between phylogenetic groups at the level of individual AA within the TAA pool were reflected in very high levels of similarity for the corresponding TAA profiles. All pairwise comparisons between groups remained within a narrow similarity range, between 90.26 % and 92.45 %, for the TAA profile (Table 1). Despite this high similarity, PERMANOVA analysis indicated that the Plantae group presented a TAA profile significantly different from that shown by Cyanobacteria, Haptophyta, Cryptophyta and Bacillariophyta. Cryptophyta was the only group in which the PERMDISP test revealed a lower dispersion of results compared to Plantae. Different AA were selected by SIMPER analysis as those contributing the most to TAA profile dissimilarity within each of the four pairwise comparisons (Table 1). However, no AA stood out from the others for an especially greater contribution to dissimilarity, reaching 90 % cumulative contribution to dissimilarity

Table 1
Similarity (Sim%) of the total amino acid profile between the studied phylogenetic groups of microalgae. The significance of each pairwise comparison is denoted by the Permanova t-statistic (P-PM). Results of the Permdisp test are in the P-PD headed column. The three most between-groups differentiating amino acid and their % contribution to dissimilarity (Simper analysis) are also indicated. CYA: Cyanobacteria. PLA: Plantae. OCH: Ochrophyte. HAP: Haptophyte. MIO: Miozoa. CRY: Cryptophyte. BAC: Bacillariophyta. * Significant differences between phylogenetic groups at $p < 0.05$

Groups	Sim %	P-PM	P-PD	Simper analysis
CYA-PLA	90.26	**0.004***	0.890	Pro (9.5 %), His (9.4 %), Tyr (7.7 %)
CYA-OCH	90.94	0.214	0.575	His (11.9 %), Pro (10.4 %), Tyr (7.9 %)
CYA-HAP	91.20	0.745	0.806	His (10.5 %), Pro (9.7 %), Phe (8.0 %)
CYA-MIO	91.28	0.643	0.628	His (9.9 %), Thr (9.6 %), Pro (8,7 %)
CYA-CRY	92.45	0.883	0.094	His (9.4 %), Thr (9.3 %), Tyr (9.0 %)
CYA-BAC	90.89	0.300	0.921	His (9.4 %), Tyr (8.9 %), Phe (8.6 %)
PLA-OCH	91.26	0.158	0.484	His (9.5 %), Met (8.2 %), Tyr (8.1 %)
PLA-HAP	90.74	**0.029***	0.870	Trp (8.5 %), His (8.3 %), Phe (8.0 %)
PLA-MIO	91.18	0.188	0.556	His (10.2 %), Met (9.2 %), Pro (8.3 %)
PLA-CRY	90.65	**0.005***	**0.037***	Thr (9.5 %), His (8.7 %), Tyr (8.2 %)
PLA-BAC	90.69	**0.014***	0.973	Phe (9.2 %), His (8.6 %), Tyr (8.4 %)
OCH-HAP	91.75	0.951	0.328	His (8.7 %), Trp (8.7 %), Phe (8.5 %)
OCH-MIO	91.46	0.401	0.681	Met (12.3 %), His (11.2 %), Pro (8.8 %)
OCH-CRY	91.45	0.120	0.023	Met (10.6 %), His (10.2 %), Pro (8.5 %)
OCH-BAC	91.72	0.806	0.444	His (9.8 %), Met (9.7 %), Tyr (9.3 %)
HAP-MIO	91.22	0.519	0.324	Met (10.1 %), His (9.8 %), Trp (8.8 %)
HAP-CRY	91.77	0.345	**0.012***	His (9.4 %), Met (8.4 %), Tyr (8.3 %)
HAP-BAC	90.94	0.377	0.865	Trp (9.3 %), Phe (9.1 %), His (8.0 %)
MIO-CRY	92.05	0.249	**0.020***	Thr (12.0 %), His (10.2 %), Tyr (9.4 %)
MIO-BAC	91.10	0.330	0.524	His (10.0 %), Thr (9.7 %), Trp (9.6 %)
CRY-BAC	91.73	0.243	0.088	Tyr (9.7 %), Thr (9.0 %), His (8.9 %)

with up to 14 AA in most instances.

Similarity between the FAA profile of microalgae groups was much lower and more variable than that corresponding to the TAA profile (Table 2). The highest similarity between FAA profiles from different groups occurred for the pairs Cryptophyta-Bacillariophyta (81.87 %) and Cyanobacteria-Bacillariophyta (83.79 %), while the least similar pairs were Cyanobacteria-Ochrophyta (16.43 %) and Ochrophyta-Bacillariophyta (26.28 %). The PERMANOVA analysis indicated the existence of strong significant differences in the FAA profile for all possible combinations between groups of microalgae (Table 2). Bacillariophyta had the lowest intra-group dispersion (mean distance to centroid ± standard error: 5.50 ± 0.55) and showed the greatest difference ($P < 0.05$) in the dispersion of results compared to other more disperse groups such as Plantae (12.74 ± 1.21), Haptophyta (16.98 ± 3.56) and Miozoa (8.58 ± 1.04). Unlike in the case of TAA, the SIMPER analysis identified AA that presented a high percentage of contribution to the dissimilarity for the FAA profile between groups of microalgae. The most striking value was for proline, a FAA that presented the maximum contribution to the dissimilarity (14.6 % to 26.1 %) between Ochrophyta and all other groups (Table 2). Tyrosine was the second FAA in importance to differentiate the FFA profile between groups, due to its contribution (13.2 %–15.1 %) to the dissimilarity between the FAA profile of Haptophyta with respect to all other groups, except for Miozoa (Table 2).

The existence of possible patterns of variation for the AA profile depending on the phylogenetic diversity of microalgae can be optimally detected by performing multivariate analysis. In the present study, the application of CAP to the set of variables (AA) that describe the TAA profile in the species configuring the different established groups, indicated a weak sample arrangement, with only 41.5 % of total variation explained by the two main axes (Fig. 5, PS). The samples were dispersedly distributed in the multivariate space, with only the Plantae samples scoring on the left side of axis 1 suggesting a mild clustering pattern. The main variables correlated with this side of axis 1 (glutamic acid, proline, valine) had a low coefficient, while the negative

Table 2
Similarity (Sim%) of the free amino acid profile between the studied phylogenetic groups of microalgae. The significance of each pairwise comparison is denoted by the Permanova t-statistic (P-PM). Results of the Permdisp test are in the P-PD headed column. The three most between-groups differentiating amino acid and their % contribution to dissimilarity (Simper analysis) are also indicated. Microalgae group abbreviation as in Table 1. * Significant differences between phylogenetic groups at $p < 0.05$

Groups	Sim %	P-PM	P-PD	Simper analysis
CYA-PLA	58.20	**0.001***	**0.026***	Asp (21.1 %), Glu (15.8 %), Ala (9.9 %)
CYA-OCH	16.43	**0.004***	0.812	Pro (21.5 %), Glu (21.2 %), Ser (8.5 %)
CYA-HAP	29.39	**0.001***	**0.031***	Glu (13.9 %), Tyr (13.2 %), Val (7.9 %)
CYA-MIO	44.60	**0.002***	0.622	Phe (12.5 %), Trp (11.0 %), Leu (10.9 %)
CYA-CRY	70.11	**0.004***	0.998	Ala (14.1 %), Glu (13.2 %), Asp (12.6 %)
CYA-BAC	83.79	**0.005***	0.940	Trp (14.0 %), Glu (10.5 %), Asp (10.5 %)
PLA-OCH	35.81	**0.002***	0.139	Pro (22.2 %), Asp (15.1 %), Glu (13.4 %)
PLA-HAP	47.43	**0.001***	0.199	Tyr (15.1 %), Asp (10.0 %), Ile (8.2 %)
PLA-MIO	60.56	**0.001***	0.192	Phe (12.7 %), Leu (11.2 %), Trp (10.5 %)
PLA-CRY	77.86	**0.006***	**0.027***	Asp (15.6 %), Met (11.9 %), Ser (9.2 %)
PLA-BAC	69.53	**0.001***	**0.006***	Asp (19.2 %), Glu (12.6 %), Ser (9.9 %)
OCH-HAP	39.39	**0.005***	0.172	Pro (17.6 %), Tyr (15.0 %), Phe (7.4 %)
OCH-MIO	32.70	**0.008***	0.422	Pro (14.6 %), Phe (11.8 %), Leu (8.9 %)
OCH-CRY	32.32	**0.030***	0.135	Pro (26.1 %), Glu (17.5 %), Ser (8.9 %)
OCH-BAC	26.28	**0.002***	0.156	Pro (22.6 %), Glu (20.0 %), Ser (9.5 %)
HAP-MIO	67.49	**0.004***	0.173	Met (10.6 %), Tyr (9.1 %), Phe (9.6 %)
HAP-CRY	50.51	**0.003***	0.069	Tyr (14.1), Glu (9.9 %), Phe (8.7 %)
HAP-BAC	40.38	**0.001***	**0.001***	Tyr (14.2 %), Glu (12.3 %), Val (8.4 %)
MIO-CRY	64.11	**0.012***	**0.025***	Phe (14.5 %), Leu (11.1 %), Trp (10.9 %)
MIO-BAC	54.67	**0.002***	**0.015***	Phe (13.7), Leu (11.3 %), Val (9.6 %)
CRY-BAC	81.87	**0.003***	0.963	Ala (14.9 %), Met (13.2 %), Trp (10.9 %)

correlation of alanine was stronger. CAP results for samples of TAA profiles from the literature grouped a priori in the same way as in the present study also showed a weak arranging pattern, with axes explaining similar percentages of variation (Fig. 5, L). One noteworthy aspect in this CAP was the separation and grouping of most Cyanobacteria samples on the right side of axis 1, where isoleucine showed the highest correlation. Within the total variation for the TAA profile in the global set of microalgae, the slight differentiation of Cyanobacteria in samples from the literature and Plantae in samples from the present study justifies the significance of the trace statistic in both instances (Table 3). Nevertheless, the low first squared canonical correlation and the high misclassification error reveal a low phylogenetic clustering for the general set of microalgae based on their TAA profile.

CAP results for the FAA profile in the here studied microalgae illustrated a strong clustering of samples in the multivariate space (Fig. 5, PS). The percentage of variation explained by the first two axes increased to 77.2 % and their corresponding canonical correlations were close to 1, indicating that both axes were very good at differentiating samples. The main inertia of variation determined by axis 1 clearly separated the Haptophyta group from the rest of the groups. Miozoa samples positioned centrally on axis 1 and Haptophyta samples showed maximum distance with a homogeneous set that included all species of the Cyanobacteria, Plantae, Cryptophyta and Bacillariophyta groups. This left side of axis 1 correlated strongly with glutamic acid, while tyrosine, phenylalanine, leucine, lysine, valine and isoleucine were the AA with the highest correlation on the right side of the axis side where Haptophyta samples scored (Fig. 5, PS). The 22.5 % explained variation

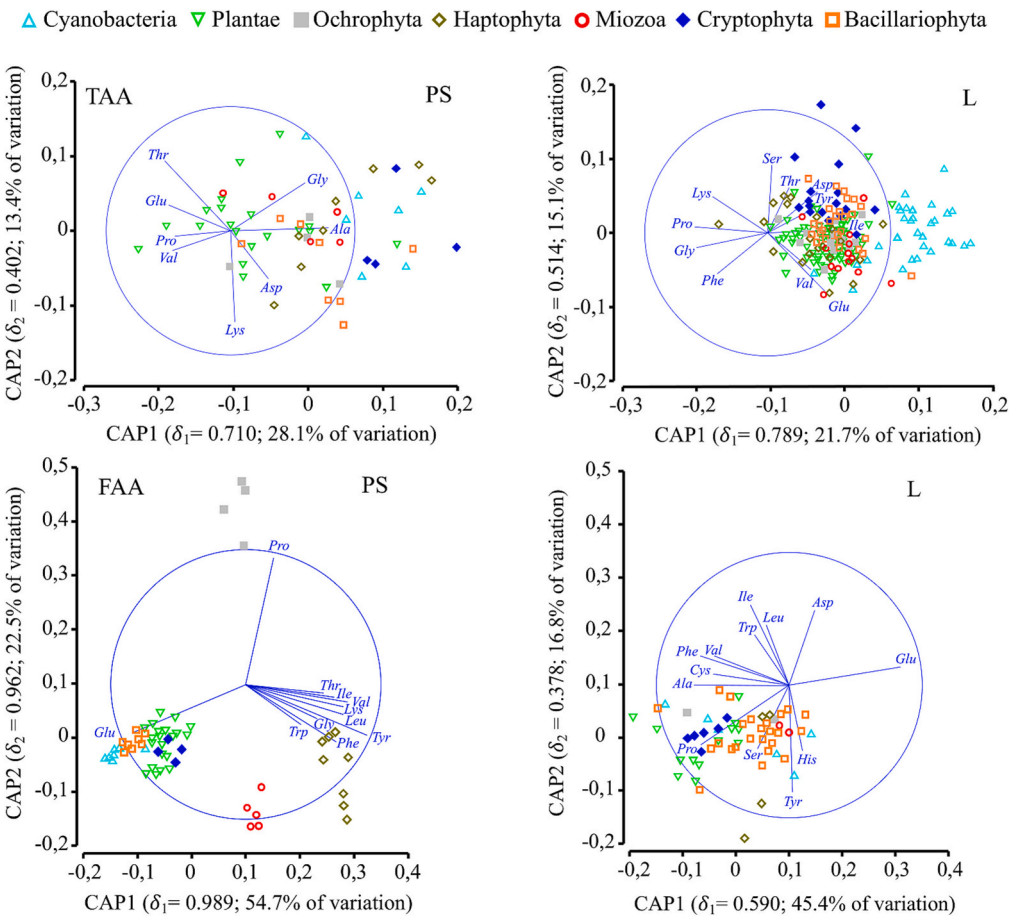

Fig. 5. Canonical analysis of principal coordinates using the phylogenetic group as a predictor variable of the total amino profile (upper graphs) and of the free amino acid profile (lower graphs) in the set of microalgae selected in present study (PS) and for the amino acid profiles available in the literature (L).

Table 3
Output of the CAP analysis for the free amino acid (FAA) and total amino acid (TAA) profile according to data from present study (PS) and from the literature (L). Results are for the trace statistic and its associated probability, the first squared canonical correlation (δ_1^2), the misclassification error (Miss E) and the percent allocation success of the analysis for each of the microalgae group. Abbreviations of phylogenetic groups as those in Table 1.

	FAA		TAA	
	PS	L	PS	L
Trace (p)	3.283 (0.001)	0.532 (0.029)	0.850 (0.003)	1.359 (0.001)
δ_1^2 (p)	0.978 (0.001)	0.348 (0.030)	0.504 (0.003)	0.623 (0.001)
Miss E	8.8	77.2	66.1	48.2
% Allocat				
CYA	85.7	0.0	14.3	85.3
PLA	85.7	53.8	70.0	50.0
OCH	100.0	0.0	0.0	33.3
HAP	87.5	25.0	12.5	20.0
MIO	100.0	100.0	0.0	53.8
CRY	100.0	16.6	25.0	52.9
BAC	100.0	8.3	25.0	44.4

by the CAP2 axis reflected the clear segregation of the Ochrophyta samples from the rest of the microalgae based on the strong positive correlation of proline with axis 2. The Cyanobacteria, Plantae, Cryptophyta and Bacillariophyta set was differentiated by the third canonical axis (CAP3), which also showed a high correlation ($\delta_3 = 0.918$), and arranged microalgae groups on their free aspartic acid content, with species of Plantae showing the highest value. The strong phylogenetic structuring of microalgae on the basis of their FAA profile was supported by the maximum values recorded for the trace statistic and the first squared canonical correlation, as well as the lowest misclassification error within the entire study (Table 3). The application of CAP on samples of FAA profiles from the literature produced a multivariate pattern with little sample clustering according to the groups configured a priori (Fig. 5, L). The variation explained by the first two axes fell to 62.2 % and the canonical correlations for axes 1 and 2 were weak. According to the minimum value of trace and δ_1^2 (Table 3), this was the least structured set of microalgae in the study.

4. Discussion

4.1. Can subtle changes in the TAA profile distinguish microalgae phylogenetic groups?

Although a high stability in the TAA profile among microalgae has been found in studies covering a relatively diversified set of microalgae [7,14], other reports described some interspecific differences [19,37,42]. Of these five studies, only the one carried out by Tibbetts et al., [42] tested significance of the results at an individual level. The possibility that subtle changes in some AA within the TAA profile may allow differentiating phylogenetic groups of microalgae has therefore hardly been explored. In this regard, the multivariate analysis carried out here provided a statistical assessment from a broader perspective than that which the individualized analysis of AA may imply. The authors are not aware of the existence of any multivariate study on microalgae AA similar to the one described here, so specific comparisons cannot be established at this respect.

While the individualized AA analysis within the TAA profile barely

allowed inferring differences between phylogenetic groups, the analysis of the TAA profile as a whole detected a slight clustering trend in the Plantae group. In this way, the CAP1 axis reflected those small changes (mostly non-significant, such as those of valine, proline and glutamic acid) of the AAs that, as a whole, better differentiated the Plantae group. This kind of descriptive approach seems promising to reveal possible patterns of AA variation among microalgae. It has been successfully applied in metabolomic studies with microalgae [31,43,44]. The closest study to the one carried out here was that of Grosse et al., [30], who were able to separate groups of natural phytoplankton based on the variation of essential and non-essential AA as a function of nutrient availability. The significance between groups, detected only for alanine in present study, was favored by the lower standard deviation for this AA, since similar absolute differences for the content of other AAs were not significant due to their higher data dispersion. Therefore, the four AAs which show the highest CAP1 correlation define better than alanine the eventual singularity in the AA profile of Plantae species. This segregation pattern for Plantae could not, however, be confirmed after the results of the multivariate analysis carried out with the published information. It could be possible that a greater data variability for the published information on Plantae had masked any eventual clustering tendency among the species of this group. This is quite plausible as AAs can differently respond to important growth factors such as nutrients [30,45,46], and culture conditions, such as light cycle [47]. The low likelihood of achieving sufficiently uniform growth conditions between works carried out by different research teams results in methodological variability that is very difficult to avoid.

Despite the assumed greater variability, analysis of TAA profiles from the literature revealed an interesting segregation pattern for Cyanobacteria. Species of Cyanobacteria formed a main cluster that was even better differentiated than that of Plantae in the species of the present study. This more evident segregation of Cyanobacteria in samples from the literature was mainly caused by their higher isoleucine content compared to the other phylogenetic groups, something that did not occur in the microalgae here analyzed. No explanation can be found for this difference and analytical issues are ruled out, since in the other microalgae groups the results for isoleucine were similar between the study samples and those in the bibliography. Assuming the greater variability in culture conditions among works, and the finding that isoleucine can increase under nitrogen deprivation in the cyanobacterium *Synechocystis* sp. [45] but not in the Ochrophyte *Nannochloropsis oceanica* [48], it is likely that nitrogen sufficiency conditions used in the present study may have contributed to the lower isoleucine content in Cyanobacteria species. It would be interesting to carry out future research to more specifically evaluate isoleucine changes against external factors in order to confirm its possible differentiating role for Cyanobacteria.

The stable proline content in the TAA profile of all the phylogenetic groups studied contrasted with the significant increase of this AA selectively in the Ochrophyta group among species from the literature. Even within a single study, proline varied from 5.1 % in *Tetraselmis chui* to 9.4 % in *Chlorella* sp. [49]. A high variability can be expected for the proline content in microalgae, since the synthesis of this AA from glutamic acid depends largely on the availability of nitrogen, with the ratio Pro/Glu being used as an indicator of nitrogen availability [50]. For this reason, it is difficult that proline can adequately differentiate phylogenetic groups of microalgae when considering the TAA profile. The stability of proline among the here studied microalgae also supports the sufficiency and uniformity in the nutrient conditions in which cultures were performed in the present work. Proline is also an AA with high physiological activity within a FAA pool that is also computed when TAA is determined. According to the scarce information available, the total FAA content can change up to two thirds, depending on nitrogen availability and culture phase [23,51]. This wide variation with respect to TAA for a fraction of FAA that usually shows a more variable AA profile represents an important issue that must be taken into account when evaluating eventual changes in the TAA profile of microalgae. To assess the impact that a variation of FAA on TAA [22,23] represent over the determination of TAA, a detailed knowledge of the factors that regulate the content of FAA in microalgae is required.. The information available in this regard is scarce and contradictory, since while the FAA content increased in the stationary phase of culture in *Phaeodactylum tricornutum* [51], the FAA of *Skelotenema costatum* decreased in stationary culture [23].

4.2. Variability in FAA strongly arrange microalgae phylogenetic groups

The discrimination of microalgae phylogenetic groups based on their FAA profile made the difference between all of them much more intense with respect to the results obtained for TAA. The CAP arrangement of phylogenetic groups reflected, however, the existence of a relatively homogeneous group encompassing species of Cyanobacteria, Plantae, Bacillariophyta and Cryptophyta, whose differentiation was little appreciable based on their FAA profile. These four groups of microalgae showed a distribution in multivariate space similar to that of these same groups when samples from the literature were considered. For these groups, both in present study and in the literature, there is a sample size that allows an acceptable interpretation of the multivariate analysis. On the contrary, the scarce number of samples available in the literature for Miozoa, Ochrophyta and Haptophyta, as well as their data variability, make their clustering difficult to interpret when applying the multivariate analysis. The greater dispersion of results revealed by the PERMDISP test for FAA profiles of microalgae in the literature also explains this situation. This effect of high dispersion obscures the results of some individual studies [25,52] in which important interspecific changes were detected for some FAAs.

In addition to more uniform culture conditions, the CAP performed in present study optimally structured microalgae groups on their FAA profile thanks to the incorporation of new samples for groups that were very poorly represented. In this way, it is possible to know the FAA variability in a phylogenetically more diversified group that better represents, therefore, the high evolutionary heterogeneity that exists among microalgae. This new, more complete information supports comments by Kuhlisch et al., [31] anticipating important differences in AA metabolism between some phytoplankton groups. Accepting that AA profiles are well conserved in structural and functional proteins throughout phylogeny, the metabolic mechanisms underlying the release or consumption of specific FAA as a function of external factors remain to be explored in most phytoplankton groups. Even for proline, an amino acid with a recognized osmoregulatory function in plants (Yoshiba et al., 1997), its response to salt stress is hardly known among microalgae (Shetty et al., 2019). In addition, the few studies available on proline changes with salinity in microalgae show inconsistent results. In diatoms, proline was found to increase with salinity in *Fragilariopsis cylindrus* [53] and remained stable during longer adaptation to high salinity in *Cyclotella cryptica* [54]. Among some halophile chlorophytes, proline cell content increased with salinity in *Dunaliella salina* [55], while in other *Dunaliella* strain, no link between proline and salinity was found [56]. Present results for free proline content in the Ochrophyta, about an order of magnitude higher compared to that in the other groups, cannot be related to a specific role in salt adaptation, since all marine microalgae species were grown under the same salinity. However, the high content of free proline in *Nannochloropsis gaditana*, together with the minimum content of its precursor glutamic acid, suggests a rather constitutive role of free proline in *N. gaditana* that deserves further study. There is some likelihood that proline plays an osmoregulatory role in marine species of *Nannochloropsis* under natural salinity values, since the relative proline level decreased markedly in *Nannochloropsis oceanica* following a salinity downshift [57]. The approximately 60 % free proline in *N. gaditana* cannot be compared to an absolute value for *N. oceanica*, since Pal et al. (2013) did not quantify proline content, but it is much higher than the 5 % proline described in

Nannochloropsis sp. [58].

Unlike the CAP2 axis, which segregated the Ochrophyta based on a non-essential AA (proline), although with high physiological functionality, the CAP1 axis, which explained the highest percentage (54.7 %) of the total variation, distinguished Miozoa and Haptophyta species mainly by their higher contents in essential AAs. Among these, it is worth noting the higher levels of free phenylalanine and lysine in Miozoa and Haptophyta, with respect to the other phylogenetic groups, something that had not been described to date. This may represent interesting implications in order to differentiate the nutritional value of both phytoplankton groups, given the importance of FAA in trophic transmission to primary consumers [28]. The high free lysine content found in the Cryptophyte *Rhodomonas salina* was associated to the relative abundance of lysine in copepod nauplii [59]. It is becoming increasingly apparent that nutritional limitation is more accurately perceived from changes in specific AA than from bulk nitrogen, as indicated by [60]. The hypothetical higher nutritional value based on the FAA profile for microalgae belonging to the Miozoa and Haptophyta groups is also supported by the comparatively higher contents found for other essential AAs such as leucine, isoleucine, methionine and tryptophan. A similar comparison could not be deduced from results in the literature, in which information on methionine, phenylalanine and tryptophan was lacking. Miozoa and Haptophyte species have been postulated as high nutritional value microalgae based on their essential fatty acid content [5].

Miozoa and, especially Haptophyte microalgae, were characterized by their higher free tyrosine content. Although not recognized as essential AA, tyrosine has been considered a semi-essential AA due to its high bioactivity as a precursor of thyroid hormones [61]. This condition is especially important when it comes to early stages of species low in the food web that directly consume phytoplankton [62]. Tyrosine-rich foods may thus contribute to supporting optimal zooplankton development and consequently lead to more efficient energy transfer to upper trophic levels. The tyrosine stability around 12 % of the total FAA for the five studied representatives of Miozoa contrasts with the 1 % tyrosine known to date as the only data available for a dinoflagellate [24]. On the other hand, the also relatively stable 29 % tyrosine among the eight Haptophyta studied contrasts both above and below with the results of other studies. Thus, in *Isochrysis galbana*, the highest content described for tyrosine varied between 68 % [25] and 47 % [52], while the minimum value was around 5 % [24]. Given the current variability and scarcity of available results for the content of certain FAAs that are determinant for the development of consumers, it is anticipated that future studies will be needed to better understand how these compounds are distributed among the different groups of microalgae.

5. Conclusions

After the analysis of the largest and most taxonomically-diverse set of microalgae studied to date, it can be concluded that there is no clear evidence to distinguish phytoplankton phylogenetic groups on the basis of their TAA profile. It is likely that the differences suggested in some works are related to the use of high stress culture conditions. Conversely, the multivariate analysis of the FAA profile allows to strongly differentiate phylogenetic groups of microalgae. It is thought that this variability may be a consequence of a highly variable AA metabolism within the wide phytoplankton phylogenetic diversity. Current results on FAA content in some microalgae groups suggest specific implications of interest in physiological (proline in Ochrophyta), nutritional (phenylalanine and lysine in Miozoa and Haptophyta) and functional (tyrosine in Haptophyta) aspects. In all phylogenetic groups of microalgae analyzed, the percentage of tryptophan within the FAA pool was lower than that detected for TAA.

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CRediT authorship contribution statement

Antonio León-Vaz: Conceptualization, Methodology, Validation, Resources, Investigation, Funding acquisition. **Inmaculada Giráldez:** Resources, Methodology, Validation, Investigation, Data curation. **Ignacio Moreno-Garrido:** Resources, Methodology, Investigation. **João Varela:** Resources, Funding acquisition, Investigation. **Javier Vigara:** Resources, Methodology, Investigation. **Rosa León:** Resources, Methodology, Investigation. **José Pedro Cañavate:** Conceptualization, Formal analysis, Resources, Investigation, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no know competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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