Comparative lipidomic analysis of *Chlorella stigmatophora* and *Hemiselmis* cf. *andersenii* in response to nitrogen-induced changes

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ARTICLE INFO

ABSTRACT

The current focus of algae biotechnology is the production of high-value lipids, and its improvement by employing abiotic perturbations such as nitrogen-induced changes. In the present study, the growth dynamics, nitrogen uptake, pigments, and lipid composition of *Chlorella stigmatophora* and *Hemiselmis* cf. *andersenii* were studied, in response to low (LN), medium (MN) and high (HN) nitrogen supplementations. Both microalgae responded to increased nitrogen levels by increasing their nitrogen uptake rate and pigment content. However, for lipid accumulation, *C. stigmatophora* presented a different pattern (LN: 16.56% > MN: 11.51% > HN: 10.95%) to that of *H.* cf. *andersenii* (MN: 15.37% > HN: 13.06% > LN: 6.71%). Untargeted gas chromatography–mass spectrometry analysis allowed the visualization of the biochemical diversity of *C. stigmatophora* and *H.* cf. *andersenii*, as well as differences in lipid regulation upon nitrogen-induced changes among species. For instance, glycosyl sterols were only detected for *C. stigmatophora* samples grown under MN and HN conditions. Moreover, lipid analysis of *H.* cf. *andersenii*, before and after alkaline hydrolysis, suggests that wax esters play a key role in the response of this microalga to high nitrogen levels. The cultivation of *H.* cf. *andersenii* at MN and HN was shown to be ideal for providing a rich source of ω3 and polyunsaturated fatty acids for nutraceutical purposes. The hierarchical cluster analysis showed the differential intra- and interspecific effects of nitrogen on lipid composition. The diverse ways by which both microalgae responded to nitrogen-induced changes highlighted the influence of phylogeny on the carbon flux through metabolic networks, and accumulation.

1. Introduction

The active search by consumers for health-conscious foodstuffs has been a driving force for studying the chemical composition of microalgae. According to Bhattacharya and Goswami [1], worldwide small scale algal growth is estimated to generate US$6.5 billion, of which US $2.5 billion comes from the health food segment. However, one of the major limiting factors for the production of microalgae continues to be the low yield of biomass and extracted products [2]. To overcome this challenge, several strategies have been studied. For example, genetic engineering techniques, like introduction of new genes, elimination of non-essential assimilatory pathways, and biochemical engineering approaches, such as optimization of nutrient formulations, study of innovative cultivation techniques — utilization of plant growth regulators [3,4].

Microalgal cells provide a biotechnological platform to modify, control and maximize the production of a targeted compound [5]. When considering algae as potential new food sources, one critical factor is their composition and nutritional content. Among lipids, the nutritional quality of microalgal species is positively associated with high levels of polyunsaturated fatty acids (PUFA), phytosterols and long-chain aliphatic alcohols (LCAA), which, in turn, can be transferred up the food-chain, adding commercial value to feeds and human dietary supplements [6–8]. Besides non-acylglycerol neutral lipids (hydrocarbons, free fatty acids, ketones), microalgae comprise polar (phospholipids and glycolipids) and acylglycerol lipids [9]. As with non-acylglycerol lipids, the quality of microalgae esterified lipid fraction is crucial for the selection of cultivation and processing strategies [10].

Microalgal lipid synthesis and accumulation can be enhanced through abiotic perturbations such as nitrogen-induced changes [11]. The main chemical forms by which photosynthetic organisms can acquire nitrogen include nitrate (NO_3^-), nitrite (NO_2^-), and ammonium

(NH_4^+) [12]. Although the information on the regulation of nitrogen acquisition and assimilation is limited to few microalgal species, previous studies have shown that NO_3^- is involved in the regulation of carbon-rich pools, such as carbohydrates and lipids [13]. In algae, NO_3^- acquisition and assimilation seem to be positively associated with its presence in the surrounding environment [13].

At the current stage of algal development, the production of biofuel from microalgal fatty acids is still a challenge due to high production costs. Therefore, the current focus of algae biotechnology is the production of high-value lipids [14]. Cryptophyte species have been shown to be rich sources of high-value products such as ω3-PUFA, carotenoids, phycobiliproteins, and sterols [15,16]. Belonging to the Cryptophyta division, *Hemiselmis* species lack heavy cell wall structures made of silica or cellulose, which, in turn, makes these microalgae easier to break down and process for commercial purposes [16,17]. In aquaculture industry, *Hemiselmis* is used as feed for fish hatcheries to improve growth and survival rates [18]. Although previous studies [17,19] have reported Cryptophyte *Hemiselmis* species as promising candidates for the sustainable and profitable production of health-promoting lipids, the modulation of *Hemiselmis* lipid quantity and composition is understudied. In this context, lipidomic studies are essential not only to reveal potential targets that may be of commercial interest, but also to understand lipid regulation in microalgae [20].

The Chlorophyta division contains the *Chlorella* genus, which is well-known to generally possess rigid, recalcitrant cell walls [21]. Species from the *Chlorella* genus are known for their health benefits when consumed as a whole, and represent the first microalgal species to be produced on a large scale [22]. Thus, species from this genus have been extensively studied, comprising models when trying to understand the mechanisms that underlie desired product accumulation [22]. Although the divisions of *H.* cf. *andersenii* and *C. stigmatophora* are rather distinct, the potential of *H.* cf. *andersenii* for high-value compound exploitation was compared to *C. stigmatophora* as one of the few marine *Chlorella* studied for the commercial application of microalgae.

While many studies assessing the impact of nitrogen-limiting conditions have been performed, few information is known about the effect of excess nitrogen supplementation in microalgae [23]. Thereby, the aim of the present study was to determine how two diverse microalgal species remodeled their growth dynamics, nitrogen uptake, pigments, and especially lipid composition in response to three different nitrogen supplementations (low, medium, and high), using gas chromatography–mass spectrometry (GC–MS) untargeted analysis as a robust method for the identification of multiple metabolites.

2. Materials and methods

2.1. Growth and culture conditions

The Cryptophyta *Hemiselmis* cf. *andersenii* (BEA 0118B) was obtained from the Spanish Bank of Algae (BEA), and the seawater Chlorophyta *Chlorella stigmatophora* (RCC 661) was obtained from the Roscoff Culture Collection (RCC). The f/2-Si medium [24,25] (8.82×10^{-4} M $NaNO_3$, 3.62×10^{-5} M $NaH_2PO_4 \cdot H_2O$, 1.06×10^{-4} M Na_2SiO_3, 1.17×10^{-5} M $FeCl_3 \cdot 6H_2O$, 1.17×10^{-5} M $Na_2EDTA \cdot 2H_2O$, 9.10×10^{-7} M $MnCl_2 \cdot 4H_2O$, 7.65×10^{-8} M $ZnSO_4 \cdot 7H_2O$, 4.20×10^{-8} M $CoCl_2 \cdot 6H_2O$, 3.93×10^{-8} M $CuSO_4 \cdot 5H_2O$, 2.60×10^{-8} M $Na_2MoO_4 \cdot 2H_2O$, 2.96×10^{-7} M Thiamine·HCl, 2.05×10^{-9} M Biotin, 3.69×10^{-10} M Cyanocobalamin) was used as the basal formula for the nitrogen manipulations, with an initial pH of 7, measured with a HI 2209 pH meter from Hannah instruments. The microalgae were grown under three different nutrient conditions by tailoring the nitrate concentrations: nitrogen-limited (low nitrogen — LN; 177 ± 11 μM $NaNO_3$), control (medium nitrogen — MN; 989 ± 13 μM $NaNO_3$) and supplemented (high nitrogen — HN; 1768 ± 14 μM $NaNO_3$). For the cultivation of the microalgae, 100 mL of exponentially growing microalgae was inoculated in 1 L flasks, giving the following initial cell concentrations: 4.95×10^5 cells

mL^{-1} for *H.* cf. *andersenii*, and 7.90×10^5 cells mL^{-1} for *C. stigmatophora*, and grown under 70 μmol m^{-2} s^{-1} light intensity, 16:8 h (light: dark cycles) at 25 °C. In the early stationary phase, the medium was centrifuged for 7 min at 3720 ×g, the supernatant was discarded, and the pellets were washed twice with an 0.09 g L^{-1} NaCl solution before being freeze-dried in a Labconco freeze dry system (Freezezone 4.5, Labconco) at −50 °C for nearly 3 days and stored at −20 °C until further analysis. According to Safafar et al. [26] storage of freeze-dried microalgae at low temperature is more effective for increasing storage stability of biomass than oxygen-reduced storage conditions (e.g. vacuum packaging). The number of microalgal cells was monitored daily by measuring the optical density of the microalgal culture at 550 nm (OD_{550}) with an ultraviolet/visible spectrophotometer (UV-6300PC, VWR, China), $R^2 = 0.9929$. The logistic model of Xin et al. [27] was used to describe the microalgal growth and fitted to the data with excel add-in solver.

2.2. Nitrogen ($NO_3^- - N$) determination

Nitrogen (N) was determined as nitrate (NO_3^-) by the ultraviolet spectrophotometric screening method previously reported by Wan et al. [28]. Briefly, a calibration curve ($R^2 = 0.9999$) was performed using 220 nm optical density (OD_{220}) against nitrogen concentrations (0–4.07 mg L^{-1}) from $NaNO_3$ solutions. The rate of N uptake by microalgal cells (Eq. (1)) was calculated as follows:

$$\text{Cell N uptake rate } \left(\text{pg N cell}^{-1}\,\text{d}^{-1}\right) = \left(\frac{N_i - N_f}{X_t}\right) \times 100 \tag{1}$$

where N_i is the initial nitrogen concentration (pg L^{-1}), N_f is the final nitrogen concentration (pg L^{-1}) and X_t is the cell concentration (cells L^{-1}) at time t (d).

2.3. Elemental analysis

The elemental analysis of carbon (C), hydrogen (H), nitrogen (N), and sulfur (S) was performed on an elemental analyser Truspec 630-200-200, LECO, USA. For elemental analysis, up to 10 mg of microalgal dried biomass were combusted in a furnace at 1075 °C. Then, the combustion gases were carried out using Helium, and further detected by infrared absorption (C, H, and S) and thermal conductivity (N).

2.4. Chlorophylls and carotenoids determination

Pigment extraction was performed from 10 mg freeze-dried biomass with an 80% acetone solution. Samples were ultrasonicated for 90 min, and centrifuged at 4430 ×g for 10 min. The supernatant was removed, and the pellet was continuously washed until the supernatant became colorless. The supernatant was passed through a 0.45 μm filter, and its absorbance was read in a spectrophotometer (UV-6300PC, VWR). The pigment content was calculated according to Chen and Vaidyanathan [29] by Eqs. (2)–(4), where Chl *a* is the chlorophyll *a* (μg mL^{-1}), Chl *b* is the chlorophyll *b* (μg mL^{-1}), and C_t is the carotenoids (μg mL^{-1}):

$$\text{Chl } a = 12.21 A_{663} - 2.81 A_{646} \tag{2}$$

$$\text{Chl } b = 20.13 A_{646} - 5.03 A_{663} \tag{3}$$

$$C_t = \frac{1000 A_{470} - 3.27 \text{Chl } a - 104 \text{Chl } b}{198} \tag{4}$$

Marine *Chlorella stigmatophora* belongs to the Chlorophyta division, which possesses chlorophylls *a* and *b*, whereas *Hemiselmis* cf. *andersenii* is a marine microalga belonging to the Cryptophyta division, which only possesses chlorophylls *a* and c_2 [15,30]. Thus, for Cryptophyta *H.* cf. *andersenii*, chlorophyll c_2 determination was performed using the equation described by Jeffrey and Humphrey [30]. Thus, acetone from the supernatant was evaporated on a nitrogen stream and the remaining water was removed by freeze-drying samples. Afterwards, the samples

were resuspended on 100% acetone. Chl c_2 is the chlorophyll c_2 (µg mL^{-1}), which was calculated by the following equation:

$$Chl\ c_2 = 27.09A_{630} - 3.63A_{663} \tag{5}$$

2.5. Lipid extraction

Total lipids were estimated according to Bligh and Dyer [31], with some modifications as described by Fernandes et al. [32]. Briefly, 50–100 mg of lyophilized microalgal biomass was used to estimate total lipids. Lipids were extracted with a methanol:chloroform mixture (2:1; v:v), followed by 400 µL of a saturated solution of KCl and 2 mL of chloroform. After homogenization, 2 mL of distilled water was added. The organic phase was removed, and the remaining biomass was continuously washed until the organic phase became colorless. Then, the organic phase was transferred to pre-weighted tubes and dried under a nitrogen stream. The extracted lipids were left to stabilize in a desiccator prior to gravimetric quantification and stored at −20 °C until further analysis.

2.6. Alkaline hydrolysis

Alkaline hydrolysis was performed to detect molecules in their esterified forms according to Santos et al. [33]. 0.5 M of NaOH in aqueous methanol was added to two lipid aliquots, and the mixtures were heated at 100 °C for 1 h in a nitrogen atmosphere. Then, the samples were acidified to pH 2 with 1 M HCl.

2.7. Gas chromatography–mass spectrometry (GC–MS) analysis

Prior to GC–MS analysis, bis(trimethylsilyl)trifluoroacetamide (BSTFA) silylation of lipid extracts before and after alkaline hydrolysis was performed as described by Santos et al. [33]. Then, trimethylsilyl (TMS) derivatives were analyzed in a gas chromatographer (6890N, Agilent Technologies, China) equipped with a mass selective detector (5973Network, Agilent Technologies, USA) and a ValcoBond capillary column from Vici Valco (30 m × 0.25 mm inner diameter, 0.25 µm film thickness). The chromatographic conditions were those described in Fernandes et al. [19]. Briefly, the oven was set to 80 °C for 5 min, increasing by 4 °C min^{-1} until 208 °C, then by 2 °C min^{-1} until 260 °C and by 5 °C min^{-1} until the final temperature of 300 °C, which was maintained for 4 min. The injector temperature was 250 °C, the transfer line temperature was 290 °C, and the split ratio was 33:1. Helium was used as the carrier gas at a rate of 1.0 mL min^{-1}. The identification of the extracted compounds as TMS derivatives was done by comparison of the mass spectra fragmentation with those from the Wiley-NIST library, literature [34–37], and when possible, by comparison with standards. For quantitative analysis, GC–MS was calibrated with pure reference compounds (mannose, *trans*-ferulic acid, nonadecan-1-ol, eicosan-1-ol, 5α-chlolestane, cholesterol, stigmasterol, hexadecanoic, and nonadecanoic acids) relative to tetracosane (internal standard).

The hypocholesterolaemic/hypercholesterolaemic fatty acid ratio (H/H), as in Santos-Silva et al. [38], was calculated as follows (Eq. (6)):

$$H/H = (C18:1\omega9 + C18:2\omega6 + C20:4\omega6 + C18:3\omega3 + C20:5\omega3 + C22 : 5\omega3 + C22:6\omega3)/(C14:0 + C16:0) \tag{6}$$

2.8. Statistical analysis

Statistical analysis of the data was carried out using the software IBM SPSS Statistics 24. Differences between treatments were assessed with one-way analysis of variance (ANOVA) followed by a Tukey post hoc analysis, with $p < 0.05$ considered statistically significant. Metaboanalyst 3.0 was used to perform multivariate analysis and metabolite-metabolite correlation analysis [39]. Prior to the data analysis, variables were median normalized, log$_2$ transformed and auto scaled. For hierarchical clustering heat maps, the Euclidean clustering distance and ward clustering method were used.

3. Results and discussion

3.1. Culture growth and nitrogen removal assessment

Chlorella stigmatophora and *Hemiselmis* cf. *andersenii* were grown under three different nitrogen conditions and sampled in the early stationary phase. The harvest days were determined as those on which the cell concentration was similar for 2 to 3 consecutive counting days; this was further corroborated by the non-significant differences found for the last cultivation days in both microalgae ($p > 0.05$). Fig. 1a shows the growth curves obtained for *C. stigmatophora*. Through this figure it is possible to see that the early stationary phase was reached at different days with respect to nitrogen supplementation: day 9 for LN conditions; and day 15 for MN and HN conditions. Meanwhile, in *H.* cf. *andersenii* (Fig. 1b), strong effects on microalgal cell growth were also observed, with the early stationary phase being reached at day 6 for LN conditions and day 8 for both MN and HN conditions.

Changes in total nitrogen (Fig. 1c and d) in the growth medium showed that both microalgae grown in LN conditions internalized the available nitrogen by day 1, confirming the initiation of nutrient-depleted conditions. In MN conditions, nitrogen was fully internalized by day 7 and day 6 for *C. stigmatophora* and *H.* cf. *andersenii*, respectively. These observations indicate that *C. stigmatophora* and *H.* cf. *andersenii*, under LN and MN cultivation conditions, continue to utilize the acquired nitrogen to support cell growth and division until they reach the early stationary phase. In both microalgae cultured under HN conditions, the nitrogen has not been fully consumed, which seemed to indicate that light-limitation occurred [40,41]. According to Lee et al. [41] cell concentration of a photosynthetic culture will continue to increase exponentially until all photosynthetically available photons are absorbed. Therefore, to verify if light-limitation did occur half the cells were returned to the conditioned media, and cell concentration was evaluated against time. For this assay, non-significant differences ($p > 0.05$) were found for cell concentration, and the highest variation rate observed for cell concentration was only 5%, indicating that a return to exponential growth did not occurred, which, in turn, shows that the assumption of light-limitation was not accurate. Thus, with the increase in initial nitrogen concentrations, a stoichiometric imbalance between nitrogen and other nutrients in the culture medium was created, which may have caused the primary growth limiting nutrient to be a different nutrient (like phosphorus) than nitrogen.

Nitrogen supplementation triggered changes in the elemental stoichiometry of both microalgae (Table 1). An interesting observation is that despite *H.* cf. *andersenii* under LN conditions had presented the lowest nitrogen content, this microalga presented the lowest (3.00) carbon to nitrogen ratio (C/N). This observation, along with the carbon content, indicates that LN conditions lead to reduced carbon fixation. In *C. stigmatophora*, the C/N ratio decreased by as much as 61% with nitrogen supplementation indicating that this microalga responded to higher nitrogen supplementation by allocating a larger proportion of carbon into nitrogen-rich pools like protein [32]. Changes in the elemental stoichiometry of microalgae in response to nutrient-induced have also been reported in other microalgae like *Rhodomonas marina*, *Nannochloropsis gaditana*, *Isochrysis* sp., and *Chlorella vulgaris* [32,42].

Under nitrogen-rich conditions, nitrogen is mainly integrated in the key structural components of microalgae, such as proteins and chlorophylls, needed to sustain cell growth [43]. In both microalgae under examination, chlorophylls and carotenoids displayed an increasing trend with nitrogen enrichment (Table 1). The observation that the lowest values were observed at LN conditions for both *C. stigmatophora* and *H.* cf. *andersenii* pigments seems to indicate that these microalgae respond to low nitrogen supplementations by triggering the degradation
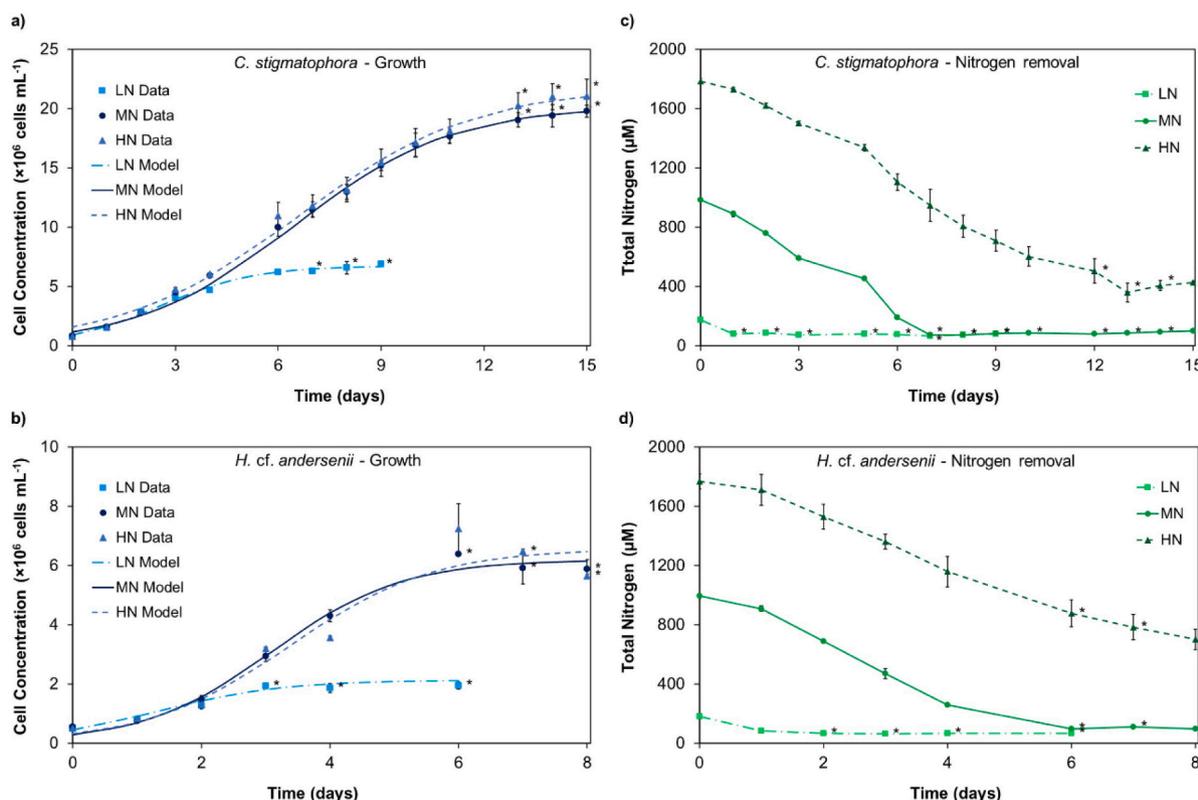
Fig. 1. Dynamics of growth and nitrogen removal for *Chlorella stigmatophora* (a, c) and *Hemiselmis* cf. *andersenii* (b, d) under low (LN), medium (MN) and high (HN) nitrogen experimental conditions. Values are expressed as mean $\pm$ standard deviation, $n = 3$ replicates. The determination coefficients (r^2) for the growth models were higher than 0.90. *Values are not significantly different ($p > 0.05$) among cultivation days, within each treatment.

of the photosynthetic machinery, in search of alternative nitrogen sources for protein synthesis to fulfil cell requirements [44].

The great biodiversity attributed to microalgae is reflected in the diverse ways by which core metabolic networks operate in microalgal species [45]. *C. stigmatophora* and *H.* cf. *andersenii* showed similar trends with respect to their cell uptake rate, which increased significantly ($p < 0.05$) with growth medium nitrogen concentrations (Table 1). For lipid accumulation, the trend displayed by both microalgae was different. For *C. stigmatophora*, the lipid content significantly ($p < 0.05$) decreased from LN (16.56% dw) to MN (11.51% dw) and HN (10.95% dw) treatments by as much as 34%, whereas for *H.* cf. *andersenii*, the lipid content significantly ($p < 0.05$) increased up to 2.74 times with increasing nitrogen concentrations, from LN (6.49% dw) to MN (15.37% dw) and HN (13.06% dw). Phylogeny determines to a large extent the efficiency of various steps involved in the desired product accumulation [45]. Moreover, intracellular organizational differences across evolutionary-distinct classes of microalgae may affect processes, such as photosynthesis, carbon flux through metabolic networks, and accumulation [45]. Thus, the highest C/N ratio (32.61) and lipid content (16.56% dw) observed at LN conditions suggests that *C. stigmatophora* responded to nitrogen-limiting conditions by increasing its intracellular composition in carbon rich pools like lipids. In contrast, *H.* cf. *andersenii* seemed to have displaced its efforts towards nitrogen storage, evidenced by the lowest C/N ratio (3.00) and lipid content (6.49%) obtained at LN conditions.

3.2. Lipid variability in response to nitrogen supplementation

Knowing that different lipid classes accumulate under specific growth stages, the early stationary phase was selected for the harvest and analysis of microalgal biomass, as it is often considered the best compromise for the simultaneous exploitation of PUFA and other high-value products, such as proteins and pigments [46]. Detailed lipidomic analysis (Fig. S1) revealed that *C. stigmatophora* and *H.* cf. *andersenii* exhibited different strategies of lipid metabolism in response to nitrogen supplementation. The lipid profiles of both microalgae revealed the presence of 66 metabolites, including fatty acids, aliphatic alcohols, sterols, monoglycerides, and other compounds (Tables 2 and 3). Significant differences ($p < 0.05$) were found for the metabolites analyzed across treatments. The analysis of *H.* cf. *andersenii* and *C. stigmatophora* samples before and after alkaline hydrolysis showed that *H.* cf. *andersenii* samples were more affected by alkaline hydrolysis than *C. stigmatophora* samples. Alkaline hydrolysis targets the ester bonds of lipids like acylglycerols, wax, and sterols esters [19]. Thus, the small changes verified for *C. stigmatophora* samples after alkaline hydrolysis might be justified by its lipid composition being richer in non-acyl lipids (like alkylglycerols) and lipid soluble compounds (such as carotenoids), which are stable to alkaline hydrolysis [19].

Through Fig. 2, it is possible to observe that the relative abundance of MUFA and PUFA significantly ($p < 0.05$) increased from LN to MN and HN treatments. This increase seems to have been achieved at the expense of SFA, which significantly ($p < 0.05$) decreased from LN to MN and HN treatments, in both microalgae. These results indicate that different nitrogen supplementations lead to membrane remodelling to respond to nitrogen-induced changes in both species studied. The degree of unsaturation of biological membranes affects their flexibility and functionality [47]. Moreover, it is known that membrane fluidity and permeability increase with the unsaturation degree of biological membranes [48]. Comparing these results with the nitrogen uptake, it is possible to observe that microalgal cell uptake in both species increased with the unsaturation degree of fatty acid, corroborating the previous assumption.

C. stigmatophora responded to increasing nitrogen supplementation by decreasing fatty acid levels (Tables 2 and 3), namely C16:0 and

Table 1

Nutrient removal, cell uptake, cell uptake rate, biomass, cell dry weight, lipid content, protein content, and elemental analysis of *Chlorella stigmatophora* and *Hemiselmis* cf. *andersenii* under low, medium, and high nitrogen supplementation.

	C. stigmatophora			*H.* cf. *andersenii*		
	Low	Medium	High	Low	Medium	High
Cell uptake rate (pg cell^{-1} d^{-1})	0.02 ± 0.00[a]	0.04 ± 0.00[b]	0.06 ± 0.00[c]	0.16 ± 0.02[A]	0.27 ± 0.01[B]	0.34 ± 0.02[C]
DCW (pg cell^{-1})	26.32 ± 3.19[a]	28.35 ± 3.31[a]	23.36 ± 1.52[a]	105.54 ± 2.66[A]	39.43 ± 3.18[B]	33.65 ± 3.04[B]
Lipid (% dw)	16.56 ± 0.75[a]	11.51 ± 1.26[b]	10.95 ± 0.52[b]	6.49 ± 1.22[A]	15.37 ± 1.38[B]	13.06 ± 0.52[C]
Chl *a* (mg g^{-1})	2.01 ± 0.03[a]	5.20 ± 0.13[b]	8.01 ± 0.48[c]	0.34 ± 0.01[A]	3.31 ± 0.01[B]	3.10 ± 0.00[C]
Chl *b* and Chl *c$_2$* (mg g^{-1})*	0.50 ± 0.02[a]	1.30 ± 0.01[b]	2.14 ± 0.13[c]	0.28 ± 0.00[A]	0.74 ± 0.10[A]	0.65 ± 0.07[A]
C$_t$ (mg g^{-1})	0.89 ± 0.02[a]	2.31 ± 0.05[b]	3.10 ± 0.22[c]	0.12 ± 0.00[A]	1.94 ± 0.01[B]	0.64 ± 0.00[C]
C (% dw)	29.70 ± 1.41[a]	30.11 ± 1.91[a]	35.98 ± 1.35[b]	4.18 ± 0.04[A]	27.29 ± 0.37[B]	24.24 ± 0.00[C]
N (% dw)	1.06 ± 0.02[a]	1.75 ± 0.03[b]	3.27 ± 0.06[c]	1.63 ± 0.10[A]	3.27 ± 0.00[B]	4.68 ± 0.02[C]
H (% dw)	4.59 ± 0.21[a]	4.48 ± 0.21[a]	4.97 ± 0.23[a]	2.12 ± 0.13[A]	4.30 ± 0.11[B]	3.81 ± 0.06[C]
C/N (mol/mol)	32.61 ± 1.57[a]	20.06 ± 0.96[b]	12.82 ± 0.24[c]	3.00 ± 0.20[A]	9.72 ± 0.19[B]	6.04 ± 0.03[C]

Values (means ± SD of at least two replicates) in the same row, not sharing a common superscript are significantly different ($p < 0.05$). Differences among treatments assessed by one-way ANOVA followed by Tukey post-hoc analysis are represented by superscript lowercase letters for *C. stigmatophora*, and by superscript uppercase letters for *H.* cf. *andersenii*. DCW: dry cell weight; Chl *a* : chlorophyll *a*; Chl *b* : C hlorophyll *b*; Chl *c$_2$* : Chlorophyll *c$_2$*; C$_t$: total carotenoids. Sulfur was not detected for *C. stigmatophora* and *H.* cf. *andersenii* samples regardless treatments applied.

* Chlorophyll *b* was estimated for *C. stigmatophora,* and chlorophyll *c$_2$* was estimated for *H.* cf. *andersenii*.

octadecanoic (C18:0) acids, which are products of *de novo* fatty acid synthesis in the chloroplast and comprise the substrates needed for MUFA, and PUFA biosynthesis [48]. Meanwhile, in *H.* cf. *andersenii*, significant shifts in fatty acid composition caused by increased nitrogen enrichment became visible in higher amounts of hexadecanoic (C16:0), 9-hexadecenoic (C16:1ω7), 9,12,15-octadecatrienoic acid (C18:3ω3; ALA), 5,8,11,14,17-eicosapentaenoic acid (C20:5ω3; EPA), and 4,7,10,13,16,19-docosahexaenoic acid (C22:6ω3; DHA).

Fatty acids were the most affected by alkaline hydrolysis, increasing up to 31 and 71% in *C. stigmatophora* and *H.* cf. *andersenii*, respectively. In *C. stigmatophora* the most affected individual fatty acids by hydrolysis were C16:0, 9-octadecenoic acid (C18:1ω9), C16:2ω6, and 9,12-octadecadienoic (LA; C18:2ω6) acids, with the biggest increase being verified for C18:1ω9, representing a fourfold increase with alkaline hydrolysis. C16:0 and C18:2 fatty acids have been reported in literature as abundant molecular species on the glycolipid's composition of *Chlorella* sp. [49]. Moreover, for other microalgae belonging to Chlorophyta division, like *Chlamydomonas reinhardtii* and *Chlamydomonas nivalis*, C18:1 fatty acids were reported to be major components of glycolipids [49]. Thus, the increase in the mentioned fatty acids after alkaline hydrolysis for *C. stigmatophora* may be because they are associated with glycolipids.

As with *C. stigmatophora*, *H.* cf. *andersenii* presented noticeable changes in fatty acid levels after hydrolysis, namely a great increase in tetradecanoic (C14:0), C16:0, ALA, and EPA contents. These variations

were more pronounced with nitrogen enrichment, with C14:0 fatty acid presenting a threefold increase. According to Guschina and Harwood [50], the plastidial glycosylglycerolipids of marine algae may contain very-long chain PUFA like EPA, in addition to ALA and C16:0. As main components of photosynthetic membranes, microalgae naturally possess high amounts of glycolipids when grown in nutrient-repleted conditions [32]. Thus, in both microalgae, the similar behavior of chlorophylls, along with the mentioned fatty acids increase after alkaline hydrolysis with nitrogen enrichment, suggests that these might be structural components of glycolipids.

A higher relative abundance of aliphatic alcohols was found for *H.* cf. *andersenii* cultured under LN conditions (up to 41% of total identified compounds). Most aliphatic alcohols occur in marine organisms as wax esters, which serve several purposes in organisms, such as energy storage and buoyancy generation [51]. Moreover, these compounds have been reported to exist in cryptophytes, namely in *Chroomonas salina* [52]. In *H.* cf. *andersenii* at MN and HN conditions, alcohols increased by as much as 39%, along with total fatty acids. These results indicate that, towards HN conditions, alcohols are mainly esterified with fatty acids (wax esters). In *C. stigmatophora* aliphatic alcohols presented a similar trend to lipids and fatty acids in response to nitrogen influence. However, after alkaline hydrolysis, a significant increase in total alcohols was not observed, indicating that these compounds are not in wax esters forms, in contrast to *H.* cf. *andersenii*.

3,7,11,15-Tetramethyl-2-hexadecen-1-ol (phytol) was found in its lowest amount in *H.* cf. *andersenii* under LN conditions (0.98 mg g^{-1} dw). When comparing samples before and after alkaline hydrolysis, this compound was the most affected by alkaline hydrolysis, increasing up to 145%. According to Yao et al. [53], this aliphatic alcohol can be used as an indicator of chlorophylls, enabling inspection of the algal cell response when exposed to low nitrogen supplementation [44]. However, in *C. stigmatophora*, the phytol content before and after alkaline hydrolysis did not present the same behavior as chlorophylls. In addition to chlorophylls, phytol is a component of other biological molecules, namely vitamin K, vitamin E, and other tocopherols [54]. As *α*-tocopherol was detected in *C. stigmatophora* (Tables 2 and 3), this could be a reasonable explanation for the different behavior shown by *C. stigmatophora*. Therefore, these results indicate that phytol is a good indicator of chlorophyll content for *H.* cf. *andersenii* but not for *C. stigmatophora*.

Monoglyceride content displayed a similar trend to lipid content with nitrogen supplementation, showing, once more, that both microalgae differently modified their lipid quantity and quality in response to nitrogen. That is, *H.* cf. *andersenii* monoglyceride content displayed a positive trend with nitrogen supplementation, in contrast to *C. stigmatophora*, which responded to increasing nitrogen supplementation by decreasing monoglyceride levels. According to Zienkiewicz et al. [55], two major pathways of triacylglycerol synthesis have been proposed to function in microalgae: *i)* Kennedy or glycerol phosphate pathway, and *ii)* monoacylglycerol pathway. The observed similar trend of lipids with monoacylglycerides upon treatments suggests that these molecules are important intermediates in anabolic fatty acid ester pathways in the microalgae studied. Thus, at LN conditions *C. stigmatophora* seemed to store carbon as triacylglycerols, in opposition to *H.* cf. *andersenii*.

Glycosyl sterols were only found for *C. stigmatophora* before hydrolysis under MN and HN conditions. These biomolecules have unique amphiphilic properties that make them suitable to be applied in food and pharmaceutical fields [34,56]. Moreover, glycosyl sterols have been reported to exist for other microalgae species belonging to Chlorophyta division, namely *Tetraselmis chui*, *Scenedesmus coastatum,* and *Platymonas helgolandica* [56,57]. Thus, the presence of glycosyl sterols under MN and HN conditions revealed the potential of these conditions for food and pharmaceutical applications.

In *H.* cf. *andersenii*, the increases in 24*α*-methylcholest-5-en-3*β*-ol and 24*α*-ethylcholest-5-en-3*β*-ol sterols by 30 and 45%, respectively,

Table 2

Lipid profile (mg g^{-1} of microalgal dry weight) of *Chlorella stigmatophora* and *Hemiselmis* cf. *andersenii* grown in three different nitrogen supplementations before alkaline hydrolysis.

No.	Identified Compounds	*Chlorella stigmatophora*			*Hemiselmis* cf. *andersenii*		
		Low	Medium	High	Low	Medium	High
	Fatty acids	*42.97 ± 1.00*[a]	*32.38 ± 1.27*[b]	*31.33 ± 0.96*[b]	*26.64 ± 0.26*[A]	*53.92 ± 0.37*[B]	*40.09 ± 1.15*[C]
	Saturated	*26.03 ± 0.23*a	*17.75 ± 0.73*[b]	*17.19 ± 0.43*[b]	*17.49 ± 0.12*[A]	*27.68 ± 0.43*[B]	*20.43 ± 0.64*[C]
1	Nonanoic acid	1.53 ± 0.01[a]	1.03 ± 0.01[b]	1.02 ± 0.01[b]	1.04 ± 0.00[A]	1.55 ± 0.00[B]	1.03 ± 0.01[C]
3	Decanoic acid	1.53 ± 0.01[a]	1.03 ± 0.01[b]	1.01 ± 0.00[c]	1.04 ± 0.00[A]	1.55 ± 0.02[B]	1.02 ± 0.01[A]
6	Undecanoic acid	1.52 ± 0.01[a]	1.03 ± 0.02[b]	1.00 ± 0.00[c]	n.d.	n.d.	n.d.
8	Dodecanoic acid	1.53 ± 0.01[a]	1.03 ± 0.01[b]	1.01 ± 0.00[c]	1.03 ± 0.00[A]	1.54 ± 0.01[B]	1.02 ± 0.01[A]
11	Tetradecanoic acid	1.55 ± 0.01[a]	1.08 ± 0.01[b]	1.05 ± 0.01[c]	1.10 ± 0.00[A]	1.97 ± 0.17[B]	1.22 ± 0.01[A]
12	Pentadecanoic acid	1.54 ± 0.01[a]	1.05 ± 0.01[b]	1.02 ± 0.01[c]	1.06 ± 0.01[A]	1.59 ± 0.01[B]	1.06 ± 0.02[A]
20	Hexadecanoic acid	3.34 ± 0.17[a]	2.43 ± 0.47[b]	2.31 ± 0.25[b]	2.69 ± 0.07[A]	5.67 ± 0.19[B]	5.44 ± 0.34[B]
22	Heptadecanoic acid	1.71 ± 0.04[a]	1.18 ± 0.05[b]	1.16 ± 0.00[b]	1.28 ± 0.01[A]	1.80 ± 0.09[B]	1.23 ± 0.05[A]
29	Octadecanoic acid	2.36 ± 0.12[a]	1.51 ± 0.21[b]	1.44 ± 0.10[b]	1.82 ± 0.02[A]	2.45 ± 0.01[B]	2.02 ± 0.09[C]
36	Eicosanoic acid	1.64 ± 0.07[a]	1.11 ± 0.06[b]	1.04 ± 0.03[c]	1.06 ± 0.00[A]	1.57 ± 0.01[B]	1.05 ± 0.01[A]
43	Docosanoic acid	1.59 ± 0.01[a]	1.08 ± 0.01[b]	1.06 ± 0.01[c]	1.12 ± 0.01[A]	1.64 ± 0.04[B]	1.10 ± 0.03[A]
45	Tricosanoic acid	1.54 ± 0.00[a]	1.04 ± 0.00[b]	1.02 ± 0.01[c]	1.05 ± 0.01[A]	1.58 ± 0.02[B]	1.04 ± 0.01[A]
49	Tetracosanoic acid	1.54 ± 0.00[a]	1.05 ± 0.00[b]	1.02 ± 0.01[c]	1.05 ± 0.00[A]	1.57 ± 0.00[B]	1.05 ± 0.02[A]
56	Octacosanoic acid	1.54 ± 0.01[a]	1.04 ± 0.00[b]	1.01 ± 0.01[c]	1.07 ± 0.01[A]	1.59 ± 0.01[B]	1.07 ± 0.01[A]
60	Triacontanoic acid	1.55 ± 0.01[a]	1.04 ± 0.01[b]	1.02 ± 0.01[b]	1.07 ± 0.01[A]	1.61 ± 0.02[B]	1.08 ± 0.01[A]
	Monounsaturated	*8.33 ± 0.63*[a]	*7.52 ± 0.49*[ab]	*7.20 ± 0.37*[b]	*4.37 ± 0.03*[A]	*11.40 ± 0.03*[B]	*8.06 ± 0.13*[C]
17	7-Hexadecenoic acid	1.63 ± 0.02[a]	1.21 ± 0.02[b]	1.11 ± 0.02[c]	1.05 ± 0.00[A]	3.17 ± 0.03[B]	2.11 ± 0.02[C]
18	9-Hexadecenoic acid[1]	n.d.	1.03 ± 0.01[a]	1.01 ± 0.01[b]	1.03 ± 0.00[A]	1.58 ± 0.01[B]	1.12 ± 0.02[C]
27	9-Octadecenoic acid	3.40 ± 0.54[a]	3.04 ± 0.46[a]	2.89 ± 0.28[a]	1.20 ± 0.01[A]	1.77 ± 0.04[B]	1.21 ± 0.03[A]
28	11-Octadecenoic acid	1.75 ± 0.10[a]	1.19 ± 0.05[b]	1.18 ± 0.05[b]	1.09 ± 0.02[A]	1.78 ± 0.02[B]	1.50 ± 0.04[C]
30	10-Nonadecenoic acid	n.d.	n.d.	n.d.	n.d.	1.56 ± 0.01[A]	1.07 ± 0.02[B]
32	12-Nonadecenoic acid	n.d.	n.d.	n.d.	n.d.	1.56 ± 0.01[A]	1.05 ± 0.01[B]
35	11-Eicosenoic acid	1.55 ± 0.01[a]	1.04 ± 0.01[b]	1.01 ± 0.01[c]	n.d.	n.d.	n.d.
	Polyunsaturated	*8.61 ± 0.24*[a]	*7.12 ± 0.20*[b]	*6.94 ± 0.17*[b]	*4.78 ± 0.16*[A]	*14.84 ± 0.11*[B]	*11.59 ± 0.39*[C]
14	C16:2ω6	1.64 ± 0.02[a]	1.35 ± 0.03[b]	1.26 ± 0.02[c]	n.d.	n.d.	n.d.
15	C16:3ω3	1.62 ± 0.01[a]	1.29 ± 0.01[b]	1.20 ± 0.02[c]	n.d.	n.d.	n.d.
16	7,12-Hexadienoic acid	n.d.	n.d.	n.d.	n.d.	1.60 ± 0.02[A]	1.17 ± 0.02[B]
19	Methyl-4,7,10,13-hexadecatetraenoate	n.d.	n.d.	n.d.	1.04 ± 0.01[A]	1.59 ± 0.01[B]	1.09 ± 0.01[C]
25	9,12-Octadecadienoic acid	1.95 ± 0.10[a]	1.82 ± 0.12[a]	1.86 ± 0.08[a]	1.14 ± 0.09[A]	1.89 ± 0.05[B]	1.28 ± 0.03[C]
26	9,12,15-Octadecatrienoic acid	1.85 ± 0.14[a]	1.60 ± 0.06[b]	1.59 ± 0.03[b]	1.45 ± 0.08[A]	3.83 ± 0.05[B]	3.16 ± 0.16[C]
33	5,8,11,14,17-Eicosapentaenoic acid	1.55 ± 0.01[a]	1.06 ± 0.00[b]	1.03 ± 0.01[c]	1.14 ± 0.00[A]	2.65 ± 0.02[B]	2.51 ± 0.13[C]
38	4,7,10,13,16,19-Docosahexaenoic acid	n.d.	n.d.	n.d.	n.d.	1.62 ± 0.01[A]	1.16 ± 0.02[B]
39	7,10,13,16,19-Docosapentaenoic acid	n.d.	n.d.	n.d.	n.d.	1.64 ± 0.01[A]	1.24 ± 0.03[B]
	Alcohols	*23.84 ± 1.00*[a]	*17.79 ± 1.21*[b]	*17.59 ± 0.30*[b]	*19.91 ± 0.20*[A]	*27.07 ± 2.53*[B]	*19.74 ± 1.21*[A]
2	Decanol	1.24 ± 0.01[a]	0.85 ± 0.02[b]	0.82 ± 0.01[b]	0.95 ± 0.01[A]	1.34 ± 0.02[B]	0.96 ± 0.04[A]
4	Undecanol	1.27 ± 0.01[a]	0.88 ± 0.04[b]	0.86 ± 0.02[b]	1.04 ± 0.02[A]	1.36 ± 0.02[B]	0.99 ± 0.03[C]
7	Dodecanol	1.26 ± 0.01[a]	0.88 ± 0.04[b]	0.86 ± 0.04[b]	0.95 ± 0.02[A]	1.32 ± 0.02[B]	0.92 ± 0.02[A]
9	Tridecanol	1.31 ± 0.01[a]	0.95 ± 0.04[b]	0.95 ± 0.06[b]	1.07 ± 0.06[A]	1.34 ± 0.05[B]	0.94 ± 0.02[C]
10	Tetradecanol	1.35 ± 0.02[a]	0.92 ± 0.02[b]	0.92 ± 0.00[b]	0.99 ± 0.02[A]	1.46 ± 0.05[B]	0.98 ± 0.04[A]
13	Hexadecanol	2.55 ± 0.28[a]	1.94 ± 0.39[b]	1.98 ± 0.08[b]	2.68 ± 0.12[AB]	3.10 ± 0.66[A]	2.21 ± 0.25[B]
21	Octadece-9-nol	3.51 ± 0.47[a]	2.64 ± 0.65a	2.68 ± 0.09[a]	3.70 ± 0.09[A]	4.22 ± 1.09[A]	3.06 ± 0.40[A]
23	Octadecanol	2.12 ± 0.18[a]	1.51 ± 0.23[b]	1.51 ± 0.04[b]	1.88 ± 0.05[A]	2.33 ± 0.37[A]	1.67 ± 0.18[B]
34	Eicosanol	1.29 ± 0.01[a]	0.87 ± 0.01[b]	0.86 ± 0.00[b]	0.90 ± 0.01[A]	1.32 ± 0.02[B]	0.88 ± 0.01[A]
40	Docosanol	1.25 ± 0.00[a]	0.84 ± 0.00[b]	0.82 ± 0.01[c]	0.85 ± 0.00[A]	1.28 ± 0.01[B]	0.86 ± 0.01[A]
50	Hexacosanol	n.d.	0.84 ± 0.00[a]	0.82 ± 0.00[b]	n.d.	n.d.	n.d.
52	Octacosanol	1.49 ± 0.05[a]	1.04 ± 0.06[b]	1.03 ± 0.01[b]	1.08 ± 0.02[A]	1.53 ± 0.12[B]	1.04 ± 0.03[A]
58	Octacosane-1,3-diol	1.31 ± 0.03[a]	0.89 ± 0.01b	0.87 ± 0.00[b]	0.94 ± 0.01[A]	1.37 ± 0.06[B]	0.92 ± 0.01[A]
59	Triacontanol	1.38 ± 0.03[a]	0.93 ± 0.02[b]	0.92 ± 0.00[b]	1.00 ± 0.02[A]	1.43 ± 0.08[B]	0.97 ± 0.03[A]
61	Dotriacontanol	1.27 ± 0.01[a]	0.85 ± 0.00[b]	0.83 ± 0.01[c]	0.88 ± 0.01[A]	1.29 ± 0.01[B]	0.86 ± 0.02[A]
24	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	1.26 ± 0.01[a]	0.96 ± 0.12[b]	0.86 ± 0.01[b]	0.98 ± 0.01[A]	2.39 ± 0.03[B]	2.49 ± 0.18[A]
	Sterols	*2.15 ± 0.20*[a]	*1.69 ± 0.28*[b]	*1.56 ± 0.04*[b]	*1.40 ± 0.09*[A]	*2.95 ± 0.39*[B]	*2.65 ± 0.15*
53	24β-Methylcholesta-5,22E-dien-3β-ol	n.d.	n.d.	n.d.	0.33 ± 0.02[A]	1.60 ± 0.08[B]	1.67 ± 0.09[B]
54	24-Methylcholesta-5,24(28)-dien-3β-ol	0.27 ± 0.01[a]	0.21 ± 0.01[b]	0.20 ± 0.01[b]	n.d.	n.d.	n.d.
55	24α-Methylcholest-5-en-3β-ol	0.31 ± 0.02[a]	0.26 ± 0.03[b]	0.25 ± 0.01[b]	0.20 ± 0.01[A]	0.28 ± 0.03[B]	0.20 ± 0.01[A]
57	24α-Ethylcholest-5-en-3β-ol	1.27 ± 0.16[a]	1.02 ± 0.22[ab]	0.89 ± 0.03[b]	0.62 ± 0.06[A]	0.73 ± 0.25[A]	0.53 ± 0.05[A]
64	Unidentified Sterol	0.28 ± 0.03[a]	0.20 ± 0.02[b]	0.21 ± 0.01[b]	0.25 ± 0.01[A]	0.34 ± 0.04[B]	0.26 ± 0.02[A]
	Monoglycerides	*0.79 ± 0.08*[a]	*0.68 ± 0.07*[b]	*0.32 ± 0.01*[b]	*0.14 ± 0.04*[A]	*0.66 ± 0.04*[B]	*1.58 ± 0.26*[C]
31	2,3-Dihydroxypropyl dodecanoate	0.06 ± 0.01[a]	0.06 ± 0.01[a]	0.05 ± 0.00[b]	0.06 ± 0.02[A]	0.07 ± 0.03[A]	0.09 ± 0.06[A]
41	1,3-Dihydroxy-2-propanyl palmitate	0.02 ± 0.01[a]	0.03 ± 0.00[b]	n.d.	n.d.	0.05 ± 0.00[A]	0.10 ± 0.02[B]
42	2,3-Dihydroxypropyl palmitate	0.35 ± 0.04[a]	0.28 ± 0.03[b]	0.11 ± 0.01[c]	0.05 ± 0.02[A]	0.26 ± 0.01[B]	0.67 ± 0.09[C]
44	3-Octadecoxypropane-1,2-diol	0.04 ± 0.01[a]	0.02 ± 0.00[b]	0.02 ± 0.00[b]	0.03 ± 0.01[A]	0.07 ± 0.02[B]	0.08 ± 0.01[B]
46	1,3-Dihydroxy-2-propanyl stearate	n.d.	0.02 ± 0.00[a]	0.01 ± 0.00[b]	n.d.	0.07 ± 0.01[A]	0.09 ± 0.02[B]
47	2,3-Dihydroxypropyl 9-octadecenoate	n.d.	0.02 ± 0.00[a]	0.03 ± 0.00[b]	n.d.	n.d.	n.d.
48	2,3-Dihydroxypropyl stearate	0.32 ± 0.03[a]	0.24 ± 0.04[b]	0.10 ± 0.01[c]	n.d.	0.15 ± 0.01[A]	0.55 ± 0.08[B]
	Others	*0.37 ± 0.04*[a]	*0.57 ± 0.09*[b]	*1.51 ± 0.09*[c]	*0.08 ± 0.05*[A]	*0.27 ± 0.01*[B]	*0.18 ± 0.06*[B]
5	2,6-bis(1,1-Dimethylethyl)phenol	0.34 ± 0.04[a]	0.19 ± 0.05[b]	0.17 ± 0.04[b]	0.08 ± 0.05[A]	0.27 ± 0.01[B]	0.18 ± 0.06[B]
51	α-Tocopherol	0.03 ± 0.01[a]	0.08 ± 0.02[b]	0.06 ± 0.01[b]	n.d.	n.d.	n.d.
62	C28:2 Steryl glycoside	n.d.	0.10 ± 0.01[a]	0.55 ± 0.01[b]	n.d.	n.d.	n.d.
63	C28:3 Steryl glycoside	n.d.	0.11 ± 0.01[a]	0.43 ± 0.04[b]	n.d.	n.d.	n.d.
65	C28:2 Steryl glycoside	n.d.	0.08 ± 0.02[a]	0.17 ± 0.01[b]	n.d.	n.d.	n.d.

Table 2 (*continued*)

No.	Identified Compounds	*Chlorella stigmatophora*			*Hemiselmis* cf. *andersenii*		
		Low	Medium	High	Low	Medium	High
66	C28:3 Steryl glycoside	n.d.	n.d.	0.13 ± 0.02	n.d.	n.d.	n.d.
	Total	*70.12 ± 1.78ᵃ*	*53.11 ± 2.86ᵇ*	*52.31 ± 0.76ᵇ*	*48.17 ± 0.42ᴬ*	*84.87 ± 3.25ᴮ*	*64.24 ± 2.67ᶜ*

Values (means ± SD of four replicates) in the same row, not sharing a common superscript are significantly different ($p < 0.05$). Differences among treatments assessed by one-way ANOVA followed by Tukey post-hoc analysis are represented by superscript lowercase letters for *C. stigmatophora*, and by superscript uppercase letters for *H.* cf. *andersenii*. All the compounds containing hydroxyl and/or carboxyl groups are identified as the correspondent trimethylsilyl (TMS) derivatives. [1]Contains *cis* and *trans* isomers. n.d. — non detected.

with alkaline hydrolysis at HN conditions, seem to indicate the presence of sterol esters in its lipid composition. Sterol esters have been reported to occur in other microalgae which, like *H.* cf. *andersenii*, belong to the kingdom Chromista, namely *Isochrysis* aff. *galbana* and *Nannochloropsis salina* [57,58]. Several assumptions have been made with respect to the physiological role of sterol conjugates, like: *i*) sterol conjugates are sterol storage pools which can be depleted when *de novo* synthesis of sterols is not sufficient, or to maintain the organization and fluidity of membranes; *ii*) the esterification of sterols and fatty acids is essential to avoid membrane perturbations from elevated sterols or free fatty acid levels resulting in a critical homeostatic response [57]. Considering this, the possibility of the production of sterol esters by *H.* cf. *andersenii*, at HN conditions, seems to be related with the first assumption, since the highest fatty acid and sterol contents before hydrolysis were found in MN conditions instead of in HN conditions.

3.3. Variations in microalgal nutritional value related to nitrogen supplementation

Fig. 3 displays the changes in the fatty acid nutritional indexes in free and hydrolysable lipid fractions of the *C. stigmatophora* and *H.* cf. *andersenii* cultured in different nitrogen supplementations. Fatty acid ratios were calculated considering their functional effects. PUFA are one of the key functional ingredients synthesized by microalgae [47]. These essential nutrients play key roles as structural components of cell membranes and cytokine precursors [47,59]. Although these fatty acids have crucial roles in animals' growth and development, PUFA precursors (LA and ALA) can only be obtained through dietary intake, and further elongation and desaturation is not enzymatically efficient [59]. Moreover, as SFA are synthesized by animals, a high PUFA intake is recommended for the diet [47].

When analysing the polyunsaturated to saturated fatty acid ratio (PUFA/SFA) against the different nitrogen supplementations (Fig. 3), it was possible to observe that both microalgae responded to increasing nitrogen levels by increasing their PUFA/SFA ratios by around double after hydrolysis. A PUFA/SFA ratio above 0.45 is recommended to induce a decrease in blood cholesterol level [38]. In *C. stigmatophora*, PUFA/SFA ratios over 0.45 were only achieved in samples cultured in MN and HN conditions after cleavage of fatty acids ester bonds. Knowing that the bioavailability of fatty acids is important for its absorption by animals, and that fatty acids in its free form have increased absorption by humans [60], these results suggest that for the application of *C. stigmatophora* as a source of a healthy PUFA/SFA ratio, a pretreatment (e.g. chemical or enzymatic hydrolysis) of *Chlorella* lipids has to be performed. In *H.* cf. *andersenii* cultured at MN and HN conditions, PUFA/SFA ratios were higher than 0.45, indicating that this microalgal strain is suitable for nutraceutical purposes, in contrast to *H.* cf. *andersenii* cultured in LN conditions.

Although for *C. stigmatophora* the ω3 to ω6 PUFA ratio (Σω3/Σω6) remained rather stable before and after alkaline hydrolysis, in *H.* cf. *andersenii* a positive trend between the Σω3/Σω6 ratio and nitrogen supplementation was observed. In this microalgal species, the Σω3/Σω6 ratio registered more than a twofold increase in response to increased nitrogen levels, suggesting that nitrogen supplementation affected the biosynthesis of PUFA by shifting enzyme specificity towards ω3 fatty

acids synthesis instead of ω6 fatty acids [48]. The same trend was displayed for another Cryptophyta (*Rhodomonas marina*) in nutrient replete conditions [48]. The high values observed for ω3 fatty acids make *H.* cf. *andersenii* at high nitrogen supplementation a suitable microalgal strain for ω3 fatty acid supplementation. ω6 predominantly dominates Western diets, with its consumption being 20 times higher than that of ω3 fatty acids [19]. However, as main precursors of pro- and anti- inflammatory molecules (e.g. prostaglandins, and leukotrienes), a balanced Σω3/Σω6 ratio close to 1:1 is recommended for the mitigation of inflammatory and chronic diseases [19]. Thus, the cultivation of *H.* cf. *andersenii* at MN and HN conditions is ideal to provide a rich source of ω3 fatty acids for nutraceutical purposes.

When stating that a product is a source of ω3 PUFA, one of the following requirements must be fulfilled: *i*) the product should have 0.3 g of C18:3ω3 per 100 g of product; or *ii*) EPA + DHA must be equal to or higher than 40 mg per 100 g of product [19]. Without hydrolysis (Table 2), *H.* cf. *andersenii* samples under MN and HN conditions presented 383 and 316 mg of C18:3ω3 per 100 g of dried algal biomass; and 427 and 367 mg of EPA + DHA per 100 g of dried algal biomass, respectively. After alkaline hydrolysis processing (Table 3), *H.* cf. *andersenii* presented 994 and 616 mg of C18:3ω3 per 100 g of dried algal biomass; and 849 and 673 mg of EPA + DHA per 100 g of dried algal biomass, at MN and HN conditions, respectively. These observations reinforce the previous assumption that *H.* cf. *andersenii* cultured at MN and HN condition is suitable for nutraceutical purposes. *C. stigmatophora* samples across treatments did not meet the above-mentioned criteria for C18:3ω3 or for EPA + DHA.

Another indicator of the nutritional quality of lipids in food is the hypocholesterolaemic/hypercholesterolaemic fatty acids ratio (H/H). This nutritional index is calculated based on the knowledge of functional effects of individual fatty acids in the cholesterol metabolism, and higher values are considered to be beneficial for human health [38]. H/H values observed for *C. stigmatophora* ranged from 1.79 to 2.73, with the main contributors go the higher H/H values being the high amounts of C18:1ω9, in combination with low levels of C16:0. The major change in H/H index found in *C. stigmatophora* was a significant ($p < 0.05$) increase (41%) observed among LN and MN conditions after hydrolysis. In the *H.* cf. *andersenii* samples, the H/H ratio varied between 1.00 and 1.76; in this microalgal species, the factor that contributed the most to the highest H/H index was the increase in unsaturated fatty acid content, namely C18:3ω3 and C20:5ω3. Moreover, the greatest increase of H/H value for *H.* cf. *andersenii* was between LN and MN conditions after hydrolysis (37%). Since the hypocholesterolemic fatty acids reduce the low-density lipoproteins (bad cholesterol) [61], samples with the highest H/H values (>1) may be important as cholesterol-lowering agents. Moreover, the ability to improve this ratio by changing initial nitrogen concentrations comprises an advantage for the biotechnological exploitation of these microalgae.

Through the above-mentioned observations, it was possible to visualize that the way in which *C. stigmatophora* and *H.* cf. *andersenii* respond to nitrogen-induced changes was species-specific and affected their nutritional value. Moreover, the analysis after and before hydrolysis proved to be crucial, since it is a rapid and high throughput methodology that provides information about the desired product bioavailability (non-esterified or esterified), which influences not only the selection of

Table 3
Lipid profile (mg g^{-1} of microalgal dry weight) of *Chlorella stigmatophora* and *Hemiselmis* cf. *andersenii* grown in three different nitrogen supplementations after alkaline hydrolysis.

No.	Identified compounds	*Chlorella stigmatophora*			*Hemiselmis* cf. *andersenii*		
		Low	Medium	High	Low	Medium	High
	Fatty acids	*48.01 ± 2.09^a*	*42.40 ± 3.61^b*	*40.05 ± 1.23^b*	*25.87 ± 2.20^A*	*85.84 ± 4.40^B*	*68.66 ± 4.67^C*
	Saturated	*22.31 ± 0.54^a*	*14.59 ± 0.82^b*	*13.95 ± 0.54^b*	*15.35 ± 1.22^A*	*41.19 ± 3.27^B*	*32.66 ± 1.75^C*
1	Nonanoic acid	2.01 ± 0.03^a	1.26 ± 0.02^b	1.19 ± 0.07^b	1.03 ± 0.00^A	2.02 ± 0.04^B	1.53 ± 0.02^C
3	Decanoic acid	2.01 ± 0.03^a	n.d.	1.19 ± 0.08^b	1.01 ± 0.00^A	2.02 ± 0.04^B	1.52 ± 0.00^C
6	Undecanoic acid	2.02 ± 0.02	n.d.	n.d.	n.d.	n.d.	n.d.
8	Dodecanoic acid	2.04 ± 0.01^a	1.27 ± 0.03^b	1.21 ± 0.07^b	1.01 ± 0.00^A	2.06 ± 0.04^B	1.54 ± 0.01^C
11	Tetradecanoic acid	2.08 ± 0.05^a	1.33 ± 0.04^b	1.29 ± 0.05^b	1.33 ± 0.01^A	5.25 ± 0.12^B	2.89 ± 0.39^C
12	Pentadecanoic acid	2.02 ± 0.03^a	1.28 ± 0.03^b	1.22 ± 0.08^b	1.05 ± 0.01^A	2.21 ± 0.05^B	1.63 ± 0.03^C
20	Hexadecanoic acid	5.37 ± 0.27^a	4.95 ± 0.55a	4.82 ± 0.10^a	5.16 ± 0.08^A	14.41 ± 0.90^B	14.05 ± 2.80^B
22	Heptadecanoic acid	2.12 ± 0.05^a	1.30 ± 0.02^b	1.26 ± 0.06^b	1.15 ± 0.02^A	2.27 ± 0.07^B	1.81 ± 0.10^C
29	Octadecanoic acid	2.64 ± 0.08^a	1.93 ± 0.11^b	1.76 ± 0.04^c	2.06 ± 0.11^A	3.75 ± 0.30^{AB}	3.76 ± 0.01^B
36	Eicosanoic acid	n.d.	n.d.	n.d.	1.02 ± 0.01^A	2.04 ± 0.04^B	1.58 ± 0.04^B
43	Docosanoic acid	n.d.	1.25 ± 0.02	n.d.	1.05 ± 0.01^A	2.08 ± 0.06^B	1.58 ± 0.02^B
45	Tricosanoic acid	n.d.	n.d.	n.d.	n.d.	n.d.	1.51 ± 0.01
49	Tetracosanoic acid	n.d.	n.d.	n.d.	n.d.	2.04 ± 0.05^A	1.52 ± 0.01^A
60	Triacontanoic acid	n.d.	n.d.	n.d.	n.d.	2.05 ± 0.03	n.d.
	Monounsaturated	*15.65 ± 1.12^{ab}*	*15.93 ± 1.87^a*	*13.38 ± 0.49^b*	*5.34 ± 0.59^A*	*16.10 ± 0.34^B*	*14.93 ± 2.02^B*
17	7-Hexadecenoic acid	2.39 ± 0.07^a	2.06 ± 0.10^b	2.06 ± 0.11^b	1.06 ± 0.02^A	4.28 ± 0.09^B	3.25 ± 0.02^C
18	9-Hexadecenoic acid[1]	2.02 ± 0.04^a	1.31 ± 0.03^b	1.26 ± 0.08^b	1.04 ± 0.00^A	2.22 ± 0.04^B	1.77 ± 0.01^C
27	9-Octadecenoic acid	9.09 ± 0.99^a	9.82 ± 1.68^a	8.60 ± 0.25^a	1.29 ± 0.04^A	2.62 ± 0.07^B	2.82 ± 0.96^B
28	11-Octadecenoic acid	2.16 ± 0.04^a	1.48 ± 0.04^b	1.46 ± 0.06^b	1.19 ± 0.03^A	2.88 ± 0.08^B	3.91 ± 1.05^B
30	10-Nonadecenoic acid	n.d.	n.d.	n.d.	n.d.	2.06 ± 0.03^A	1.61 ± 0.01^B
32	12-Nonadecenoic acid	n.d.	n.d.	n.d.	1.01 ± 0.00^A	2.04 ± 0.03^B	1.57 ± 0.01^B
35	11-Eicosenoic acid	n.d.	1.26 ± 0.02	n.d.	n.d.	n.d.	n.d.
	Polyunsaturated	*10.04 ± 0.43^a*	*11.89 ± 0.94^b*	*12.72 ± 0.21b*	*5.18 ± 0.39^A*	*28.54 ± 0.88^B*	*21.07 ± 0.95^C*
14	C16:2ω6	2.34 ± 0.09^a	2.35 ± 0.15^b	2.75 ± 0.11^b	n.d.	n.d.	n.d.
15	C16:3ω3	2.38 ± 0.07^a	2.15 ± 0.10^b	2.28 ± 0.09^{ab}	n.d.	n.d.	n.d.
16	7,12-Hexadienoic acid	n.d.	n.d.	n.d.	2.26 ± 0.05^A	n.d.	1.82 ± 0.04^B
19	Methyl-4,7,10,13-hexadecatetraenoate	n.d.	n.d.	n.d.	n.d.	2.03 ± 0.04^A	1.52 ± 0.01^B
25	9,12-Octadecadienoic acid	2.82 ± 0.17^a	3.53 ± 0.43^b	3.97 ± 0.11^b	1.28 ± 0.02^A	3.26 ± 0.03^B	2.74 ± 0.61^B
26	9,12,15-Octadecatrienoic acid	2.50 ± 0.11^a	2.56 ± 0.23a	2.53 ± 0.04^a	2.58 ± 0.23^A	9.94 ± 0.46^B	6.16 ± 0.13^C
33	5,8,11,14,17-Eicosapentaenoic acid	n.d.	1.29 ± 0.04^a	1.20 ± 0.08^a	1.31 ± 0.14^A	6.05 ± 0.21^B	4.80 ± 0.14^C
38	4,7,10,13,16,19-Docosahexaenoic acid	n.d.	n.d.	n.d.	n.d.	2.44 ± 0.07^A	1.93 ± 0.06^B
39	7,10,13,16,19-Docosapentaenoic acid	n.d.	n.d.	n.d.	n.d.	2.56 ± 0.07^A	2.10 ± 0.05^B
	Alcohols	*25.49 ± 0.79^a*	*15.25 ± 0.15^b*	*13.10 ± 0.31^c*	*15.33 ± 0.46^A*	*31.95 ± 1.65^B*	*27.38 ± 0.45^C*
2	Decanol	n.d.	1.02 ± 0.01	n.d.	n.d.	1.63 ± 0.03^A	1.23 ± 0.01^B
4	Undecanol	1.62 ± 0.02^a	1.03 ± 0.00^b	n.d.	0.82 ± 0.00^A	1.63 ± 0.03^B	1.26 ± 0.04^C
7	Dodecanol	1.63 ± 0.02^a	1.02 ± 0.01^b	n.d.	0.83 ± 0.00^A	n.d.	1.24 ± 0.01^B
9	Tridecanol	1.68 ± 0.01^a	1.03 ± 0.00^b	0.98 ± 0.06^b	0.84 ± 0.00^A	1.64 ± 0.02^B	1.28 ± 0.05^C
10	Tetradecanol	1.64 ± 0.02^a	1.03 ± 0.01^b	0.97 ± 0.06^b	0.84 ± 0.00^A	1.65 ± 0.02^B	1.25 ± 0.01^C
13	Hexadecanol	2.17 ± 0.16^a	1.35 ± 0.02^b	1.31 ± 0.02^b	1.65 ± 0.02^A	2.76 ± 0.20^B	2.37 ± 0.10^C
21	Octadece-9-nol	2.83 ± 0.23^a	1.67 ± 0.04^b	1.70 ± 0.09^b	2.28 ± 0.01^A	4.02 ± 0.50^B	3.54 ± 0.16^B
23	Octadecanol	2.05 ± 0.13^a	1.27 ± 0.03^b	1.26 ± 0.02^b	1.50 ± 0.01^A	2.55 ± 0.20^B	2.06 ± 0.03^C
34	Eicosanol	1.63 ± 0.02^a	1.02 ± 0.01^b	0.97 ± 0.06^b	0.85 ± 0.02^A	1.65 ± 0.03^B	1.24 ± 0.01^C
40	Docosanol	1.62 ± 0.02^a	n.d.	n.d.	0.83 ± 0.00^A	1.64 ± 0.02^B	1.23 ± 0.00^C
50	Hexacosanol	n.d.	n.d.	0.96 ± 0.06	n.d.	n.d.	n.d.
52	Octacosanol	1.73 ± 0.05^a	1.08 ± 0.02^b	1.05 ± 0.04^b	0.99 ± 0.01^A	1.82 ± 0.09^B	1.40 ± 0.03^C
58	Octacosane-1,3-diol	1.64 ± 0.03^a	1.02 ± 0.01^b	0.98 ± 0.05^b	0.86 ± 0.01^A	1.69 ± 0.04^B	1.26 ± 0.01^C
59	Triacontanol	1.68 ± 0.04^a	1.04 ± 0.02^b	1.00 ± 0.05^b	0.94 ± 0.01^A	1.76 ± 0.06^B	1.33 ± 0.02^C
61	Dotriacontanol	1.63 ± 0.02^a	1.02 ± 0.01^b	n.d.	0.83 ± 0.00^A	1.66 ± 0.03^B	1.24 ± 0.02^C
24	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	1.92 ± 0.04^a	1.67 ± 0.04^b	1.91 ± 0.01^a	1.48 ± 0.15^A	5.86 ± 0.38^B	5.46 ± 0.02^B
	Sterols	*1.10 ± 0.07^a*	*1.19 ± 0.08^a*	*1.07 ± 0.08^a*	*1.18 ± 0.04^A*	*2.54 ± 0.21^B*	*2.71 ± 0.24^B*
53	24β-Methylcholesta-5,22E-dien-3β-ol	n.d.	n.d.	n.d.	0.32 ± 0.01^A	1.40 ± 0.10^B	1.50 ± 0.06^B
54	24-Methylcholesta-5,24(28)-dien-3β-ol	n.d.	0.19 ± 0.00a	0.19 ± 0.01^a	n.d.	n.d.	n.d.
55	24α-Methylcholest-5-en-3β-ol	0.30 ± 0.01^a	0.21 ± 0.01^b	0.23 ± 0.00^b	0.18 ± 0.01^A	0.33 ± 0.01^B	0.26 ± 0.02^C
57	24α-Ethylcholest-5-en-3β-ol	0.80 ± 0.06^a	0.63 ± 0.06^b	0.66 ± 0.09^{ab}	0.50 ± 0.02^A	0.81 ± 0.11^B	0.77 ± 0.10^B
64	Unidentified Sterol	n.d.	0.16 ± 0.00^b	n.d.	0.18 ± 0.01^A	0.30 ± 0.01^A	0.23 ± 0.01^A
	Monoglycerides	*0.10 ± 0.01^a*	*0.05 ± 0.01^b*	*0.06 ± 0.01^b*	*0.18 ± 0.01^A*	*0.62 ± 0.11^B*	*0.67 ± 0.02B*
31	2,3-Dihydroxypropyl dodecanoate	0.02 ± 0.00^a	0.01 ± 0.00^b	n.d.	0.05 ± 0.00^A	0.06 ± 0.02^A	0.05 ± 0.01^A
37	3-Hexadecoxypropane-1,2-diol	n.d.	n.d.	n.d.	0.03 ± 0.01^A	0.26 ± 0.05^B	0.25 ± 0.01^B
41	1,3-Dihydroxy-2-propanyl palmitate	n.d.	n.d.	0.01 ± 0.00	n.d.	n.d.	n.d.
42	2,3-Dihydroxypropyl palmitate	0.01 ± 0.00	n.d.	n.d.	0.04 ± 0.00^A	n.d.	0.05 ± 0.01^A
44	3-Octadecoxypropane-1,2-diol	0.01 ± 0.00^a	0.01 ± 0.00^b	n.d.	0.06 ± 0.00^A	0.30 ± 0.05^B	0.33 ± 0.01^B
46	1,3-Dihydroxy-2-propanyl stearate	0.03 ± 0.00^a	n.d.	n.d.	n.d.	n.d.	n.d.
47	2,3-Dihydroxypropyl oleate	0.02 ± 0.00^a	0.03 ± 0.01^a	0.03 ± 0.01^a	n.d.	n.d.	n.d.
48	2,3-Dihydroxypropyl stearate	n.d.	tr^a	0.01 ± 0.00^b	n.d.	n.d.	n.d.
	Others	*n.d.*	*0.04 ± 0.00^a*	*0.03 ± 0.00^a*	*0.09 ± 0.00^A*	*0.15 ± 0.15^{AB}*	*0.36 ± 0.11^B*
5	2,6-bis(1,1-Dimethylethyl)phenol	n.d.	0.03 ± 0.00^a	0.02 ± 0.00^b	0.09 ± 0.00^A	0.15 ± 0.15^{AB}	0.36 ± 0.11^B
51	α-Tocopherol	n.d.	0.01 ± 0.00^a	0.01 ± 0.00^a	n.d.	n.d.	n.d.
	Total	*74.69 ± 2.92^a*	*58.94 ± 3.83^b*	*54.30 ± 1.46^b*	*42.65 ± 2.32^A*	*121.40 ± 6.39^B*	*99.78 ± 5.43^C*

Values (means ± SD of four replicates) in the same row, not sharing a common superscript are significantly different ($p < 0.05$). Differences among treatments assessed by one-way ANOVA followed by Tukey post-hoc analysis are represented by superscript lowercase letters for *C. stigmatophora*, and by superscript uppercase letters for

H. cf. *andersenii.* All the compounds containing hydroxyl and/or carboxyl groups are identified as the correspondent trimethylsilyl (TMS) derivatives. [1]Contains *cis* and *trans* isomers. n.d. — non detected.
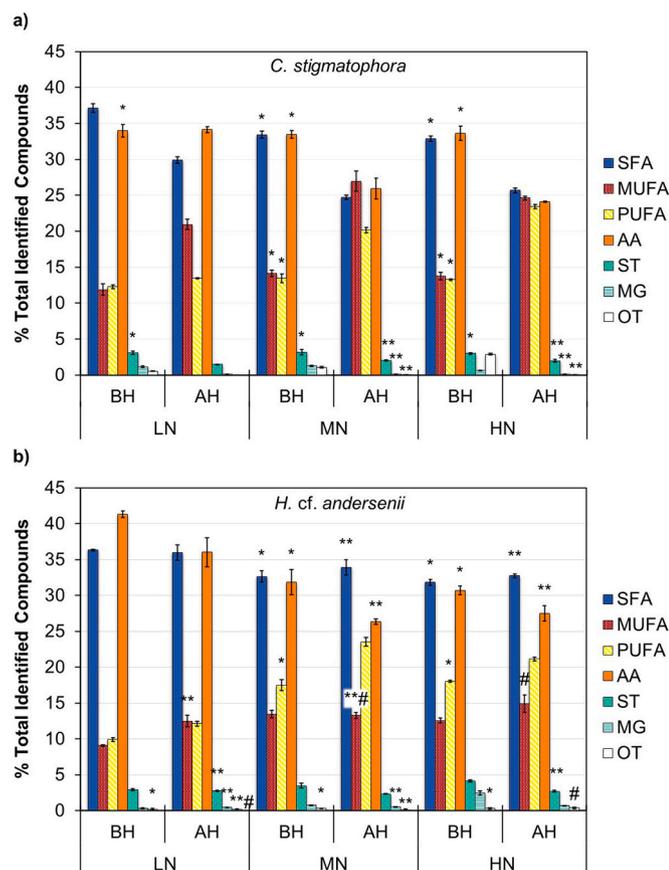

Fig. 2. Changes in main lipophilic classes before (BH) and after (AH) alkaline hydrolysis in a) *Chlorella stigmatophora* and b) *Hemiselmis* cf. *andersenii* grown under low (LN), medium (MN) and high (HN) nitrogen experimental conditions. Values are expressed as mean ± standard deviation, *n* = 4 replicates. Values from the same lipid class sharing common symbols (* before hydrolysis, and ** or # after hydrolysis) are not significantly different (*p* > 0.05) among treatments. SFA — saturated fatty acids; MUFA — monounsaturated fatty acids; PUFA — polyunsaturated fatty acids; AA — aliphatic alcohols; ST — sterols; MG — monoglycerides; OT — other compounds.

Fig. 3. Variations in a) *Chlorella stigmatophora* and b) *Hemiselmis* cf. *andersenii* nutritional values in response to nitrogen-induced changes, *n* = 4 replicates. Values from the same nutritional ratio sharing common symbols (* before hydrolysis, and ** after hydrolysis) are not significantly different (*p* > 0.05) among treatments. H/H — hypocholesterolaemic/hypercholesterolaemic fatty acids ratio; PUFA/SFA — polyunsaturated to saturated fatty acids ratio; LN — low nitrogen supplementation; MN — medium nitrogen supplementation; HN — high nitrogen supplementation; BH — before hydrolysis; AH — after hydrolysis.

microalgae but also the processing strategies to be used by the industry. For instance, non-esterified fatty acids in fish oils are more efficiently absorbed by humans than their esterified form (triacylglycerol, and ethyl esters) [60].

3.4. Multivariate analysis of lipid profile

Hierarchical cluster analysis (HCA) of microalgal lipid extracts before hydrolysis (Fig. 4a) did not reflect the classification of *H.* cf. *andersenii* cultured under different nitrogen supplementations. Instead, *H.* cf. *andersenii* grown in LN conditions (HLN) was farthest positioned, in a different clade closest to *C. stigmatophora* group. This discrimination was promoted by the greater abundance of MUFA, PUFA, C16:0, and phytol observed for *H.* cf. *andersenii* grown in MN (HMN) and HN (HHN) conditions. Moreover, the similarity of *H.* cf. *andersenii* cultured under LN conditions with *C. stigmatophora* samples was related with low values of very-long chain PUFA (EPA, DHA) and high amounts of SFA, which were common features of these samples.

After hydrolysis (Fig. 4b), it is noteworthy that *H.* cf. *andersenii* cultured under different nitrogen supplementations are within the same

cluster, despite having a distinction among HLN and the other *H.* cf. *andersenii* samples (medium nitrogen — HMN; and high nitrogen — HHN). These observations show once more that *H.* cf. *andersenii* grown in HMN and HHN are chemically more similar than HLN. Conversely, *C. stigmatophora* grown in different nitrogen supplementations (low — CLN; medium — CMN; high — CHN) did not cluster together, with CLN presenting the most distinct metabolic features.

4. Conclusions

Chlorella stigmatophora and *Hemiselmis* cf. *andersenii* growth patterns and nitrogen uptake dynamics suggests that nitrogen was not the growth limiting nutrient under high nitrogen supplementation. Gas chromatography–mass spectrometry untargeted analysis allowed the study of the lipid remodelling of both microalgae in response to nitrogen-induced changes. Microalgae grown under medium and high nitrogen supplementation showed potential for high-value compound exploitation. For instance, glycosyl sterols, suitable to be applied in food and pharmaceutical fields, were only found for *C. stigmatophora* at medium and high nitrogen supplementation, whilst the highest contents of ω3 and
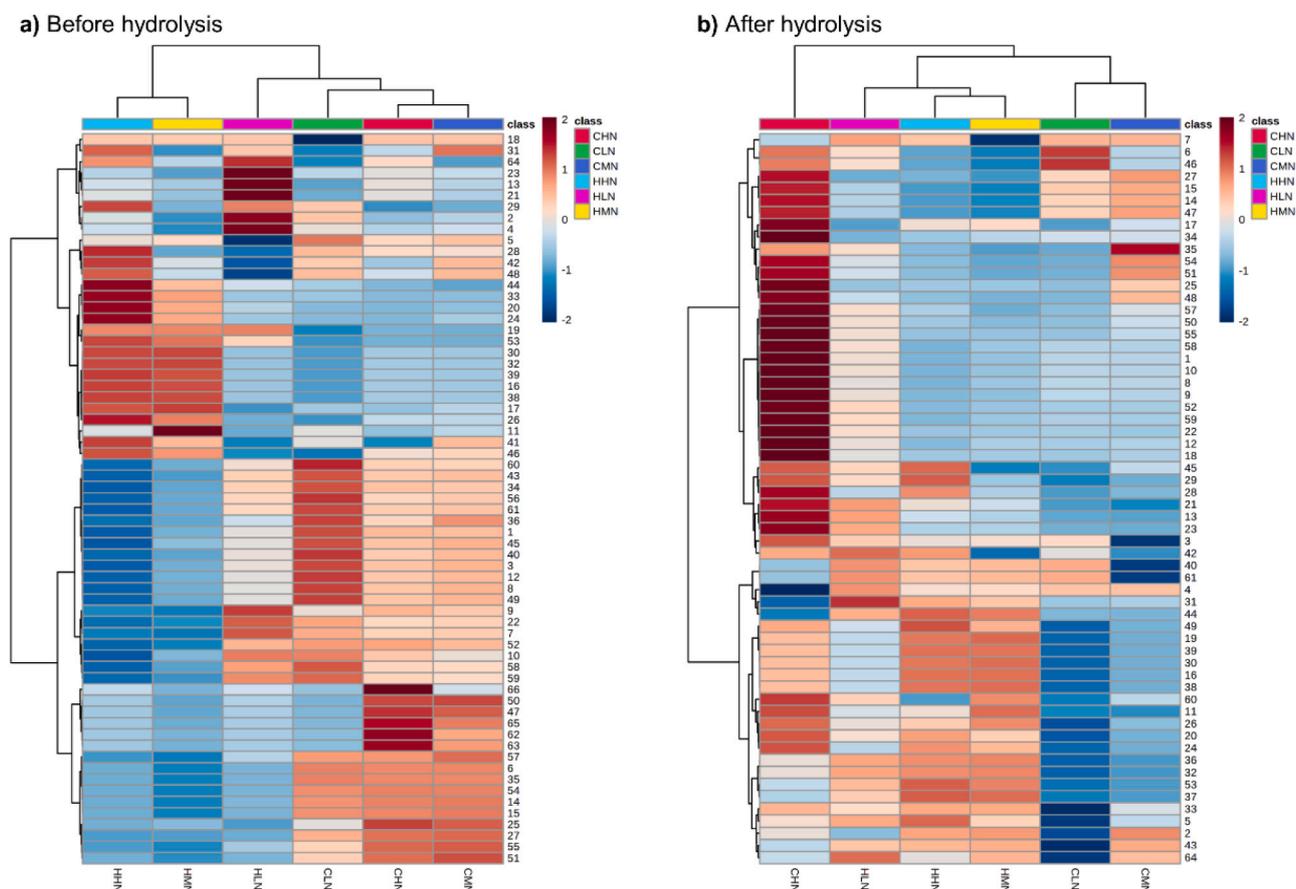
Fig. 4. Hierarchical clustering analysis heat maps based on Euclidean clustering distance and the ward clustering method, $n = 4$ replicates, for *Chlorella stigmatophora* and *Hemiselmis* cf. *andersenii* grown under different nitrogen supplementations a) before and b) after alkaline hydrolysis. Feature descriptions can be seen in Table 2 and 3. The heat maps reflect the relative levels of metabolites among different treatment groups, the color scheme is associated with the elevation and reduction in metabolite level through treatments: dark blue, lowest; dark red, highest. HLN, HMN, and HHN stands for *H.* cf. *andersenii* grown under low, medium, and high nitrogen supplementations, respectively. CLN, CMN, CHN stands for *C. stigmatophora* grown in low, medium, and high nitrogen supplementations, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

polyunsaturated fatty acids were found for *H.* cf. *andersenii* under these conditions. The analysis of chlorophyll and phytol contents show that phytol is a good indicator for the chlorophyll content of *H.* cf. *andersenii* but not for that of *C. stigmatophora*. The diverse ways by which both microalgae modulated their elemental stoichiometry and lipid content in response to the same perturbation highlighted the influence of phylogeny on the carbon flux, through metabolic networks and accumulation. However, either the use of enzyme inhibitors or genetic manipulation would be necessary to demonstrate the specific changes in metabolism.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2021.102417.

Statement of informed consent, human/animal rights

No conflicts, informed consent, human or animal rights applicable.

CRediT authorship contribution statement

Tomásia Fernandes: Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Writing – original draft, Validation. **Artur Ferreira:** Formal analysis, Project administration, Funding acquisition, Supervision, Writing – review & editing, Validation. **Nereida Cordeiro:** Conceptualization, Methodology, Project administration, Funding acquisition, Supervision, Writing – review & editing, Validation.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Nereida Cordeiro reports financial support was provided by Foundation for Science and Technology. Nereida Cordeiro reports financial support was provided by European Territorial Cooperation Programme PCT-MAC 2014-2020. Tomasia Fernandes reports financial support was provided by Regional Agency for Development of Research, Technology and Innovation of Madeira.

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