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Fatty Acids as Indicators of Environmental Condition in Microalgae

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ABSTRACT

Fatty acids (FA) were widely used as taxonomic indicators to distinguish algal groups by the presence, the abundance and the distribution of particular FA. This work aimed to compare between species FA composition of three green algae (Chlorophyceae: Chlorella vulgaris, Pseudokirchneriella subcapitata and Scenedesmus subspicatus) grown under comparable conditions, and to assess the suitability of using FA as indicators of taxonomic groups and environmental conditions for microalgae. Results showed that FA composition could not be used as a taxonomic feature of particular species because close species may present different FA composition due to their physiological state which in turn depends on environmental conditions. In this context, FA should be used as indicator at the class rather than the genus and/or species level. We propose two FA indices, the $\omega 3/\omega 6$ polyunsaturated FA ratio and an unsaturation index as indicators of physiological state and the growth phase of algae and a signs of changing environment.

Key words: Chlorophyceae, growth, unsaturation index, $\omega 3/\omega 6$ polyunsaturated fatty acids

INTRODUCTION

In aquatic ecological research, identifying the source and sinks of organic matter is an important step towards understanding trophodynamics within an ecosystem. Many techniques were used to investigate the trophic transfer between aquatic organisms, from stomach content and faecal analyses to biochemical and stable isotope analyses. It has been proposed that fatty acids (FA) may be used as indicators of energy transfers through the food chain, thus helping to understand foraging ecology and food webs (Kirsch *et al.* 1998; Iverson *et al.* 2004).

As the principal primary producers in aquatic ecosystems, algae play an essential role on the ecosystem (Pauly and Christensen 1995). Microalgae are a diverse and highly specialized group of micro-organisms adapted to various ecological and environmental conditions.

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Field as well as laboratory studies reveal that biochemical composition of algae, particularly their lipids and FA composition, varies as a function of their taxonomical affiliation and responds to a variety of physical and chemical factors such as light (McLarnon-Riches *et al.* 1998; Liang *et al.* 2006; Solovchenko *et al.* 2008), temperature (Thompson *et al.* 1992; McLarnon-Riches *et al.* 1998; Thompson 1999), aeration (Chen and Johns 1991), nutrient concentrations (Ahlgren and Hyenstrand 2003, Solovchenko *et al.* 2008) and toxicants (McLarnon-Riches *et al.* 1998; De Schamphelaere *et al.* 2007; Rocchetta *et al.* 2007), influencing both the biochemical composition and physiological status of micro- and macroalgae. Thus, FA are widely used as indicators of trophic transfer in food web studies and indicators of environmental conditions.

FA features of common algae classes are described in (Maazouzi *et al.* 2007). For instance, Chlorophyceae are characterised by a high proportion of 16:0, C16 polyunsaturated fatty acid (PUFA), essentially 16:2 ω 6, 16:3 ω 3 and 16:4 ω 3, and C18 PUFA, principally 18:2 ω 6 and 18:3 ω 3, but only trace levels or complete lack of eicosapentaenoic acid (EPA, 20:5 ω 3) and docosahexaenoic acid (DHA, 22:6 ω 3) (Cranwell *et al.* 1990; Ahlgren *et al.* 1992; Zhukova and Aizdaicher 1995). Some Chlorophyceae have been reported to have a high content of long chain PUFA (Yongmanitchai and Ward 1991; Bigogno *et al.* 2002), but this is generally rare. Different case studies and different laboratory and/or growing conditions (Wright *et al.* 1980; De la Peña 2007) can all lead to discrepancies between reported biochemical compositions for algae. For instance, where grown at different temperature, the green algae *Chlorella vulgaris* (20, 25, and 30°C) and *Botryococcus braunii* (18, 25 and 32°C) decreased its unsaturated FA content at higher temperature (Sushchik *et al.* 2003). Similarly, nitrogen starvation and high irradiance induce accumulation of arachidonic acid (20:4 ω 6) in the green alga *Parietochloris incisa* (Solovchenko *et al.* 2008; Khozin-Goldberg *et al.* 2002). In contrast, in *B. braunii*, nitrogen starvation leads to the decrease of PUFA and the increase of saturated fatty acids (Zhila *et al.* 2005). Wright *et al.* (1980) found different FA profiles in six green algae (including *Chlorella* species) when grown under three different growth conditions. Hence, a complication in any comparative study of the FA in microalgae on the basis of literature data is that FA composition of the same species can vary between studies because of growth conditions and other factors were not comparable. In this study, we aimed to compare between species FA composition of three microalgae grown under similar conditions: *Chlorella vulgaris*, *Scenedesmus subspicatus* and *Pseudokirchneriella subcapitata* (formerly known as *Selenastrum capricornutum* and *Raphidocelis subcapitata*). The three algal species studied in this work belong to the class of Chlorophyceae. These green algae are (1) widely studied for their nutritional value and (2) commonly used as target organisms and test species in ecotoxicological bioassays. To our knowledge, this work was the first study undertaken to compare FA composition of these algae, which would assess the stability of FA composition in algae from the same taxonomic group and hence the suitability of using FA as trophic indicators at the specific level in food web studies.

MATERIAL AND METHODS

Microalgae Culture

The green algal strain used, *C. vulgaris* (CCAP 211/11b), *S. subspicatus* (Göttingen 86-81) and *P. subcapitata* (ATCC 22662), were obtained from the Collection of Algal Cultures, Institute of Plant Physiology, University of Göttingen, Göttingen, Germany. Each species was grown in 2-L Erlenmeyer flasks containing 1.5 L of the same culture medium. Cells were cultivated in autoclaved Lefevre-Czarda medium which was enriched with various nutrients and microelements (I^{-1}): $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 40 mg; KNO_3 100 mg; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 30 mg; K_2HPO_4 40 mg; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.015 mg; $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, 0.03 mg; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.03 mg; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.03 mg; $\text{Mn}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 0.03 mg; $\text{C}_6\text{H}_8\text{O}_2 \cdot 7\text{H}_2\text{O}$, 0.03 mg; H_3BO_3 , 0.03 mg; $\text{C}_6\text{H}_5\text{FeO}_7 \cdot 5\text{H}_2\text{O}$, 0.8125 mg; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.3125 mg; $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 0.3125 mg; $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 40 mg.

Each flask was illuminated by fluorescent lights at an exposure intensity of 3000 lux on a 12:12 h light/dark cycle. The culture was continuously aerated and the temperature was controlled at $25 \pm 1^\circ\text{C}$. To minimize evaporation, experimental flasks were covered and the volume of the culture medium was constantly monitored. Cultures were harvested during the lighting period.

Cells were measured and their volume was calculated according to (Hillebrand *et al.* 1999). Growth rate (μ) was measured by determining cell number, using a Thoma counting chamber, and calculated as $\mu = \ln (F_1 / F_0) / (t_1 - t_0)$, where F_1 and F_0 =biomass at time t_1 and t_0 respectively (Guillard 1973).

Lipid and Fatty Acid Analyses

Samples of 40 ml algal culture were harvested and concentrated by filtering through Whatman GF/F (nominal pore size 0.7 μm) glass fiber filters. The filters were precombusted at 500°C for 48h to avoid sample's contamination by external organic compounds. All filters were added separately to glass flasks containing chloroform and stored at -80°C in an N_2 atmosphere until processed. Total lipids were extracted homogenizing filters in a mixture of chloroform–methanol (2:1, v/v) according to (Folch *et al.* 1957). Heneicosanoic acid (21:0) was added as an internal standard. Fatty acids were analyzed as FA methyl esters as described in (Maazouzi *et al.* 2008) and results were given as percentage of total fatty acid content (TFA).

To compare the unsaturated degree of FA profile between algae species, an unsaturation index (UI) was calculated as $\text{UI} = \sum A_i N_i$ where A_i = molar percentage of the total fatty acid (TFA) for the FA “ A_i ” and N_i = number of double bounds of the FA “ A_i ”. Student's t-test allowed comparison of the FA composition between species. FA distributions between species were compared using chi-square tests. Data analysis was performed using R (R Development Core Team 2007).

RESULTS

The three species were differentiated by their shape, size and growth rate. The lowest individual biovolume was observed for *P. subcapitata* ($44.9 \pm 13.4 \mu\text{m}^3$) and the highest for *C. vulgaris* ($168.3 \pm 57.3 \mu\text{m}^3$); *S. subspicatus* showed an intermediate biovolume ($59.6 \pm 15.3 \mu\text{m}^3$). *S. subspicatus* showed the highest specific growth rate ($1.01 \pm 0.1 \text{ d}^{-1}$) in comparison to *C. vulgaris* ($0.36 \pm 0.06 \text{ d}^{-1}$) and *P. subcapitata* ($0.26 \pm 0.05 \text{ d}^{-1}$) suggesting that our culture conditions were more favorable for *S. subspicatus* than for the other studied species.

The three studied green microalgae grown under similar conditions showed a related FA pattern (Table 1; χ^2 test $p > 0.23$). FA profiles showed lower proportions of saturated fatty acid (SAFA) for *C. vulgaris* ($21.78 \pm 0.92\%$ TFA) than for *P. subcapitata* ($31.51 \pm 1.08\%$ TFA) and *S. subspicatus* ($39.51 \pm 10.92\%$ TFA). Palmitic acid (16:0) and stearic acid (18:0) were the major SAFA in all samples. Similar monounsaturated fatty acid (MUFA) proportions were obtained for the three algae: $22.76 \pm 2.43\%$ TFA, $21.27 \pm 3.07\%$ TFA and $23.95 \pm 09.04\%$ TFA for *C. vulgaris*, *S. subspicatus* and *P. subcapitata* respectively. We found almost the same predominant MUFA: oleic acid (18:1 ω 9), palmitoleic acid (16:1 ω 7) and cis-vaccenic acid (18:1 ω 7). PUFA were amounted to $55.45 \pm 2.47\%$ TFA in *C. vulgaris*, $44.54 \pm 8.78\%$ TFA in *P. subcapitata* and $39.21 \pm 11.17\%$ TFA in *S. subspicatus*. PUFA were principally present as ω 3 series except for *S. subspicatus* which presented relatively close values of ω 6 PUFA compared to ω 3 PUFA. Under our experimental conditions, linoleic acid (LA, 18:2 ω 6) was the most abundant ω 6 PUFA in all samples examined, followed by arachidonic acid (ARA, 20:4 ω 6) and eicosadienoic acid (20:2 ω 6). In *C. vulgaris*, the ω 3 series were essentially represented by DHA, followed by EPA and linolenic acid (LNA, 18:3 ω 3). *S. subspicatus* and *P. subcapitata* showed the same major ω 3 PUFA but lower DHA and LA percents. *P. subcapitata* was distinguished from the two other chlorophytes by higher percents of 18:4 ω 3.

Table 1. Average level (mean \pm SE, N = 3) of major fatty acid (as percentage of total fatty acids) in the studied green algae. UI: unsaturation index. SAFA: sum of saturated fatty acids. MUFA: sum of monounsaturated fatty acids. PUFA: sum of polyunsaturated fatty acids.

Algae	<i>Chlorella vulgaris</i>	<i>Scenedesmus subspicatus</i>	<i>Pseudokirchneriella subcapitata</i>
14:0	1.55 \pm 0.51	1.89 \pm 0.33	2.05 \pm 0.78
14:1	1.79 \pm 0.61	0.70 \pm 0.19	0.87 \pm 0.47
16:0	14.02 \pm 0.39	28.26 \pm 9.01	17.39 \pm 3.50
16:1 ω 9	2.62 \pm 0.54	1.77 \pm 0.70	1.91 \pm 0.54
16:1 ω 7	4.40 \pm 0.92	2.86 \pm 1.32	8.80 \pm 6.61
16:2 ω 4	0.41 \pm 0.13	0.33 \pm 0.17	0.55 \pm 0.22
16:3 ω 4	0.59 \pm 0.09	0.39 \pm 0.06	0.34 \pm 0.09
16:4 ω 3	0.16 \pm 0.01	0.18 \pm 0.02	0.72 \pm 0.44
16:4 ω 1	1.24 \pm 0.38	0.33 \pm 0.06	0.76 \pm 0.39
18:0	3.25 \pm 0.32	4.48 \pm 0.92	3.91 \pm 0.76
18:1 ω 9	7.15 \pm 0.15	7.82 \pm 1.08	4.84 \pm 0.95
18:1 ω 7	2.32 \pm 0.17	2.36 \pm 0.17	4.38 \pm 2.26
18:2 ω 6	10.24 \pm 4.06	9.38 \pm 5.11	5.36 \pm 2.01
18:3 ω 3	3.7 \pm 0.39	2.12 \pm 0.38	2.59 \pm 0.50
18:4 ω 3	0.95 \pm 0.15	0.90 \pm 0.20	1.89 \pm 0.51
18:4 ω 1	1.74 \pm 0.65	1.18 \pm 0.35	1.49 \pm 0.68
20:1	2.03 \pm 0.61	1.81 \pm 1.09	2.03 \pm 1.25
20:2 ω 6	2.18 \pm 0.82	1.98 \pm 0.63	2.07 \pm 0.91
20:4 ω 6	0.81 \pm 0.07	0.60 \pm 0.18	0.92 \pm 0.04
20:5 ω 6	2.67 \pm 0.97	2.64 \pm 0.88	3.76 \pm 0.14
20:5 ω 3	9.24 \pm 0.72	4.64 \pm 1.59	8.25 \pm 1.50
22:0	0.19 \pm 0.04	0.27 \pm 0.07	1.19 \pm 0.56
22:1	1.43 \pm 0.81	1.44 \pm 1.13	0.33 \pm 0.13
22:2	2.64 \pm 0.97	2.77 \pm 0.91	2.62 \pm 1.28
22:5 ω 6	1.92 \pm 0.72	2.08 \pm 0.63	1.97 \pm 0.98
22:5 ω 3	1.00 \pm 0.07	0.67 \pm 0.18	1.67 \pm 0.44
24:0	0.44 \pm 0.15	0.68 \pm 0.25	1.17 \pm 0.59
22:6 ω 3	14.23 \pm 0.69	7.27 \pm 2.95	7.99 \pm 4.60
SAFA	21.78 \pm 0.92	39.51 \pm 10.92	31.51 \pm 1.08
MUFA	22.76 \pm 2.43	21.27 \pm 3.07	23.95 \pm 9.04
PUFA	55.45 \pm 2.47	39.21 \pm 11.17	44.54 \pm 8.78
UI	251.1 \pm 14.8	169.4 \pm 43.5	205.4 \pm 54.9
ω 3 PUFA	30.04 \pm 1.78	16.68 \pm 5.03	23.97 \pm 4.66
ω 6 PUFA	18.37 \pm 1.82	17.13 \pm 6.04	13.69 \pm 4.78
ω 3/ ω 6	1.69 \pm 0.27	1.05 \pm 0.23	2.39 \pm 0.92

A statistically significant difference was observed only for unsaturated FA (as the sum of MUFA and PUFA) between *C. vulgaris* and *P. subcapitata* ($p < 0.01$) and for LNA between *C. vulgaris* and *S. subspicatus* ($p = 0.04$). The difference on the FA classes (i.e. saturated vs. unsaturated FA) was reflected in the unsaturation index where UI was similar for *C. vulgaris* (251.1 ± 14.8) and *P. subcapitata* (205.4 ± 54.9) but higher than for *S. subspicatus* (169.4 ± 43.5). In spite of the distinctions between species for some FA percentages, the lack of statistically significant differences may be due to the high variability of our data.

DISCUSSION

The three green algae showed considerable proportions of C20 and C22 PUFA while C16 PUFA remained in low percentages under our experimental conditions which contrasted with literature data regarding FA pattern of Chlorophyceae (Cranwell *et al.* 1990; Ahlgren *et al.* 1992; Zhukova and Aizdaicher 1995). Discrepancies observed in the FA composition between our results and those for other green microalgae from the literature may originate from (a) species /strain-specific lipid composition (b) the culture and growth of algae under different conditions. The ability of algae to modify their lipid metabolism in response to changing environmental conditions can be interpreted as an adaptive mechanism ensuring their growth and survival (Guschina and Harwood 2006). These adaptations are often species specific and generalised responses are rarely applicable to all members of the group because the intrinsic ability to produce large quantities of lipid is species/strain-specific rather than genus-specific (Hu *et al.* 2006).

Many eukaryotic algae change their fatty acid composition drastically during growth in order to fabricate more cellular membranes (McLarnon-Riches *et al.* 1998), and show high abundance of PUFA (Volkman *et al.* 1989). Under optimal conditions of growth, algae synthesize fatty acids, principally for esterification into glycerol-based membrane lipids, and include medium-chain (C10 – C14), long-chain (C16 – C18) and very-long-chain (\geq C20) fatty acids and fatty acid derivatives. Thus, the lipid composition of microalgae can change with their growth rate and/or environmental condition as well as with the phase of their life cycle. We assume that both *C. vulgaris* and *P. subcapitata* were growing in exponential phase under non-limiting conditions which was reflected in the abundance of PUFA and a high UI and $\omega 3/\omega 6$ PUFA ratios. Ahlgren *et al.* (1992) reported that the $\omega 3/\omega 6$ ratio is mostly higher when algae are growing in exponential phase than under limiting conditions.

In this study, we found a highest $\omega 3/\omega 6$ PUFA ratio for *C. vulgaris* and *P. subcapitata* (up to 2.4) supporting our previous assumption. In contrast, *S. subspicatus* may deal with nutrient limitation, mainly nitrogen, since nitrogen limitation in *Scenedesmus* led to a reduction of LNA and consequently a decrease of $\omega 3/\omega 6$ PUFA ratio (Ahlgren and Hyenstrand 2003). Indeed, when bulk nutrients or micronutrient become limiting, leading to the cessation of cellular division (e.g. stationary phase), neutral lipids are accumulated mainly in the form of triacylglycerols (Hodgson *et al.* 1991; Ahlgren *et al.* 1992; Chu *et al.* 1997) with the increase of relative percentage of SAFA (Chu *et al.* 1997; Alonso *et al.* 2000).

Furthermore, nutrient limitation may prevent the production or activity of enzymes involved in the desaturation processes contributing in the accumulation of SAFA and MUFA., Hence, our data suggest the onset of the stationary phase for *S. subspicatus* where the tendency was the increase of SAFA at the detriment of PUFA and consequently low values of UI (169) and $\omega 3/\omega 6$ PUFA ratio (≈ 1). Hayakawa *et al.* (2002) suggest that the $(18:3\omega 3 + 18:4\omega 3) / (18:2\omega 6 + 18:3\omega 6)$ ratio could be used as an indicator of the physiological state of the *Microcystis* cells; they found a positive correlation between such FA ratio and growth rate of this cyanobacteria. Looking for this relationship in our data set, we found a positive correlation between this FA ratio and the growth rate of studied green microalgae but it was not significant ($n=9$, $R^2 = 0.18$, $p = 0.26$). However, $\omega 3/\omega 6$ PUFA ratio was significantly and inversely correlated to growth rate ($n = 9$, $R^2 = 0.51$, $p = 0.03$). Thus, on the basis of our results, we

suggest the $\omega 3/\omega 6$ PUFA ratio and to a lesser extent the UI as indicators of the physiological state and the growth phase of microalgae.

Fatty acids have previously been widely used as indicators to distinguish algal groups by the presence, the abundance and the distribution of specific FA. However, as shown in this work, FA composition should not be used as a taxonomic feature of particular species because related species have similar FA distributions and even the same species may present different FA compositions due to their physiological state which in turn depends on environmental conditions. The relationship between FA composition and the taxonomic position of algae is complex, and the indicator approach of FA should be used to distinguish algae at the class level rather than the genus and/or species.

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