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The high content of polyunsaturated fatty acids in *Nannochloropsis limnetica* (Eustigmatophyceae) and its implication for food web interactions, freshwater aquaculture and biotechnology

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Abstract

In the eustigmatophycean *Nannochloropsis limnetica* the content of polyunsaturated fatty acids (PUFA) is extremely high in comparison to different planktonic green algal taxa in freshwater ecosystems. The sums of n-6 and n-3 fatty acids in *N. limnetica* were ten-fold higher than in the other picoplankton *Choricystis minor* and *Pseudodictyosphaerium jurisii*, and higher than in the nanoplanktonic green algae *Chlorella vulgaris*, *Monoraphidium neglectum* and *Scenedesmus obtusiusculus*. The content of fatty acids in *N. limnetica* was highly variable under different culture conditions. The highest concentrations of PUFA in *N. limnetica* were found in non-aerated suspension cultures, with a high content of phosphate (40 mg l^{-1} K_2HPO_4) in the culture medium: linoleic acid 22.19 mg g^{-1} DW, arachidonic acid 10.52 mg g^{-1} DW, and eicosapentaenoic acid 55.56 mg g^{-1} DW. *N. limnetica* represent a high-quality food resource in freshwater food chains. Furthermore, cultures of this eustigmatophycean alga have a high potential for use in biotechnology and aquaculture.

Keywords: Chlorophyceae; Eustigmatophyceae; Trebouxiophyceae; *Nannochloropsis*; Polyunsaturated fatty acids; Food web interactions; Picoplankton; Aquaculture; Biotechnology

Introduction

Members of the genus *Nannochloropsis* (Eustigmatophyceae) are widely distributed in the plankton of marine ecosystems, particularly in coastal waters; until now, five different *Nannochloropsis* species are recognized in saline habitats (Andersen, Brett, Potter, & Sexton, 1998; Hibberd, 1981; Karlson, Potter, Kuylenstierna, & Andersen, 1996; Suda, Atsumi, & Miyashita, 2002). *Nannochloropsis* is successfully used as a food in marine aquaculture (Watanabe, Kitajima, & Fujita, 1983) and is cultured on an industrial scale (Chini Zitelli, Rodolfi, & Tredici, 2004), due to the high content of fatty acids, especially eicosapentaenoic acid (Sukenik, 1999; Volkman, Jeffrey, Nichols, Rogers, & Garland, 1989).

Observations on *Nannochloropsis* in freshwaters are very scarce. Krienitz, Hepperle, Stich, and Weiler (2000) described the first freshwater species: *N. limnetica* which was found in a highly productive village pond in Germany. Later, this species was also found in different

Abbreviations: PUFA, polyunsaturated fatty acids; DW, dry weight

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types of lakes in Germany, North America and in Russia (Fawley & Fawley, 2004; Fawley, et al., 2004; Fietz et al., 2005; Krienitz et al., 2000).

N. limnetica is a member of the picoplanktonic size class (smaller than 3 µm in diameter). Due to a very effective volume-to-surface ratio phytoplankton of this size fraction contribute to a high amount of the primary production of inland lakes and ponds of different trophic state (Hepperle & Krienitz, 2001; Stockner, 1991). The picoplankton populations in stagnant inland waters are dominated by the green algae *Choricystis minor* (Trebouxiophyceae) and *Pseudodictyosphaerium jurisii* (Chlorophyceae) (Fawley, Fawley, & Owen, 2005; Hepperle & Krienitz, 2001; Krienitz, Takeda, & Hepperle, 1999; Padisák, Krienitz, Koschel, & Nedoma, 1997). However, in some cases the dominating taxon is *N. limnetica*, which was documented for the hypertropic village pond Dorfteich Schwarz, the polytrophic Lake Roter See in Germany (Krienitz et al., 2000), the mesotrophic Lake Itasca, Minnesota, USA (Fawley et al., 2005) and the oligotrophic Lake Baikal, Russia (Fietz et al., 2005). These populations of *Nannochloropsis* in waters of very different trophic state and morphometry were accompanied by subdominating populations of green algal picoplankton.

The rapid reproduction and strong growth of *N. limnetica* both under field and culture conditions and the high content of fatty acids, comparable to that of the marine *Nannochloropsis* species (Krienitz et al., 2000) make the alga interesting for food web studies, freshwater aquaculture and biotechnology.

The aim of the present study was to compare the content of fatty acids in *N. limnetica* with that of two highly abundant and productive green algal picoplankton species from freshwater lakes (*Choricystis minor* and *Pseudodictyosphaerium jurisii*) and three key taxa of importance for biotechnology and aquaculture (*Chlorella vulgaris*, *Scenedesmus obtusiusculus*, *Monoraphidium braunii*).

Material and methods

The unialgal strains of the six algae species used in this study (Table 1) were maintained as agar cultures in a modified Bourrelly medium (Table 2) in the strain collection of the Leibniz-Institute of Freshwater Ecology and Inland Fisheries (IGB). Duplicates of these strains are available from the Sammlung von Algenkulturen der Universität Göttingen (SAG) and the Culture Collection of Algae and Protozoa of the Scottish Association for Marine Science (CCAP). The modified Bourrelly medium (without agar) was used to cultivate the algae in suspension in order to investigate their different concentrations of fatty acids in the strains. To study the influence of different concentrations of phosphate on fatty acid production in *N. limnetica*, the K_2HPO_4 content of the liquid culture medium was graduated to 100% (40 mg l^{-1}), 50% (20 mg l^{-1}), 25% (10 mg l^{-1}) and 10% (4 mg l^{-1}).

To study the influence of different culture conditions on the fatty acid content of *N. limnetica*, the algae were transfered from agar into two different types of suspension cultures: (i) aerated suspensions (without CO_2 enrichment) in 300 ml culture tubes, under continuous illumination, and (ii) non-aerated suspensions in 100 ml Erlenmeyer flasks, shaken twice a day and under a light/dark regime of 14 h/10 h. Cultures were hatched at room temperature (about 20 °C) and a light intensity of ~180 µmol photons m^{-2} s^{-1}. After transferring the algae from stock agar cultures to experimental suspensions, 1 week was allowed to enable the organisms to adapt to the new culture conditions before experiments began.

At the starting time, in the different treatments the biomass (fresh weight) of the algae was 10 mg l^{-1}. From the aerated cultures the algal biomass was harvested in the exponential phase of growth (after 3 days) and in the stationary phase (after 1 week). From the non-aerated suspensions the fresh cell mass was harvested after 3 weeks. At the time of harvest, the suspensions of the different algae contained about 100–400 mg l^{-1} fresh weight. All cultures were run in five parallels and pooled before harvest. The biomass was determined after counting of the cell density in a Thoma counting chamber (Feinoptik GmbH Bad Blankenburg, Germany). For calculation of biomass by geometrical approximations, the computer programme Opticount was used (Hepperle, 2000).

Table 1. Algal strains used in this study

Species	IGB strain number	SAG/CCAP strain number
Nannochloropsis limnetica Krienitz, Hepperle, Stich et Weiler	KR 1998/3	SAG 18.99
Monoraphidium braunii (Nägeli in Kützing) Komárková-Legnerová	KR 1980/22	SAG 2006
Scenedesmus obtusiusculus Chodat	KR 1979/301	CCAP 276/25
Chlorella vulgaris Beijerinck	KR 1979/36	CCAP 211/81
Choricystis minor (Skuja) Fott	KR 1979/121	CCAP 6055/1
Pseudodictyosphaerium jurisii (Hindák) Hindák	KR 1996/1	CCAP 260/1

Table 2. Culture medium for algal cultivation

	Stock solution (g 100 ml^{-1})	Applied solution (ml l^{-1})	Final concentration (mg l^{-1})
KNO_3	10	2	200
K_2HPO_4	1	4	40 [a]
$MgSO_4 \cdot 7H_2O$	1	3	30
$Ca(NO_3)_2$	1	3	30
$NaHCO_3$	1.68	10	168
FeDTA-solution:			
$FeSO_4 \cdot 7H_2O$	0.7	0.5	3.5
EDTA (Titriplex III)	0.9	0.5	4.5
Vitamins:			
Biotin	0.0033	10	0.33
Vitamin B12	0.0005	10	0.05
Thiamin	0.0005	10	0.05
Trace elements (the following components were given together in 100 ml for the stock solution, and 0.5 ml of the stock solution was used in 1 l of medium)			
$MnCl_2 \cdot 4H_2O$	0.00990		4.95
$CoSO_4 \cdot 7H_2O$	0.00028		0.14
$CuSO_4 \cdot 5H_2O$	0.00005		0.025
$ZnSO_4 \cdot 7H_2O$	0.00073		0.36
H_3BO_3	0.00031		0.155
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.00018		0.09
$NiSO_4 \cdot 6H_2O$	0.00263		0.1315
NH_4VO_3	0.00015		0.075

[a]Gradually decreased from 40 to 4 mg l^{-1}.

For determination of fatty acid content, the algal suspensions were freeze-dried. The samples were weighted and transferred into a plastic tube to which 5 ml chloroform/methanol (2:1 v/v) and 1 ml internal standard (0.4 mg tricosan acid) were added. The samples were held for 90 min in an ultrasonic device (water bath) for lipid extraction. The extraction products were filtered and washed twice with the extraction solvent. The samples were evaporated, and 5 ml methanol/sulphuric acid (95:5 v/v) was added to convert the fatty acids into the methyl esters. After heating the samples for 4 h at 80 °C, 10 ml water was added. The fatty acid methyl esters were extracted twice with 2 ml *n*-hexane. Subsequently, the fatty acids were analysed by gas–liquid chromatography (GC 3600, Varian) as described in Wirth, Steffens, Meinelt, and Steinberg (1997). The separation of fatty acids was performed using a 30 mm capillary column (Omegawax 320, Supelco). The fatty acids were identified by comparison of retention times with retention times of a calibration standard solution (Supelco), and quantified by comparing the peak areas with the peak area of the internal standard.

Results

The fatty acid contents varied greatly in the six different algae species grown under identical culture conditions (Table 3). Among the six species, the saturated fatty acids palmitic (16:0) and stearic acid (18:0), and the monounsaturated palmitoleic (16:1 n-7) and oleic acid (18:1 n-9) were dominant (Table 3). In comparison to the five different planktonic green algal taxa in the eustigmatophycean alga *N. limnetica* the concentration of PUFA is high. The essential fatty acids linoleic (18:2 n-6) and α-linoleic acid (18:3 n-3) were found in highest concentrations in *N. limnetica* and *Scenedesmus obtusiusculus*. In contrast to these observations, *Choricystis minor* and *Monoraphidium neglectum* are rich in 18:3 n-3. Arachidonic acid (20:4 n-6) was detected in the highest concentrations in *N. limnetica* and in lower concentrations in *M. neglectum* and *S. obtusiusculus*. Furthermore, the results indicate that *N. limnetica* is an important source for eicosapentaenoic acid (20:5 n-3). Relatively high contents of this fatty acid were also found in *M. neglectum*. The content of docosahexaenoic acid (22:6 n-3) is very low in *N. limnetica*, although this alga is rich in n-3 fatty acids. Under the same culture conditions we have found the highest content of 22:6 n-3 in *S. obtusiusculus*.

The content of fatty acids in *N. limnetica* was variable under different culture conditions (Table 4). In aerated suspensions a slightly increased content of fatty acids was observed during the stationary phase of growth. A comparison of aerated (fast growing) versus non-aerated (slower growing) cultures showed a remarkably higher content of fatty acids in the non-aerated cultures.

Table 3. Selected fatty acids content (in mg g^{-1} DW) of *Nannochloropsis limnetica* in comparison to five different green algal species under aerated suspension cultures in the stationary phase of growth

Fatty acids	*Nannochloropsis* KR1998/3	*Choricystis* KR1979/121	*Pseudodictyosph.* KR1996/1	*Chlorella* KR1979/21	*Monoraphidium* KR1980/22	*Scenedesmus* KR1979/307
14:0	6.30	1.09	0.20	1.17	0.39	0.57
16:0	36.06	6.59	3.13	8.60	13.14	27.53
16:1 n-7	37.10	0.64	0.10	0.18	1.13	0.38
18:0	0.68	0.95	0.40	0.10	0.41	2.56
18:1 n-9	30.14	0.68	0.45	0.90	6.53	11.95
18:2 n-6	3.83	0.90	0.85	0.40	0.80	1.17
18:3 n-6	0.18	0.04	0.08	0.07	0.09	0.12
18:3 n-3	0.37	1.80	0.77	0.13	3.17	0.56
20:4 n-6	5.26	0.14	0.14	0.09	0.68	0.78
20:5 n-3	28.06	0.46	0.17	0.55	3.26	0.55
22:5 n-3	0.08	0.04	0.15	0.02	0.30	0.02
22:6 n-3	0.06	0.22	0.15	0.10	0.75	2.37
$\sum$n-6	10.12	1.75	1.43	0.80	3.64	2.88
$\sum$n-3	28.98	3.69	2.35	1.53	9.00	3.98

Table 4. Selected fatty acids content (in mg/g^{-1} DW) of *Nannochloropsis limnetica* in suspension cultures in aerated and non-aerated culture conditions and different phosphate concentrations

Fatty acids	Aerated culture in exponential phase	Aerated culture in stationary phase	Not aerated culture, 40 mg l^{-1} K_2HPO_4	Not aerated culture, 20 mg l^{-1} K_2HPO_4	Not aerated culture, 10 mg l^{-1} K_2HPO_4	Not aerated culture, 4 mg l^{-1} K_2HPO_4
14:0	7.50	6.30	22.37	3.66	4.97	0.92
16:0	30.85	36.06	125.07	17.84	20.94	4.17
16:1 n-7	38.34	37.10	120.62	16.08	17.25	3.00
18:0	2.31	0.68	11.70	2.00	2.44	1.11
18:1 n-9	22.15	30.14	98.89	1.60	12.94	2.03
18:2 n-6	2.78	3.83	22.19	4.08	4.81	1.03
18:3 n-6	0.28	0.18	0.41	0.11	0.34	0.14
18:3 n-3	0.54	0.37	3.19	3.42	4.59	0.81
20:4 n-6	2.73	5.26	10.52	2.03	5.34	1.14
20:5 n-3	18.65	28.06	55.56	15.71	14.72	2.22
22:5 n-3	0.22	0.08	1.70	0.32	0.66	0.22
22:6 n-3	0.04	0.06	6.89	0.47	0.50	0.36
$\sum$n-6	7.50	10.12	50.48	10.03	14.81	3.47
$\sum$n-3	20.38	28.98	71.59	23.18	22.91	4.89

The phosphate concentration in the medium strongly influenced the fatty acid content in *N. limnetica* (Table 4). High phosphate concentration induced high concentration of fatty acids. The fatty acid content was highest at phosphate concentrations of 40 mg l^{-1} while phosphate concentrations of 4 mg l^{-1} induced a very low fatty acid content of *N. limnetica*. It was shown that high phosphate concentration led to a higher total content of saturated, monounsaturated as well as PUFA. In detail, the saturated palmitic acid was increased from 4.2 to 125.1 mg and the monounsaturated oleic acid from 2.0 to 98.9 mg g^{-1} DW. Furthermore, the polyunsaturated arachidonic acid was increased by nearly ten times and eicosapentaenoic acid almost 25 times. The docosahexaenoic acid increased from 0.4 to 6.9 mg g^{-1} DW.

Discussion

It was shown that the content of fatty acids in six species of planktonic freshwater algae was notably

different (Table 3). The sums of n-6 and n-3 fatty acids in the eustigmatophycean alga *N. limnetica* were three- to ten-fold higher than in five green algal species. These results suggest distinct differences in the food quality of different planktonic algae, which have consequences for the consumer populations in the food web. Among organisms in aquatic food webs, algae possess the highest ability to synthesize long-chain PUFA (Brett & Müller-Navarra, 1997). In contrast, most animals are not able to synthesize essential fatty acids. PUFA in the food of zooplankton predict the success of the life cycle of consumer populations, for example, the development and ontogenetic cycle of zooplankton (Müller-Navarra, 1995; Park, Brett, Müller-Navarra, & Goldman, 2002; Von Elert, 2004) and fishes (Meinelt, Schulz, Wirth, Kürzinger, & Steinberg, 1999). Linoleic (18:2 n-6) and α-linoleic acid (18:3 n-3) are essential fatty acids that can be converted into long-chain PUFA. Arachidonic (20:4 n-6) and eicosapentaenoic (20:5 n-3) acid of the membrane phospholipids are precursors in prostaglandins synthesis. The prostaglandins are themselves precursors for a number of compounds known as tissue hormones. The biological functions are described by Weber (1989). Docosahexaenoic acid (22:6 n-3) plays an important role in the membrane lipids (Henderson & Tocher, 1987). Krienitz et al. (2000) did not detected this fatty acid in *N. limnetica*. In the present study, docosahexaenoic acid was found, however, only in low concentrations. Wacker, Becher, and Von Elert (2002) found *N. limnetica* to be similar to the haptophyte *Isochrysis galbana* as a high-quality food source for the zebra mussel *Dreissena polymorpha* whereas several chlorophyte algae were classified as low-quality food source. Based on the low content of PUFA the green algae studied here, can be grouped to the algae with low food quality, and vice versa, based on the high content of PUFA, *N. limnetica* can be grouped to the algae with excellent food quality in freshwater ecosystems.

It was shown that especially the members of the picoplankton size class, the eustigmatophyte *N. limnetica* and the green algae *Choricystis minor* (Trebouxiophyceae) and *Pseudodictysphaerium jurisii* (Chlorophyceae), which form competitive dominance structures in stagnant water bodies remarkably differ in fatty acid content. Despite the different fatty acid content and food quality, the differentiation of these picoplankton algae by light microscopy is very difficult because of their similar morphology. It can be suspected that *Nannochloropsis* is much more distributed in inland waters than documented until now; however, it is hidden by the other picoplanktont. To allow a reasonable evaluation of the food quality of picoplankton populations in lakes, a clear determination of these primary producer communities is essential. Investigations in Lake Baikal have revealed that new methods are needed to better discriminate eustigmatophyte and chlorophyte picoplankton by pigment analysis and molecular approaches (Fietz et al., 2005).

Since the fatty acid content of microalgae varies greatly between taxa, rigorous taxon and strain sampling and selection is essential for algal mass culture. Few algal species are capable of rapid and high production of lipids, and especially of the important long-chain PUFA, and members of the genus *Nannochloropsis* are possible candidates for biotechnological production of these fatty acids (Suen, Hubbard, Holzer, & Tornabene, 1987). Optimization of culture conditions for the selected strains can be a challenge, because the fatty acid content of individual species can vary considerably under different environmental conditions in the field and in culture (summarized in Ahlgren, Gustafsson, & Boberg, 1992). In marine *Nannochloropsis* lipid content was promoted under nitrogen deficient conditions (Suen et al., 1987). Hu and Gao (2003) and Roncarati, Meluzzi, Acciarri, Tallarico, and Melotti (2004) reported a higher production of fatty acids under CO_2 enrichment of the air volume in aerated suspension cultures. The latter authors also found a changed proportion of short-chain vs. long-chain fatty acid content during the logarithmic and stationary phase of growth. In the present study, in *N. limnetica* the PUFA concentration was higher in the stationary phase of growth and considerably higher in non-aerated cultures (Table 4).

Furthermore, in *N. limnetica* we showed that a higher phosphate concentration induced a higher content in PUFA. These findings conflict with phytoplankton field studies in lakes of different trophic states: for example regression analyses between lake total phosphorus and sestonic n-3 PUFA showed that with increasing phosphorus concentrations there was a decrease of eicosapentaenoic acid content on a double-logarithmic scale (Müller-Navarra et al., 2003). We suggest that individual species of the phytoplankton community have a different phosphate-optimum for production of PUFA, and this can differ considerably from that of the sum of the whole phytoplankton community.

Our results confirmed the potential of a strain of *N. limnetica* to produce a high amount of valuable long-chain PUFA under freshwater conditions. This makes the strain interesting for freshwater aquaculture and biotechnology. In aquaculture *N. limnetica* could be used as food for rotifers, a usual food organism for fish larvae. One of the challenges facing aquaculture of fish is the high demand for fish meal and -oil as food for the farmed fishes (Steffens, 1997). Therefore, alternative, plant-based sources of fish food are sought for aquacultures. Positive effects of vegetable supplements based on oil from maize, sunflowers and rapeseeds, or the addition of cereals, were tested successfully (Steffens, 1996; Wirth & Steffens, 1998). An alternative supplementary source of fish food based on *N. limnetica*

biomass could partly replace the traditional fish-based food in aquaculture. Furthermore, *Nannochloropsis* biomass is an interesting alternative to fish oil produced from fatty fish, which is widely used for medical purpose in order to reduce the risk of myocardial illnesses of man (summarized in Singer & Wirth, 2004).

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