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Unusual medium-chain polyunsaturated fatty acids from the snow alga *Chloromonas brevispina*

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Summary

The gas chromatography-mass spectrometry (GC-MS) method was used to identify unusual medium-chain polyunsaturated fatty acids (PUFAs) in the snow alga *Chloromonas brevispina* collected in 2006 from surface layers of a snow field with conspicuous green patches in Bohemian Forest (Czech Republic).

PUFAs formed more than 75% total fatty acids. Among them, mass spectroscopy of picolinyl esters showed sizable proportions of medium-chain PUFA, e.g., 5,8,11-tetradecatrienoic and 6,9,12-pentadecatrienoic acids. The high relative content of PUFA indicates that PUFA are an important element ensuring cell survival. Our report appears to be the first to describe the presence of short- and medium-chain PUFAs in green psychrophilic algae of the genus *Chloromonas*.

Introduction

Snow algae grow in snow in the mountain or polar regions of the world. Their optimum growth temperatures are generally below 10 °C. To adapt to this harsh environment, they developed a number of adaptive features, which include biosynthesis of pigments, polyols, sugars and lipids, mucilage sheaths, motile stages and spore formation. Many of the snow algal species are colored green, red, orange or yellow green. The spores, which withstand subzero temperatures in winter and also high soil temperatures and desiccation in summer, usually have large amounts of lipid reserves, polyols and sugars. The protective role of polyunsaturated fatty acids (PUFAs) against the damaging effect of high light intensity and UV radiation, especially at low temperature, obviously helps the organism to survive and adapt in extreme environments (Whitelam and Codd, 1986).

The snow alga *Chloromonas brevispina* has a complex life cycle, which includes both asexual and

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sexual reproduction and formation of resting stages. The flagellates and immature zygotes are responsible for green coloration of snow. During the process of maturing, zygotes gradually accumulate carotenoids in relation to cell's exposure to sunlight, and thus may color the snow yellow, orange or even red (Hoham et al., 1979). *C. brevispina* has been recorded from all continents except Africa and Australia (Kol, 1968). In contrast to the alpine red snow alga *Chlamydomonas nivalis*, it is a typical forest species of shaded or at least partially shaded localities (Hoham and Blinn, 1979) (Table 1).

Because we were unable to find in the literature any data about fatty acids in the genus *Chloromonas*, we refer to papers concerned with FA in the closely related genus *Chlamydomonas*.

Algae of the genus *Chlamydomonas* contain a multitude of medium-chain fatty acids. For instance, *C. moewusii* was found to contain acids from 14:0 to 18:4 including PUFAs C16 acids (Tatsuzawa et al., 1996; Arisz et al., 2000). Two mutants of *Chlamydomonas reinhardtii* contained mostly polyenoic C16 and C18 fatty acids, some of which were unusual: 3t-16:1, 7,10-16:2, 4,7,10-16:3, 7,10,13-16:3, 4,7,10,13-16:4, 11-18:1, 5,12,15-18:3 and 5,9,12,15-18:4 (Sato et al., 1995). PUFA including α-linolenic acid (18:3n-3) and 16:4n-3 along with palmitic acid (16:0) being most abundant, and positional isomers 6,9,13-16:3 and 3,6,9-16:3 were also identified (Poerschmann et al., 2004).

The fatty acid composition of whole cells of *Chlamydomonas* spp. collected on the Hermit Island, Antarctica was published by Bidigare et al. (1993). In green cells, which were also analyzed in our study, the authors found fatty acids from 14:0 up to 24:1, including PUFA C16, C18, 20:4n-3 and 22:6n-3. Large differences were found between green and red cells.

Pinolenic acid (5,9,12-18:3) and coniferonic acid (5,9,12,15-18:4), Δ^5-unsaturated bis-methylene-interrupted fatty acids commonly found in pine seed oil (Wolff and Christie, 2002), were identified in *C. reinhardtii* (Kajikawa et al., 2006). They are assumed to be synthesized from linoleic acid (9,12-18:2) and α-linolenic acid (9,12,15-18:3), respectively, by Δ^5-desaturation.

Some algae were found to contain some PUFA with chains shorter than 16 carbon atoms such as the 3Z,6Z,9Z-dodecatrienoic acid in female gametes of *Analipus japonicus* (Kodama et al., 1993) or 14:1n-6, 14:3n-3, and 16:3n-6 in the golden alga (*Schizochytrium* sp.) (Michaud et al., 2002).

A recent review concerning fatty acids in psychrophilic microorganisms describes changes in fatty acids in glycolipids as dependent on temperature (Morgan-Kiss et al., 2006).

In summary of possible pathways for the biosynthetic origin of fatty acyl chains in bacterial membrane lipids (Russell and Nichols, 1999), it was proposed that the substrate for aerobic desaturation in the cytoplasm could be acyl-CoA or acyl-ACP. The resulting monounsaturated chains however, are not capable of direct conversion to

Table 1. Fatty acid composition from the snow alga *Chloromonas brevispina* as determined by GC-MS

Peak no.[a]	Fatty acid	%
1	5,8-11:2 (n-3)	0.59
2	3,6-12:2 (n-6)	0.71
3	6,9-12:2 (n-3)	0.83
4	3,6,9-12:3 (n-3)	0.48
5	4,7-13:2 (n-6)	0.12
6	4,7,10-13:3 (n-3)	0.30
7	8-14:1 (n-6)	2.38
8	5,8-14:2 (n-6)	2.91
9	5,8,11-14:3 (n-3)	4.63
10	6,9-15:2 (n-6)	0.36
11	6,9,12-15:3 (n-3)	1.37
12	16:0	2.49
13	16:1 (n-9)	8.91
14	16:1 (n-7)	2.97
15	3t-16:1(n-13)	0.89
16	5,9-16:2	2.02
17	7,10-16:2 (n-6)	2.91
18	9,12-16:2 (n-4)	2.14
19	4,7,10-16:3 (n-6)	5.76
20	6,9,12-16:3 (n-4)	3.98
21	7,10,13-16:3 (n-3)	3.15
22	9,12,15-16:3 (n-1)	2.67
23	4,7,10,13-16:4 (n-3)	4.69
24	6,9,12,15-16:4 (n-1)	4.87
25	9-17:1 (n-8)	1.19
26	5,9-17:2	0.12
27	6,9-17:2 (n-8)	0.36
28	9,12-17:2 (n-5)	0.42
29	6,9,12-17:3 (n-5)	0.53
30	8,11,14-17:3 (n-3)	0.27
31	9-18:1 (n-9)	7.13
32	5,9-18:2	0.97
33	6,9-18:2 (n-9)	1.28
34	9,12-18:2 (n-6)	5.11
35	12,15-18:2 (n-3)	1.49
36	5,9,12-18:3	2.05
37	6,9,12-18:3 (n-6)	4.06
38	9,12,15-18:3 (n-3)	6.16
39	11,14,17-18:3 (n-1)	0.75
40	5,9,12,15-18:4	0.81
41	6,9,12,15-18:4 (n-3)	2.18
42	8,11,14,17-18:4 (n-1)	1.70
43	3,6,9,12,15-18:5 (n-3)	1.29

[a]Methyl esters are shown.

PUFAs because of the need for elongation. On the other hand, shorter-chain PUFA such as tetradecatrienoic (14:3n-3) and hexadecatrienoic (16:3n-6) acids are biosynthesized through the β-oxidation of α-linolenic (18:3n-3) and γ-linolenic (18:3n-6) acids, respectively.

Based on our previous experience with analysis of FA (Rezanka et al., 1986; Rezanka and Podojil, 1984; Rezanka, 2002; Rezanka and Dembitsky, 2004; Rezanka and Sigler, 2006), we have now used it for the rapid identification and quantification of the short- and medium-chain PUFAs. FA mixture containing PUFAs isolated from the unusual natural source, i.e., snow alga was utilized to demonstrate the efficient identification of picolinyl esters of the PUFAs by means of gas chromatography-mass spectrometry (GC–MS).

Material and methods

Isolation and preparation of derivatives

The snow alga *C. brevispina* (Fritsch) Hoham, Roemer et Mullet (Chlorophyta, Chlamydomonadales) was collected from surface layers of a snow field with conspicuous green patches in the Bohemian Forest (Czech Republic), on May 18, 2006. The sampling site was located on the edge of a spruce forest within ca. 200 m northeast of the Laka Lake (1090 m a.s.l.). Snow samples were aseptically collected, transferred into laboratory at 0 °C and identified. Motile flagellated cells dominated in the sample, but a low share of young green zygospores bearing characteristic spines was also present.

Lipid extraction was done immediately after collection. Melted snow with the alga was centrifuged at 2000 rpm and the supernatant was decanted. Its lyophilization afforded 31.8 mg of the alga. The lyophilized alga was extracted overnight (three nights, every time with fresh solution) by CH_2Cl_2–MeOH (5 mL, 2:1 v/v), yielding 1.2 mg crude lipids, which was suspended in 1.5 mL of 1 M KOH in water and 2 mL methanol. The mixture refluxed 2 h at 70 °C. After cooling, the unsaponifiable material was removed from the mixture by three times 5 mL of hexane. Then the mixture was diluted to 50 mL with water and acidified by HCl to pH 2. Fatty acids were extracted by 3×10 mL of CH_2Cl_2. The yield was 0.635 mg of total FA. FAME were prepared from 0.10 mg of total free FA, by reaction with bortrifluoride–methanol solution (Christie, 2003) and were further identified by GC-MS under the conditions, see below.

The free fatty acid sample (0.50 mg) was dissolved in dichloromethane (100 μL) and a solution of 1,1′-carbonyldiimidazole (100 μL, 10 mg/mL in dichloromethane) was added. The picolinyl reagent (200 μL, i.e., 3-(hydroxymethyl)pyridine (900 μL) in dichloromethane (9 mL) and triethylamine (9 mL)) was further supplied. The mixture was agitated for 10 min at 37 °C, acetic acid (25 μL) was added in drops and the mixture was evaporated to dryness in a stream of nitrogen at 30 °C. The mixture was then extracted with hexane (5 mL) and distilled water (2 mL), the hexane layer was separated and dried with Na_2SO_4, concentrated and analyzed by GC-MS.

Analysis

GC-MS of FAME and also picolinyl esters was done on a Finnigan 1020 B single-state quadruple GC-MS instrument in the electron ionization mode. Injection temperature (on-column injection) was 100 °C, and a fused-silica capillary column (Supelcowax 10; 60 m × 0.25 mm i.d., 0.25 μm film thickness; Supelco, PA) was used. The temperature program was as follows: 100 °C for 1 min, subsequently increasing at 5 °C/min to 180 °C and at 2 °C/min to 220 °C, which was maintained for 10 min. The carrier gas was helium at a linear velocity of 60 cm/s. All spectra were scanned within the range *m/z* 50–500. The general order of elution of fatty acid methyl esters from a polar column such as Supelcowax 10 follows the increasing number of double bonds.

Analysis of fatty acid picolinyl ester mixture (prepared above) was carried out analogously, with some parameters being slightly different: The temperature program was as follows: 100 °C for 1 min, subsequently increasing at 20 °C/min to 180 °C and at 2 °C/min to 280 °C, which was maintained for 1 min.

Fatty acids are named systematically from the saturated hydrocarbon with the same number of carbon atoms, the final ''ane'' being changed to ''enoic''. Thus, the fatty acid with 18 carbon atoms is systematically named *cis*-9-octadecenoic acid (the trivial name is oleic acid). In the shorthand nomenclature, it is designated ''18:1'' (or 9-18:1 or 9c-18:1). The position of the double bond can be denoted in the form (n-*x*), where n is the chain length of the fatty acid and *x* is the number of carbon atoms from the double bond in the terminal region of the molecule. Oleic acid is then 18:1(n-9), 18:1n-9, or (in the early literature) 18:1ω9. This designation is very useful although it contradicts the convention that the position of functional groups should be related to that of the carboxyl carbon. Families of monoenoic fatty acids with

similar terminal structures, but with different chain lengths may arise from a common precursor either by chain elongation or by β-oxidation (Gunstone and Herslof, 2000).

In the shorthand nomenclature, PUFAs are designated 9,12-18:2 and 9,12,15-18:3, respectively. The number before the colon specifies the number of carbon atoms, and that after the colon, the number of double bonds. The position of the terminal double bond can also be denoted in the form (n-*x*), assuming that all the other double bonds are methylene-interrupted.

Results and discussion

We analyzed the content of FA in a far-from-common source, a snow alga. As stated in the introduction, snow algae are psychrophilic microorganisms inhabiting an extraordinarily harsh environment characterized by low temperature, nutritional limitation and high irradiation. A crucial condition for survival at temperatures around 0 °C is the maintenance of membrane fluidity even under these conditions. As shown in the chromatogram of FAME from this organism (Fig. 1), our data confirm the importance of this feature. We identified a total of 43 FA, predominantly short- and medium-chain PUFAs, the only saturated FA being palmitic acid. The high content of PUFA with chains shorter than C16 is extraordinary and, to our knowledge, no organism has as yet been described that would contain a spectrum of short- and medium-chain PUFAs of this complexity. The presence of these uncommon acids is obviously caused by the effects of the extreme environment.

FAs were identified and quantified by GC-MS as methyl esters on a polar capillary column. The analysis determined the chain length and the number of double bonds in each FA. The primary identification of positions of double bonds made use of the known fact that a peak at $m/z = 150$ is characteristic for methylene-interrupted fatty acid methyl esters with an n-6 terminal moiety, while one at $m/z = 108$ defines an n-3 terminal group (Christie, 2003). Judging from the presence and abundance of these ions and also retention characteristics, it can be provisionally stated that the methyl esters marked by asterisk in Fig. 1 have

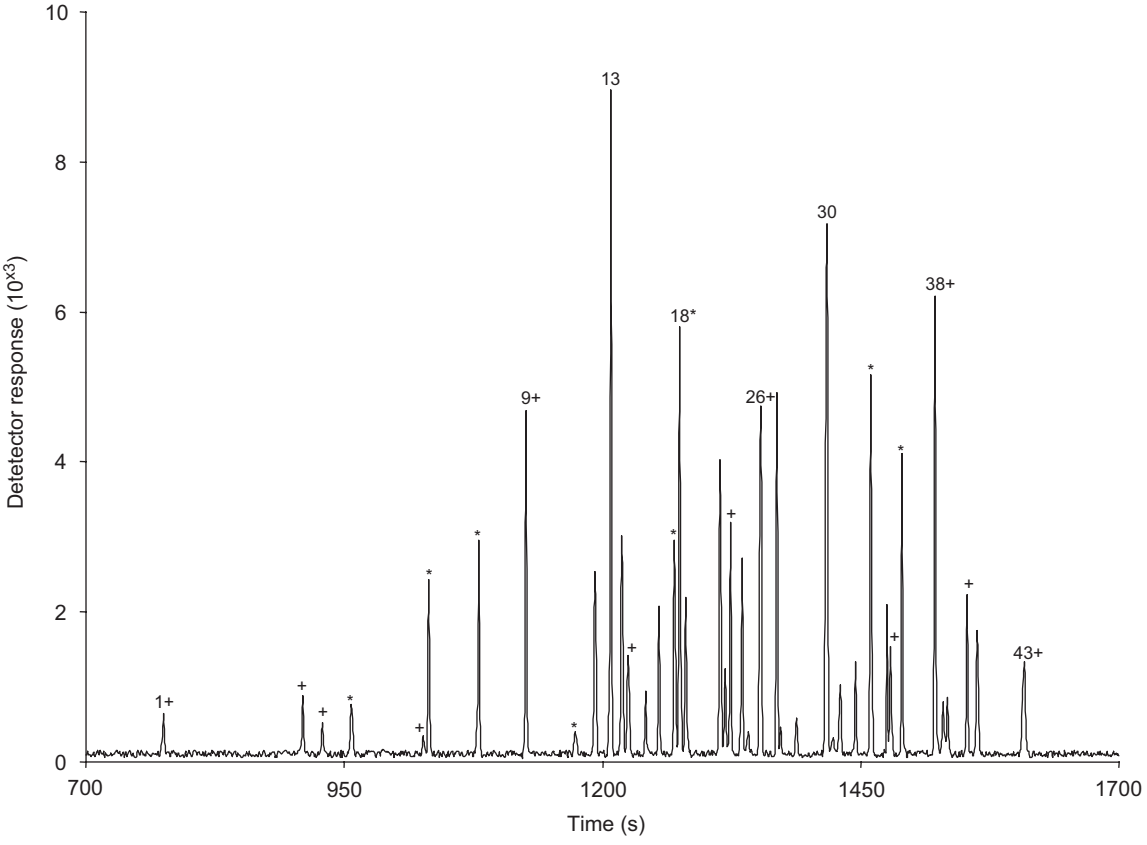

Figure 1. GC–MS chromatogram of FAME from the snow alga *Chloromonas brevispina*. Peak identification: 1 – FAME of 5,8-11:2, etc.

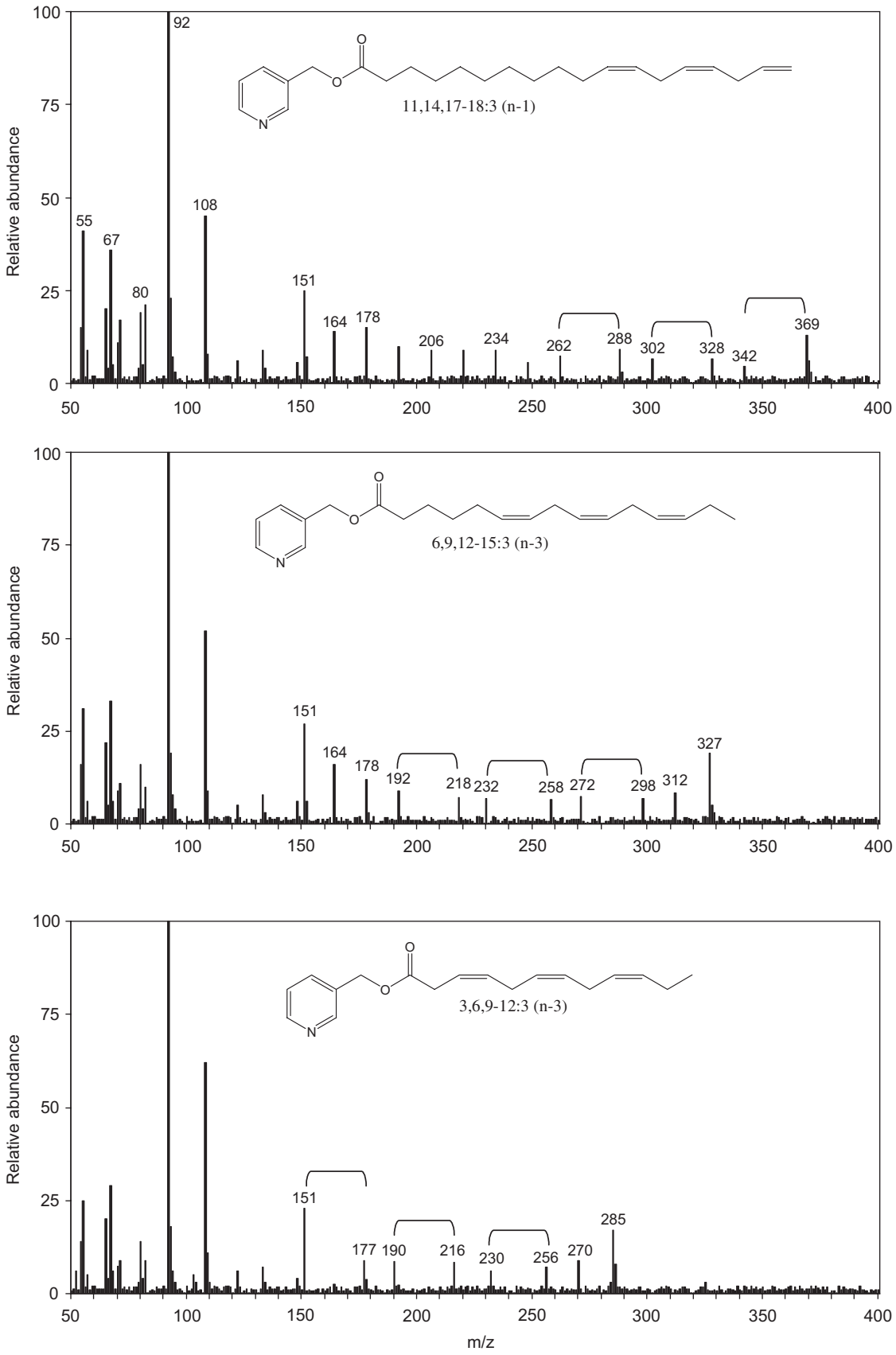

Figure 2. Mass spectra of picolinyl esters of three acids (11,14,17-18:3, 6,9,12-15:3 and 3,6,9-12:3); only important ions are shown. For explanation of values see the text.

double bonds in position n-6, those marked by a cross in position n-3. Further specification of the position of double bonds was done by using picolinyl esters, see below.

The spectra of picolinyl esters always contain a molecular ion, which is in all cases odd-numbered, because of the presence of the nitrogen atom, other ions can be even numbered. Other typical, highly abundant ions are those with $m/z = 92$, 108, 151 (the McLafferty ion) and 164, all of which are fragments from the proximity of the pyridine ring. The interpretation of MS starts from the molecular ion and continues to lower ions. These are formed due to the loss of the methyl group (ion M-15), to be followed by a series of ions with interval decrements of 14 amu down to the position of the double bond.

In this case the 14 amu interval is replaced by a 26 amu (–CH═CH–) "gap" interval. This gap can be supplemented by an auxiliary gap 40 amu (–CH_2–CH═CH–) in size, which is more prevalent especially in polyenes. Unfortunately, the geometry of the double bond (*cis-trans*) makes no readily discernible difference and we therefore used another method for its determination, see below.

The MS of picolinyl esters of dienoic, trienoic and even more multiply unsaturated fatty acids are usually sufficiently clear to permit the localization of double bonds, especially with the aid of the 40 amu gap (Harvey, 1998). Although diagnostic ions are always present, they sometimes occur in the center of clusters and are not sufficiently abundant. The main source of problems is usually the localization of the first double bond. Fortunately, different positional isomers have totally different spectra; the structure of unknown compounds can thus be determined by using tabulated spectra from the literature.

For the sake of clarity, we show the interpretation of three spectra of fatty acids found in our study (the spectra are shown in Fig. 2). The first acid (in the form of a picolinyl ester) to be interpreted is 11,14,17-18:3 (n-1), the other is 6,9,12-15:3 (n-3) and the third one is 3,6,9-12:3 (n-3).

The presence of the terminal double bond in acid 11,14,17-18:3 (n-1) cannot be directly detected since its position brings about rearrangements that confuse the interpretation. On the other hand, two double bonds were readily localized. The gap of 26 amu between $m/z = 262$ and 288 locates the first double bond rather easily, and that between $m/z = 302$ and 328 locates another in position 14, but the last double bonds are not easily seen, see above. As with trienes, it is often easier to spot gaps of 40 amu for the double bond and an associated methylene group, in this instance from $m/z = 248$–288 and 288–328.

The gap of 26 amu between $m/z = 272$ and 298 locates the terminal double bond in acid 6,9,12-15:3 (n-3) and further gap between $m/z = 232$ and 258 locates double bond in position 9, but the first double bond (delta 6) was not easy to discern and gaps of 40 amu were therefore used for the location of double bonds which are associated with methylene groups; this is seen in the spectrum. Here the gaps of 40 discussed above are between $m/z = 178$ and 218, 218 and 258, and 258 and 298.

The double bonds in acid 3,6,9-12:3 (n-3) were identified as in the previous spectrum. The gaps of 26 amu between $m/z = 151$ (see above) and 177, and 190 and 216 serve to locate the double bonds in positions 3 and 6, respectively.

All fatty acids isolated by us have a *cis* geometric configuration of the double bond since the IR spectrum of a mixture of fatty acids was not found to exhibit any deformation of the adjacent C-H bonds at a wave number of 967/cm (or, in other words, no absorbance in the region 950–1000/cm), which is taken as an evidence of the absence of *trans* double bond(s) (Christie, 2003).

Snow algae are often accompanied by bacteria. Recently, bacterial flora from snow was characterized by 16S rRNA sequencing, which confirmed the presence of both psychrophilic and psychrotrophic species (Segawa et al., 2005). Bacterial abundances are generally higher in snow containing algae than in noncolored snow, suggesting possible benefit in the form of increased nutrients (Weiss, 1983; Thomas and Duval, 1995). However, the role of PUFA in these associations is still not known.

Conclusion

The presence of short-chain PUFA has also been described by Ghazala and Shameel, (2005) who analyzed green algae and obtained rather unusual results. For instance, the alga *Tetraspora gelatinosa* was found to contain only three FA – 15:3 (18%), 17:3 (24%) and 15:4 (30%). Similarly unorthodox FA composition was found in, e.g., *Rhizoclonium hieroglyphicum* that was stated to contain tetradecapentaenoic acid, or *Chara contraria* that was declared to contain 20% of nonacosatrienoic acid.

Our analysis of the snow alga appears to be the first mention of the presence of short- and medium-chain PUFAs in green psychrophilic algae of the genus *Chloromonas*. Although changes in the content of FA are taken as a factor regulating membrane fluidity (Adlerstein et al., 1997; Morgan-Kiss et al., 2006), there are only few examples of this mechanism (Huner et al., 1993). The relatively high content of PUFA, i.e., more than

75% of total FA, indicates that PUFA are an important element ensuring cell survival.

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