

Comparative lipidomics of snow algal blooms

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Snow algae are excellent models for elucidating adaptations to abiotic stresses that characterize their extreme habitat. In cold-adapted microorganisms, changes in lipid composition represent an important strategy that enables survival at low temperatures. However, our knowledge in this field remains fragmentary. Using shotgun lipidomics, we identified 303 lipid species in field samples of snow algal blooms originating from 14 sites across a wide altitudinal gradient in mountains of Central Europe. Red, orange, and green snow blooms caused by vegetative cells or cysts of species from the genera *Sanguina*, *Chloromonas*, and *Chlainomonas* (Chlamydomonadales, Chlorophyta) were sampled. The analysis of total lipids using hydrophilic interaction chromatography showed that the samples were dominated by sphingolipids and triacylglycerols, forming 39.9–50.5% and 21.9–31.8% out of total lipids, respectively. Significant variability in lipid composition was revealed, reflecting differences in life cycle stage or metabolic setting and species composition among blooms of different color. Vegetative cells were characterized by a higher proportion of phospholipids (mean 18.0% vs. 13.5% in orange and 14.4% in red cysts) and glycolipids (mean 17.3% vs. 13.2% and 10.3%), whereas triacylglycerols were less represented compared to the other two groups (mean 22.3% vs. 30.2% and 28.3%). This pattern was in line with the assumed physiological difference between the two main stages of the life cycle in chlamydomonadacean snow algae. A higher degree of unsaturation of phospholipid species in algal cells causing red blooms suggested a better adaptation of membranes to low temperatures compared to green and orange snow blooms.

Keywords: *Chlainomonas*; Chlamydomonadaceae; *Chloromonas*; lipidomics; *Sanguina*; snow algae

Abbreviations: DGDG, digalactosyldiacylglycerol; ESI, electrospray ionization; GIPC, glycosylinositolphosphoceramide; GlcCer, glucosylceramide; HILIC, hydrophilic interaction liquid chromatography; HPLC, high-performance liquid chromatography; HR-MS, high-resolution mass spectrometry; ITS2, internal transcribed factor 2; MGDG, monogalactosyldiacylglycerol; PC, phosphatidylcholine; PCA, principal component analysis; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; TAG, triacylglycerol

INTRODUCTION

Cold ecosystems cover a substantial part of the Earth's surface and are often dominated by microorganisms from various groups (bacteria, archaea, fungi, and pro-

tists) that were able to adapt to the extreme conditions of mountain, polar, or deep-sea habitats (Morgan-Kiss et al. 2006). Snow algae are a highly specialized group of mi-



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croorganisms growing in the challenging habitat of melting snow in mountain or polar regions worldwide. Their habitat is characterized by many stresses, namely low temperature, high and variable irradiance, and freeze-thaw cycles. Under favorable conditions, they form intensively colored patches on snow. Most snow algae belong to the order Chlamydomonadales (Chlorophyta), and the genera *Chloromonas* and *Sanguina* are the most represented (Procházková et al. 2019a, Hoham and Remias 2020). Their life cycles include flagellates that can migrate through liquid water films between snow crystals and formation of resistant cysts on the surface during ongoing melting. Cysts, which withstand sub-zero temperatures in winter and, in contrast, higher temperatures in soil in combination with desiccation stress after complete snow melt, usually have large amounts of lipid reserves, polyols and sugars (Remias 2012).

In relation to their taxonomic affiliation and life cycle stage, snow algal cells from Chlamydomonadales can be colored green, orange, or pink to red. In green cells that are represented by flagellates, swarmer-like stages, or young cysts, chlorophylls dominate in the pigment profile. Depending on the degree of accumulation of secondary carotenoids (namely astaxanthin), snow blooms formed by cysts are colored orange, pinkish, or red. The high concentration of red cells causes the striking phenomenon of ‘red’ or ‘watermelon’ snow that is frequently reported from polar and alpine sites (Remias 2012).

Lipids are currently considered not only as energy depots and structural components of cells, but also play an important role in responding to environmental stresses (Hölzl and Dörmann 2007, Li et al. 2017). Depending on the taxonomic affiliation and environment, their composition may vary significantly. One of the main causes of variability is the ambient temperature, which causes changes in the physical properties of membranes. The detrimental effects of low temperature on membrane functions have been thoroughly documented (Los and Murata 2004). As maintenance of membrane fluidity and its integrity is essential for any organism, it represents a major adaptation of metabolic function that influences growth in permanently cold environments. In particular, the extent of unsaturation of fatty acids in membrane lipids plays a major role in avoiding membrane rigidification (Morgan-Kiss et al. 2006). In line with this, fatty acid analyses of snow algae pointed to their efficient adaptation to low temperatures by the abundant presence of polyunsaturated fatty acids (e.g., Řezanka et al. 2008, Procházková et al. 2019b). Analysis of the few sequenced genomes of cold-adapted microalgae revealed character-

istics connected with adaptation to their extreme environment (Zhang et al. 2020).

In recent years, lipidomic analysis enabled to define quantitatively the lipid classes, including their molecular species. Shotgun lipidomic uses direct injection of total lipids into the mass spectrometer, which is very fast and allows the identification of hundreds of molecular species, especially when using high-resolution tandem mass spectrometry (HR-MS) (Řezanka et al. 2014). Of course, it also has limitations, e.g., identification of regioisomers of triacylglycerols (TAG) is very difficult, and enantiomers of TAG cannot be identified. The second method uses lipid separation by high-performance liquid chromatography (HPLC) and their identification by mass spectrometry (MS). However, it is more time-consuming, depending on the use of the column for HPLC; the separation of regioisomers and enantiomers is also possible (Palyzová et al. 2021).

Lipidomics of microalgae has been performed before; for an overview, see, e.g., Kato et al. (2022). Lipidomic analysis was performed on commonly cultivated green algae of the genus *Chlorella* (Couto et al. 2022) and *Chlamydomonas*, where examples include, e.g., data on a strain isolated from snow under different stresses (Lu et al. 2012, 2013). Although snow algae represent an excellent model for the study of metabolic adaptations to low temperatures, the study of their lipids is currently underrepresented. The first study to demonstrate differences between the TAG profiles of green and red snow was by Bidigare et al. (1993) and later by Spijkerman et al. (2012), who used the same classical gas chromatography / mass spectrometry protocol to screen strains of snow algae. However, there are only a few examples of complex lipid analyses of snow algae. Řezanka et al. (2014) used an integrated approach based on both lipidomic profiling using Orbitrap HR-MS and the traditional method of analysis by silver ion liquid chromatography and non-aqueous reversed-phase liquid chromatography of TAG to study flagellates of a *Chloromonas* species that cause green snow. Lu et al. (2012) focused on the putative snow algal strain UTEX LB2824, which was, however, wrongly identified and does not form blooms (i.e., does not represent a true snow alga). The comparison of green and red communities of polar snow algae (i.e., dominated by vegetative and cyst stages, respectively) showed significant differences in metabolomics, but the data related to lipid metabolism were limited (Lutz et al. 2015, Davey et al. 2019).

As noted above, lipidomic analysis of snow algae has been performed only on samples containing one cell

type of a single species from a limited geographical area (Řezanka et al. 2014). Here, we performed a comprehensive comparison of lipidomic profiles of snow algae differing in taxonomic classification, life cycle stage, and geographic origin.

MATERIALS AND METHODS

Chemicals and standards

For a full description of all chemical compounds, see the Supplementary Text S1. All other reagents used were of analytical grade and purchased from Merck (Prague, Czech Republic).

Sample collection and characteristics

Unialgal field samples of snow algae (green, orange to pinkish, and red snow) were collected in the Jeseníky Mts., Jizera Mts., Krkonoše Mts. (Czech Republic), High Tatra Mts. (Slovakia), and European Alps (Austria) in May 2019, June 2020, and June 2021 (Table 1). Surface snow was harvested with a sterile shovel, placed in 1 L thermos bottles or 5 L buckets, and transported the same day to the laboratory. To ensure the unialgal character of the samples (i.e., only one species appeared to be present), snow bloom samples were checked using a portable microscope in the field and / or light microscope in the laboratory. For taxonomic identification and determination of the life cycle stage of snow algae, light microscopy (magnification 1,000×) was performed under oil immersion using an Olympus BX43 microscope (Olympus Corporation, Tokyo, Japan) with Nomarski contrast and an Olympus DP27 camera, and the algal biomass was kept frozen for further analysis. In addition, molecular internal transcribed factor 2 (ITS2) metabarcoding / Sanger sequencing of selected samples was performed for determination of algae from the genus *Chloromonas* (Lutz et al. 2019). However, identification to the species level was not possible in some cases, as some taxa have not been formally described yet.

The sampling sites were sun-exposed above the timberline (LP07, LP11, WP235, WP242, WP244, WP245, and WP266) or in the middle part of the avalanche slope (Krk19-2), partly shaded next to *Pinus mugo* vegetation (LP13), next to a spruce tree (Jes19-3 and Jes19-5), in broad leaf forest (still without leaves, Jiz 19-3) or shaded in spruce forest (Krk19-7 and Mil19-5). Electrical conductivity and pH of the meltwater were obtained with

HANNA (Combo EC, Cluj Napoca, Romania) or with WTW Instruments (Cond 340i; Inolab, Weilheim, Germany). Green blooms were formed entirely by vegetative flagellates of various species of the genus *Chloromonas*, while red and orange (or pinkish) blooms consisted of the immotile cysts from the genera *Chloromonas*, *Sanguina*, and *Chlainomonas* (Table 1, Supplementary Fig. S1).

Extraction of lipids

Lipid extraction followed a modified protocol from Palyzová et al. (2021). The dried biomass underwent cryogenic grinding with glass beads under liquid nitrogen. The ground material was then mixed with a dichloromethane-methanol solution (2 : 1 v/v) and stirred for 30 min. Following this, additional dichloromethane and water were introduced to the mixture. The dichloromethane phase was collected and concentrated to dryness using vacuum evaporation. The resulting total lipid fraction was solubilized in acetonitrile for subsequent analysis. For polar lipid extraction, we adapted the method of Markham and Jaworski (2007) with modifications as described in Vítová et al. (2022). The water phase underwent three sequential extractions using an isopropanol / hexane / water bottom phase mixture (55 : 25 : 20 v/v/v). These extracts were combined, evaporated to dryness, and redissolved in pure acetonitrile. Lipid extraction followed a modified protocol from Palyzová et al. (2021). The dried biomass underwent cryogenic grinding with glass beads under liquid nitrogen. The ground material was then mixed with a dichloromethane-methanol solution (2 : 1 v/v) and stirred for 30 minutes. Following this, additional dichloromethane and water were introduced to the mixture. The dichloromethane phase was collected and concentrated to dryness using vacuum evaporation. The resulting total lipid fraction was solubilized in acetonitrile for subsequent analysis. For polar lipid extraction, we adapted the method of Markham and Jaworski (2007) with modifications as described in Vítová et al. (2022). The water phase underwent three sequential extractions using an isopropanol / hexane / water bottom phase mixture (55 : 25 : 20 v/v/v). These extracts were combined, evaporated to dryness, and redissolved in pure acetonitrile.

Shotgun lipidomics

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Table 1. Snow algal samples with codes, collection date, sampling site, altitude (m a.s.l.), geographic position (GPS), taxonomic identification of snow algae, life cycle stage, snow / slush colour of field sample, and abiotic habitat parameters

Sample	Date	Mountain range	Location	Altitude m a.s.l.	GPS	Species	Life cycle stage	Snow / slush colour	pH	EC
Jes19-3	May 8, 2019	Jeseníky Mts. (CZ)	Close to Ovčárna chalet, shaded by spruce tree	1,316	50°04'24.12" N, 17°14'02.16" E	<i>Chloromonas</i> sp. 1	Swarmer-like stages	Green snow	7.84	18
Jes19-5	May 8, 2019	Jeseníky Mts. (CZ)	Close to ski lift to Ovčárna, shaded by spruce tree	1,287	50°04'20.04" N, 17°14'10.20" E	<i>Chloromonas</i> sp. 1	Swarmer-like stages	Green snow	7.8	41
Jiz19-3	May 1, 2019	Jizera Mts. (CZ)	Broad leaf forest	920	50°48'42.5" N, 15°21'05.7" E	<i>Chloromonas hindakii</i>	Cysts	Orange snow	6.73	30
Krk19-2	May 16, 2019	Krkonoše Mts. (CZ)	Avalanche slope in Malá kotelní jáma	1,150	50°44'53.70" N, 15°32'02.16" E	<i>Chloromonas hindakii</i>	Cysts	Pinkish snow	7.06	13
Krk19-7	May 16, 2019	Krkonoše Mts. (CZ)	Shaded field depression next to a road	1,054	50°44'16.26" N, 15°33'51.60" E	<i>Chloromonas</i> sp. 2	Swarmer-like stages	Green snow	7.94	158
LP07	Jun 24, 2020	High Tatras (SK)	Western slope above Červené Lake in Červená Valley	1,861	49°12'48.6" N, 20°12'45.3" E	<i>Chloromonas</i> cf. <i>miwae</i>	Flagellates	Green snow	6.3	89
LP11	Jun 26, 2020	High Tatras (SK)	At a plateau below the peak Jahňací štít	1,908	49°12'55.5" N, 20°12'31.6" E	<i>Chloromonas</i> cf. <i>muramotoi</i>	Swarmer-like stages	Green snow	6.31	41
LP13	Jun 26, 2020	High Tatras (SK)	Close to the lake Čierne pleso kežmarské	1,598	49°12'26.4" N, 20°13'27.1" E	<i>Chloromonas hindakii</i>	Cysts	Orange snow	5.93	119
Mil19-5	May 5, 2019	Krkonoše Mts. (CZ)	Next to pedestrian pathway in forest	1,068	50°44'42.3" N, 15°32'24.3" E	<i>Chloromonas brevispina</i>	Cysts	Red snow	n.d.	n.d.
WP235	Jun 1, 2020	Austrian Alps (AT)	Finstertal valley	2,342	47°11'39.5" N, 11°01'53.4" E	<i>Sanguina nivaloides</i>	Cysts	Red snow	6.53	10.8
WP242	Jun 3, 2020	Austrian Alps (AT)	Skiing slope Schwarzmoos	2,428	47°13'38.9" N, 11°00'45.4" E	<i>Sanguina nivaloides</i>	Cysts	Red snow	6.52	3.2
WP244	Jun 3, 2020	Austrian Alps (AT)	Slush from partly frozen lake Gossenkölle	2,410	47°13'45.7" N, 11°00'46.6" E	<i>Chlainomonas</i> sp.	Cysts	Red slush	6.31	3.2
WP245	Jun 3, 2020	Austrian Alps (AT)	Lake shore of Gossenkölle- see	3,426	47°13'46.5" N, 11°00'45.7" E	<i>Chloromonas nivalis</i>	Cysts	Orange snow	6.7	3.7
WP266	Jun 14, 2021	Austrian Alps (AT)	Near small river flowing to Hundsfeldsee	1,887	47°15'51.0" N, 13°33'30.8" E	<i>Sanguina nivaloides</i>	Cysts	Red snow	6.19	21

CZ, Czech Republic; SK, Slovakia; AT, Austria; EC, electrical conductivity ($\mu\text{S cm}^{-1}$); n.d., not determined.

gen. The ground material was then mixed with a dichloromethane-methanol solution (2 : 1 v/v) and stirred for 30 min. Following this, additional dichloromethane and water were introduced to the mixture. The dichloromethane phase was collected and concentrated to dryness using vacuum evaporation. The resulting total lipid fraction was solubilized in acetonitrile for subsequent analysis. For polar lipid extraction, we adapted the method of Markham and Jaworski (2007) with modifications as described in Vítová et al. (2022). The water phase underwent three sequential extractions using an isopropanol / hexane / water bottom phase mixture (55 : 25 : 20 v/v/v). These extracts were combined, evaporated to dryness, and redissolved in pure acetonitrile.

Hydrophilic interaction liquid chromatography

Hydrophilic interaction liquid chromatography (HILIC) was conducted following Vítová et al. (2022) using two Ascentis Express HILIC columns (2.7 μ m particle size, 150 mm $\times$ 4.6 mm L $\times$ I.D.) connected in series. The separation employed a flow rate of 0.95 mL min⁻¹ with a 60-min linear gradient between two mobile phases: solvent A (methanol : acetonitrile:aqueous 1 mM ammonium acetate, 50 : 30 : 20 v/v/v) and solvent B (methanol : acetonitrile:aqueous 1 mM ammonium acetate, 10 : 70 : 20 v/v/v). Of the total HPLC flow, 10% was directed to the electrospray ionization (ESI) source for detection, while the remaining 90% containing lipid class fractions was collected manually. Complete experimental details are available in the Supplementary Text S1.

Lipid nomenclature, data visualization and analyses

The lipid nomenclature was applied according to Liebisch et al. (2013). To perform multivariate statistical analyses, the program CANOCO 5 (Microcomputer Power, Ithaca, NY, USA) was used. Because the gradient lengths in the data were short, a principal component analysis (PCA) was performed to visualize the variability within the dataset.

RESULTS AND DISCUSSION

Altogether, 14 unialgal samples of snow blooms of different colors collected in five mountain ranges of Central Europe were analyzed. They were formed by nine species from the genera *Sanguina*, *Chlainomonas*, and *Chlo-*

romonas (Chlamydomonadales, Chlorophyta). The altitude of the sites was 920–3,426 m a.s.l. and included both forested and open exposure habitats. The red and orange snow blooms were formed by a cyst stage, while the green snow blooms contained flagellates and swarmer-like stages (Table 1).

Shotgun lipidomic analysis

Total lipids were obtained by two-step extraction from individual snow algae samples (Table 1). First, it was a classical extraction according to Palyzová et al. (2021) followed by an extraction performed according to Markham and Jaworski (2007) with some modifications (Vítová et al. 2022). Two different extraction methods allowed to extract even very polar lipids, which are in the aqueous phase when only the first method is used. These included numerous classes of polar plant sphingolipids, such as glycosphingolipids, glucosylceramides (GlcCer), and glycosylinositol phosphoceramides (GIPC). Because the solubility of GIPC in organic solvents is poor (Vítová et al. 2022) due to high hydrophilicity of polar heads, GIPC are not extracted and consequently not determined.

The use of ESI allowed us to analyze both highly polar lipids (GIPC and / or GlcCer) and moderate polar lipids (Vítová et al. 2022) (Fig. 1). Furthermore, the use of HR-MS allowed for identification of such molecular species that differ in the values of $[M+H]^+$ only in hundredths of Da. As an example, three molecular species of lipids, i.e., phosphatidylserine (40:3-PS), phosphatidylcholine (40:2-PC), and GlcCer (t18:1/h24:1-GlcCer) at m/z 842.5908, 842.6635, and 842.6717, were identified earlier. Tandem MS enabled the determination of the long-chain base (sphingoid bases) and acyl(s) of individual lipid classes. Although the use of shotgun lipidomic facilitates the detection of up to thousands of molecular species, as demonstrated by Lu et al. (2012), the latter authors actually identified only about one percent of the total molecular species (4,975 vs. 35). Thanks to the use of a high-resolution mass spectrometer and, at the same time, a tandem MS, more than 300 molecular species have been identified in this study (Supplementary Table S1). Below are examples of the analysis of two important classes of lipids found in snow algae (Supplementary Fig. S1).

Glucosylceramides. In positive tandem MS mode, fragmentation of $[M+NH_4]^+$ ions from GlcCer (d18:1/16:0) at m/z 810.6821 resulted in a fragment ion at m/z 792.6715 due to the loss of water $[M+H-H_2O]^+$. The precursor ion $[M+H]^+$ of GlcCer produced also fragment ions $[Glc+H]^+$ (often referred to as C1 ion) at m/z 169.0683 and

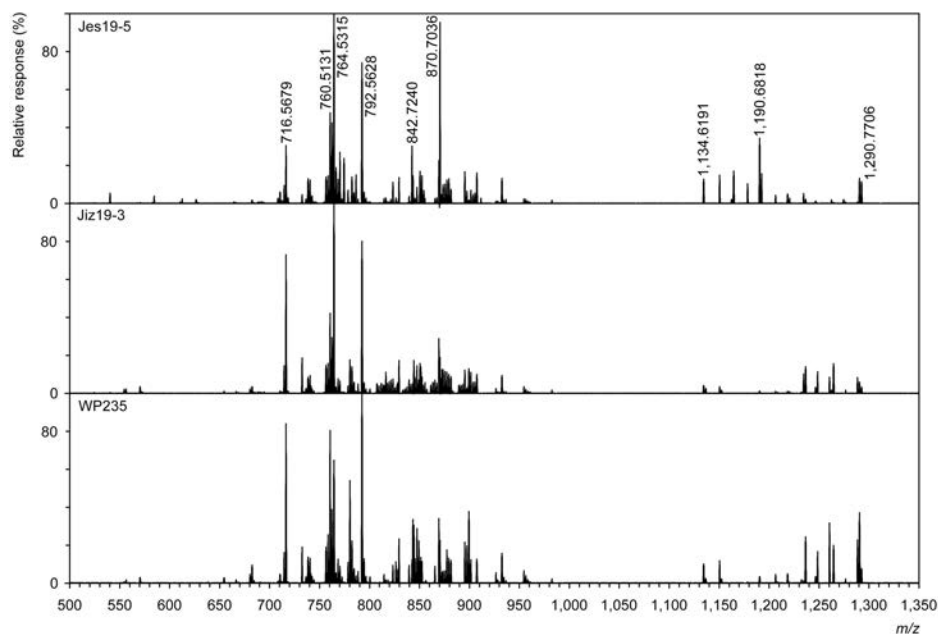


Fig. 1. Mass spectra (positive ion mode) of total lipids from three types of snow algal blooms (Jes19-5, green snow; Jiz19-3, orange snow; WP235, red snow), acquired by high mass resolution electrospray ionization mass spectrometry. Numbers correspond to the accurate mass values.

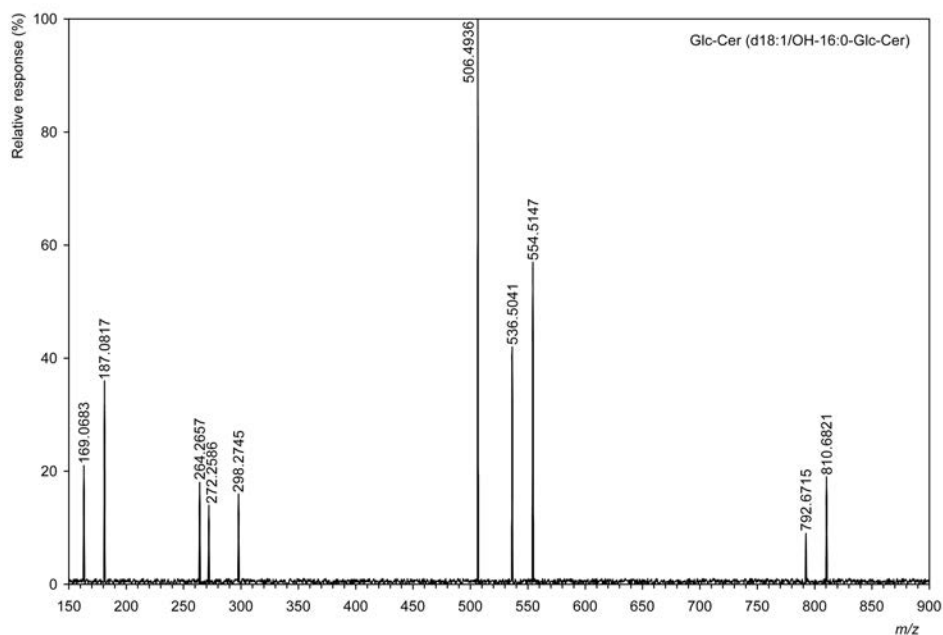


Fig. 2. The collision-induced dissociation tandem mass spectrum of ion at m/z 810.6821 (glucosylceramide, GlcCer; d18:1/OH-16:0-Glc-Cer) from sample WP235.

$[\text{Glc-H}_2\text{O}+\text{H}]^+$ at m/z 181.0717 (ion B1). Fragments at m/z 298.2745 and 272.2586 (the nomenclature of ions was described by Li et al. (2017) provide information of the *N*-acyl chain (Ann and Adams 1992). In addition, the precursor ion $[\text{M}+\text{H}]^+$ of GlcCer produced a fragment ion of long-chain base type at m/z 264.2657. Furthermore, the

identification of head polar groups of glycosphingolipids was performed. In the tandem MS spectrum, the product ion Y_0 is formed by the loss of one saccharide ($[\text{M}+\text{H}-\text{Glc}]^+$ from the precursor ion of monosaccharide in ceramide. Likewise, water is lost from the ion Y_0 to form ions Z_0 at m/z 554.5147 and further loss of formaldehyde is formed

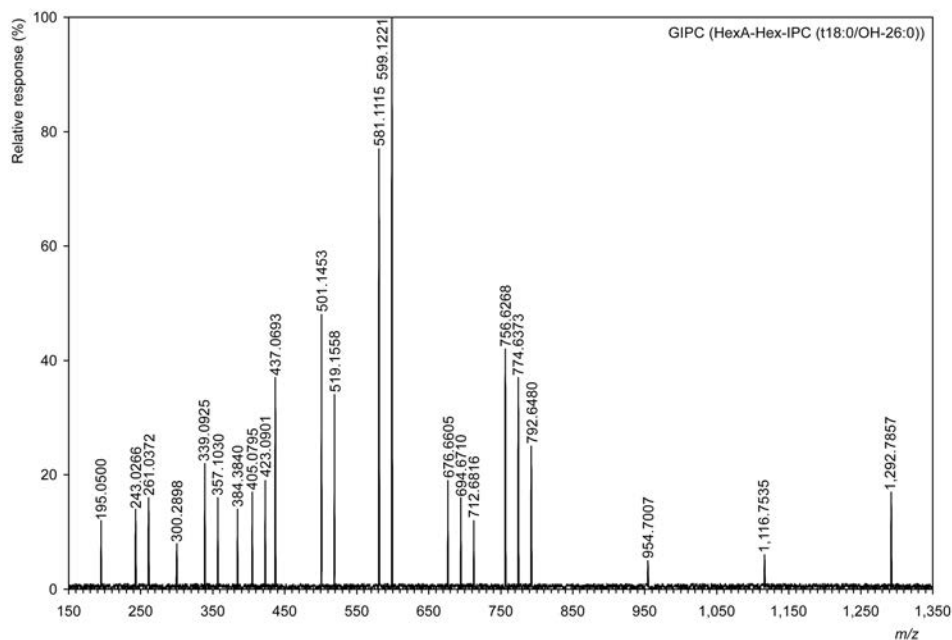


Fig. 3. The collision-induced dissociation tandem mass spectrum of ion at m/z 1,292.7857 (glycosylinositolphosphoceramide, GIPC; HexA-Hex-IPC [t18:0/OH-26:0]) from sample WP235.

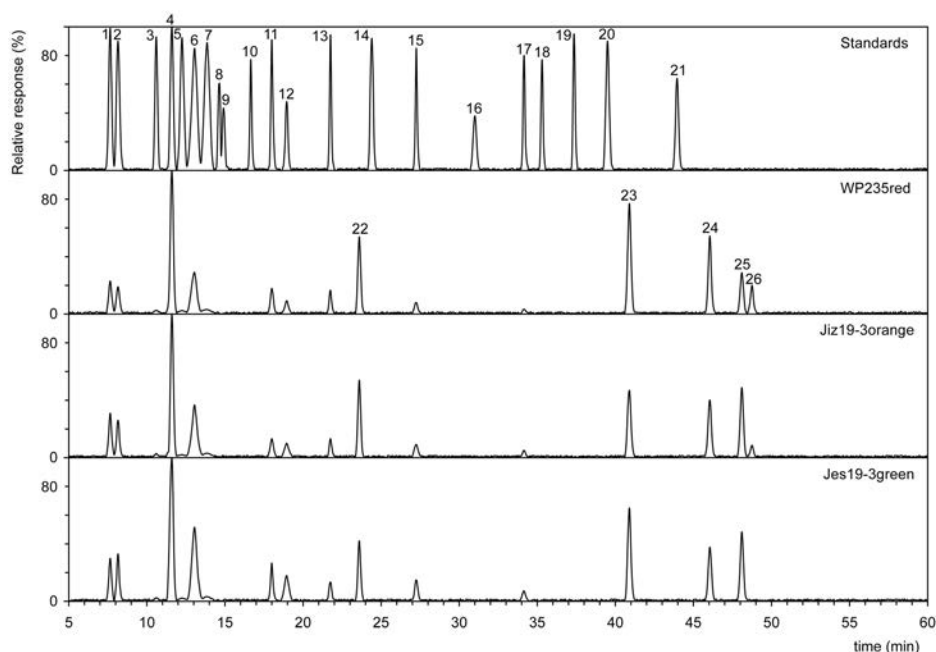


Fig. 4. The separation of total lipids in the three types of snow algal blooms (Jes19-3, green snow; Jiz19-3, orange snow; WP235, red snow) by hydrophilic interaction liquid chromatography (HILIC). Two Ascentis Express HILIC column were connected in series, 2.7 μ m particle size, $L \times I.D.$ 150 mm $\times$ 4.6 mm were used. For the identification of peaks, see Supplementary Table S2.

by the ion $Z_0\text{-CH}_2\text{O}$ at m/z 506.4936. This proved the structure of one of the major GlcCer, see tandem MS spectrum shown in Fig. 2.

Glycosylinositol phosphoceramides. The $[M+H]^+$ ion at m/z 1292.7857 corresponds to predominant molecular species containing t18:0 with OH-26:0 fatty-*N*-acyla-

tion. The most abundant fragments in positive tandem ESI were ceramide ions $[Y_0\text{PO}_3+H]^+$ at m/z 792.6480 and $[Z_0\text{PO}_3+H]^+$ at m/z 774.6373 and the pair of glycosylinositol phosphate ions $[B3\text{PO}_3+H]^+$ and $[C3\text{PO}_3+H]^+$ at m/z 599.1221 and at m/z 581.1115, respectively. Furthermore, minor ions of the Y2 and Y1 type are present in the tan-

dem MS at m/z 1,116.7535 and at m/z 954.7007, respectively. The tandem MS spectrum is shown in Fig. 3 that fully confirmed the unusual structure of HexA-Hex-IPC where the ceramide part is formed t18:0 with OH-26:0 fatty acid. Tandem mass spectra of three commonly occurring lipids, namely Po/Po/Ln-TAG (Supplementary Fig. S2), L/St-MGDG (Supplementary Fig. S3), and Po/Ln-PG (Supplementary Fig. S4), all molecular species were from sample WP235, are presented in Supplementary Table S1.

HILIC. Due to the different relative hydrophobicity of lipid molecules, it is impossible to extract all kinds of lipids from plant samples (de Jesus and Filho 2020). It has already been published several times (Markham and Jaworski 2007, Shiva et al. 2018) that the chloroform / methanol mixture is not suitable, for example, for plant sphingolipids such as GIPC. Therefore, a mixture of isopropanol, hexane, and water (e.g., lower phase of isopropanol / hexane / water (55 : 20 : 25 v/v/v)) is used for the extraction of plant sphingolipids (Markham and Jaworski 2007). Therefore, after extraction according to Palyzová et al. (2021), the aqueous phase was still extracted according to Markham and Jaworski (2007).

Total lipids were extracted both with a dichloromethane-methanol mixture (2 : 1, v/v) and with an isopropanol / hexane / water bottom phase (55 : 25 : 20, v/v/v). The separation of total lipids by HILIC is shown in Fig. 4 (for the identification of peaks, see Supplementary Table S2) and the values are shown in Table 2. The content of sphingolipids, i.e., GlcCers and GIPCs reaches one-half of total lipids in WP235 sample. Similar results were published by, for example, Cacas et al. (2016), who describe in their publication that the content of sphingolipids in tobacco leaves reaches up to 70% of total lipids (see his fig. 4A).

Main lipid groups and species in snow algae

Regarding the main lipid groups, all the samples were dominated by sphingolipids and TAG, forming 39.9–50.5% and 21.9–31.8% out of total lipids, respectively. Phospholipids and glycolipids were less abundant with the ranges of 11.8–20.0% and 9.5–18.5% (Table 2, Fig. 5). This is in line with a previous lipidomic analysis of snow algae from the genus *Chloromonas* (Řezanka et al. 2014). In contrast, glacier ice algae from the genus *Ancyronema*, which represent another group of extremophilic cold-adapted microorganisms forming blooms (though on glacier surfaces), lipidomic analysis revealed a slightly different lipid composition with a higher proportion of TAG and lower abundance of sphingolipids and phospholipids (Procházková et al. 2021). Like snow algae, they

Table 2. Relative proportions of the main lipid classes (in % of total lipids)

Compound	Type of the snow algal bloom														
	Green					Orange					Red				
	Krk19-7	Jes19-3	Jes19-5	LP11	LP07	WP245	Jiz19-3	LP13	Krk19-2	WP235	WP242	WP266	WP244	Mil19-5	
TAG	21.9	23.2	22.6	23.1	20.8	28.2	30.6	30.3	31.8	24.9	26.8	29.2	30.7	29.8	
MGDG	13.2	12.2	14.5	13.6	12.8	10.6	11.3	9.8	9.1	8.9	7.6	7.5	8.4	8.2	
DGDG	3.8	4.2	4.0	4.2	3.9	2.8	2.9	3.1	3.0	2.2	1.9	2.5	2.4	2.0	
PC	2.8	3.2	3.2	4.1	3.9	4.4	4.1	4.8	4.2	5.3	5.0	6.8	5.9	4.9	
PE	4.6	3.5	4.8	4.5	5.9	3.8	2.5	3.5	3.2	2.0	2.7	2.9	2.2	1.9	
PG	7.5	7.2	8.1	8.4	7.9	4.3	3.5	4.8	3.9	5.2	4.9	5.8	5.0	5.5	
PI	2.0	1.7	2.1	2.2	2.3	1.9	1.7	1.6	1.8	1.0	1.3	1.5	1.3	1.1	
GIPs1	14.9	15.3	15.8	16.1	15.7	11.7	11.8	12.6	12.4	21.3	22.9	21.0	19.6	20.8	
GIPs2	10.7	11.4	12.4	8.6	8.1	9.7	8.9	9.0	6.7	7.3	7.4	5.8	6.2	8.3	
GIPs3	9.8	8.6	1.2	3.3	8.0	5.2	3.8	2.8	4.4	9.4	5.8	4.7	3.2	2.9	
GlcCers	8.8	9.5	11.3	11.9	10.7	17.4	18.9	17.7	19.5	12.5	13.7	12.3	15.1	14.6	
Phospholipids	16.9	15.6	18.2	19.2	20.0	14.4	11.8	14.7	13.1	13.5	13.9	17.0	14.4	13.4	
Glycolipids	17.0	16.4	18.5	17.8	16.7	13.4	14.2	12.9	12.1	11.1	9.5	10.0	10.8	10.2	
Triacylglycerols	21.9	23.2	22.6	23.1	20.8	28.2	30.6	30.3	31.8	24.9	26.8	29.2	30.7	29.8	
Sphingolipids	44.2	44.8	40.7	39.9	42.5	44.0	43.4	42.1	43.0	50.5	49.8	43.8	44.1	46.6	

TAG, triacylglycerols; MGDG, monogalactosyldiacylglycerols; DGDG, digalactosyldiacylglycerols; PC, phosphatidylcholines; PE, phosphatidylethanolamines; PG, phosphatidylglycerols; PI, phosphatidylinositols; GIPs1–3, glycosyl inositol phosphoryl ceramides; GlcCers, glucosylceramides.

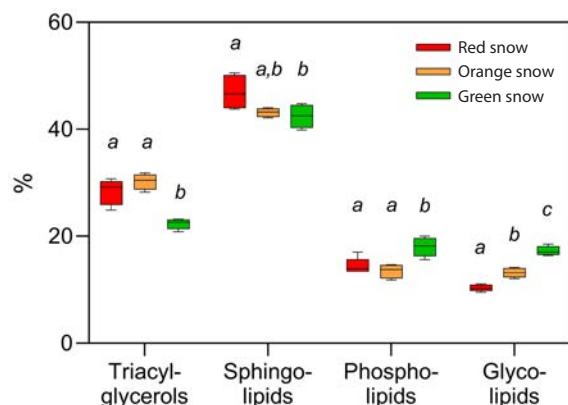


Fig. 5. Main lipid groups – differences among snow blooms of different color (red, orange, and green). The letters *a*, *b*, and *c* indicate statistically significant difference within each lipid group. Boxes with no common letters are significantly different ($p < 0.05$).

belong to Viridiplantae, but to different infrakingdom Streptophyta, class Zygnematomphyceae. However, more testing will be needed to confirm whether this pattern is a characteristic one, or if it is influenced by environmental conditions, sampling date, or other factors.

In line with the previously published data (Řezanka et al. 2014), TAG in snow algae were represented by species with even number of acyl carbons from 48 to 54 and 0–12 double bonds. The most abundant TAGs were 50:7, 54:8, and 50:4 that formed on average >30% of the total TAG. Among sphingolipids, GIPC were the most abundant (particularly those with sphingoid bases t18:1/h24:0, t18:1/h24:1, and t18:1/h26:0), followed by GlcCer, where t18:1/h24:1, t18:1/h24:0 and t18:1/h26:1 were the most frequent. GIPC have been detected in fungi and the green lineage (Vítová et al. 2022), and are considered the most abundant sphingolipids in the biosphere with multiple roles connected with the plasma membrane (Gronnier et al. 2016). The major glycolipids were monogalactosyldiacylglycerol (34:6-MGDG) and digalactosyldiacylglycerol (36:6-DGDG). However, a small proportion of sulfoquinovosyldiacylglycerols has been already also detected in snow algae (Řezanka et al. 2014). On the contrary, the latter lipid group was rather important in the previously mentioned glacier ice algae (Procházková et al. 2021). The main phospholipids were phosphatidylglycerols (PG) (namely 34:4) and PC (36:4, and 36:5), which was again in agreement with a previous study (Řezanka et al. 2014).

Challenges arise when trying to compare our results with existing lipidomic data from related algal species. Unfortunately, the data for the model chlamydomonadacean alga *Chlamydomonas* (*C.*) *reinhardtii* do not include a quantitative analysis of the relevant mo-

lecular species. For example, Yang et al. (2013) identified 35 molecular species of lipids, without any quantitative analysis. They reported the presence of e.g. 16:1/18:2-MGDG, 16:1/18:3-PG, or 16:0/18:1-PI. Tietel et al. (2020) described the effect of hyperosmotic stress on metabolomic changes in the biosynthesis of complex lipids. Again, many tens of molecular species of lipids were identified, especially TAG (18:2/18:2/18:3-TAG, 16:0/16:1/18:3-TAG, or 18:1/22:1/22:1-TAG). Polar lipids of different classes such as 18:3/18:4-DGDG, 16:3/18:5-MGDG, 18:2/20:4-PG were also identified. Unfortunately, this work did not provide the primary data. Jüppner et al. (2017) were able to quantify 212 lipid species from nine lipid classes. In contrast to our results, glycolipids (MGDG and DGDG) were dominant and TAG formed only a minor proportion of total lipids in mesophilic *C. reinhardtii*, which points to high metabolic variability and thus ecological flexibility within Chlamydomonadaceae. Lu et al. (2012, 2013) described the content of molecular species of lipids, e.g., MGDG, DGDG, phosphatidylethanolamines, PG, PI in order to identify lipid biomarkers in a chlamydomonadacean alga isolated from snow. They focused on the trend followed by the selected lipid biomarkers as a result of cultivation at different conditions, making any comparison with our data difficult.

Comparison of different types of snow blooms

The field samples were classified into three groups based on the pigmentation of the snow bloom (green, orange to pinkish, and red) that reflected the life cycle stage of these algae (flagellates or swarmer-like stages vs. cysts) and their taxonomic affiliation. The most common genera of snow algae (*Chloromonas*, *Sanguina*, and *Chlainomonas*) were covered (Table 1). In general, the diversity of snow algae below the genus level is still poorly known and many species await their formal taxonomic description, which is the case for samples labeled ‘sp.’ in Table 1. The use of ITS2 ribosomal DNA metabarcoding enables to discern even among closely related species with very similar morphologies (Lutz et al. 2019). In this study, green snow was caused exclusively by flagellates from the genus *Chloromonas*, while cysts of this genus were found in orange to pinkish and red snow. Most of the red snow samples were made of cysts of the recently established genus *Sanguina* that is widespread in exposed high mountain habitats including the Alps (Procházková et al. 2019a), but the other two genera (*Chloromonas* and *Chlainomonas*) were also represented in red pigmented snow.

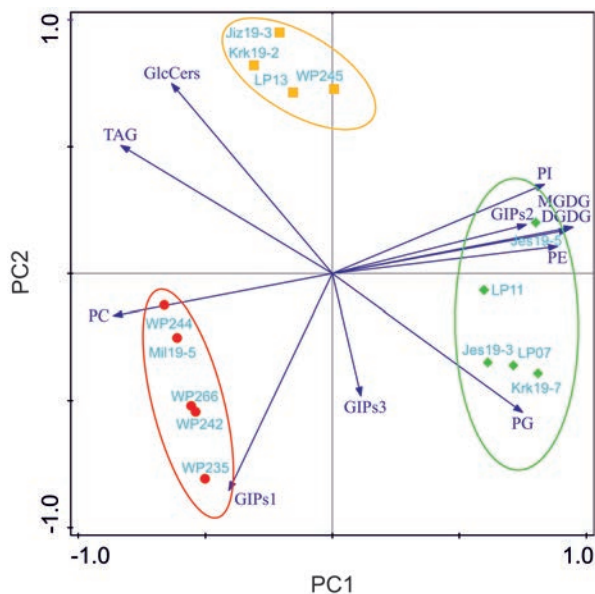


Fig. 6. Principal component analysis of the lipid groups classified based on the polar head type in the snow blooms of different color (red, orange, and green). The first two canonical axes are plotted; the first axis (PC1) explained 58.2% and the second (PC2) 21.2% of the variability. For sample codes, see Table 1. DGDG, digalactosyldiacylglycerols; GIPs1–3, glycosyl inositol phosphoryl ceramides; GlcCers, glucosylceramides; MGDG, monogalactosyldiacylglycerols; PC, phosphatidylcholines; PE, phosphatidylethanolamines; PG, phosphatidylglycerols; PI, phosphatidylinositols; TAG, triacylglycerols.

Comparison of the main lipid groups showed significant differences among bloom types (Fig. 5). Green snow made of flagellates and swarmer-like stages was characterized by a higher proportion of phospholipids (mean 18.0% vs. 13.5% in orange snow cysts and 14.4% in red snow cysts) and glycolipids (mean 17.3% vs. 13.2% and 10.3%), while TAG were less represented (mean 22.3%) compared to the other two groups, where they formed on average 30.2% and 28.3%, respectively. The proportion of sphingolipids also varied, and significant differences were found among the three sample groups (means: red 47.0%, orange 43.1, and green snow 42.4%) (Fig. 5).

A more detailed insight into the lipid variability among bloom types was provided by the principal component analyses that were performed based on the data representing 17 lipid classes (see Table 2, Fig. 6), and also based on the entire lipidomic dataset that contained 303 lipid species (Supplementary Table S1 & Fig. S5). Both analyses revealed a clear separation of the three bloom types represented in the samples with green snow having a very distinct lipid composition compared to the other two types based on the x-axis sample scores. Orange and red snow samples also formed separate groups with sig-

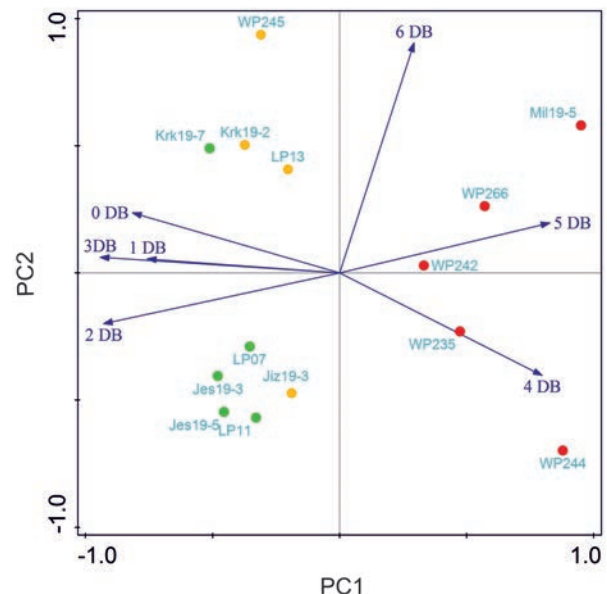


Fig. 7. Principal component analysis of the phospholipid species classified based on the degree of unsaturation of fatty acids in the snow blooms of different color (red, orange, and green). The first two canonical axes are plotted; the first axis (PC1) explained 62.7% and the second (PC2) 16.0% of the variability. For sample codes, see Table 1. DB, number of double bonds.

nificant differences in y-axis scores but were closer to each other than to the green snow samples, as this axis explained only 17.7% and 14.1% of the total variability in the two analyses, respectively. This pattern is in line with the assumed physiological difference between the two key stages of the life cycle in chlamydomonadacean snow algae (flagellates vs. cysts) (Remias 2012).

Vegetative flagellates forming green snow are considered to represent the actively growing life cycle stage, which actively migrates in the melt water film surrounding the snow crystals, reproduce and develop large populations that usually appear (on or under the snow surface) as the first type of snow coloration during the season (Hoham and Remias 2020). As the period suitable for the growth of snow algae is usually rather short in the middle latitudes (April–May at low altitudes and shifted to later periods at high altitudes), it is expected (although never measured in the field) that the growth rates of flagellates must be high, which requires a high amount of energy gained *via* intense photosynthesis. The higher proportion of phospholipids and especially glycolipids (MGDG and the DGDG) and lower proportion of TAGs compared to the orange and red snow samples are in accordance with this metabolic setting.

The results showed that cysts accumulate more TAGs

than flagellates. A high content of TAGs in the cysts of *Sanguina nivaloides* was interpreted by Ezzedine et al. (2023) as a response of the alga to a phosphate depleted environment. Although the three sample types were clearly separated based on lipid composition, the differences among them were only moderate (Fig. 5). This is possibly linked to the fact that (in contrast to common expectations) these cysts remain photosynthetically very active (Hoham and Remias 2020) and therefore membrane lipids must still be present in substantial amounts. The differences between both cyst categories (orange and red) could be associated with the taxonomic differences between the two groups (Table 1).

Another significant difference between flagellates and cysts has been represented by a contrasting pattern in the composition of GIPC, as the samples of green snow contained much more dihydrosphingosine compared to the other two groups. Regarding phytosphingosine, the pattern was inverted, although not so pronounced (Supplementary Table S3). The link of these patterns to the metabolic setting of both stages of the life cycle needs to be elucidated, however, it must be associated with shifts in membrane functions (Gronnier et al. 2016).

Carbon chain saturation within the main classes of lipids was determined by further analysis. The PCA of phospholipids as the key components of cell membranes based on the degree of unsaturation of fatty acids revealed a clear separation of green vs. orange and red snow (Fig. 7). Red snow samples showed more unsaturation, indicating better adaptation of membranes to low temperatures (Morgan-Kiss et al. 2006, Ezzedine et al. 2023). Generally, fatty acid composition and extent of unsaturation were shown to vary considerably in snow algae depending on species, life cycle stage, and nutritional regime (Spijkerman et al. 2012). However, a high proportion of polyunsaturated fatty acids has often been found reflecting the adaptation to low temperatures in snow.

CONCLUSION

Snow algae are a unique group of photoautotrophic microorganisms adapted to the extreme environment of melting snow. Although the phenomenon of colored snow has been known for a very long time, there are still major gaps in our understanding of their physiology in relation to the abiotic stresses that characterize their habitat. Shotgun lipidomic analysis can provide valuable insights into the metabolic adaptations of these extremophiles, but it has only been applied rarely so far. An exten-

sive data set of 303 molecular species of snow algae lipids, based on the analysis of 14 samples of different types of snow blooms originating from mountains in Central Europe, is presented (details see the Supplementary Table S1). Samples characterized by different degree of secondary carotenoid accumulation (red, orange, or green blooms) were clearly separated on the basis of their lipid composition. For the first time, lipidomic profiles of the two main life cycle stages of chlamydomonadacean snow algae (flagellates and swarmer-like stages vs. cysts) were compared. Significant differences in the proportion of the main lipid classes and lipid species composition were found, and they are in accordance with the metabolic demands of the two cell types (growth vs. reproductive inactivity).

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CONFLICTS OF INTEREST

The authors declare that they have no potential conflicts of interest.

SUPPLEMENTARY MATERIALS

Supplementary Text S1. Additions to the materials and methods, results and discussion (<https://www.e-algae.org>).

Supplementary Table S1. List of lipid species from snow algae analysed by ESI-MS in positive ion mode (<https://www.e-algae.org>).

Supplementary Table S2. The description of the peaks in Fig. 4 (<https://www.e-algae.org>).

Supplementary Table S3. Relative proportion of dihydrosphingosine and phytosphingosine (main constituents of GIPs – glycosyl inositol phosphoryl ceramides) in total lipids as revealed by shot gun lipidomic analysis (<https://www.e-algae.org>).

Supplementary Fig. S1. Dominant algal species in the studied snowfield samples (<https://www.e-algae.org>).

Supplementary Fig. S2. The collision-induced dissociation tandem mass spectrum of ion at m/z 842.7243 (triacylglycerol, TAG; Po/Po/Ln) from sample WP235 (<https://www.e-algae.org>).

Supplementary Fig. S3. The collision-induced dissociation tandem mass spectrum of ion at m/z 792.5630 (monogalactosyldiacylglycerol, MGDG; L/St/MGDG) from sample WP235 (<https://www.e-algae.org>).

Supplementary Fig. S4. The collision-induced dissociation tandem mass spectrum of ion at m/z 760.5134 (phosphatidylglycerol, PG; Po/Ln/PG) from the sample WP235 (<https://www.e-algae.org>).

Supplementary Fig. S5. Principal component analysis based on all the identified lipid species in the snow blooms of different colour (red, orange and green) (<https://www.e-algae.org>).

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