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Impact of trophic state on the distribution of intact polar lipids in surface waters of lakes

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Abstract

We characterized the intact polar lipid (IPL) composition in the surface waters of 22 lakes from Minnesota and Iowa, ranging in trophic state between eutrophic and oligo-mesotrophic, to investigate the impact of trophic state on IPL composition. A high diversity of IPL classes was detected. Most IPL classes were detected in all lakes, but the eutrophic lakes contained a significantly higher relative abundance of lyso-phosphatidylcholine (PC) than the oligo-mesotrophic lakes, which in turn were characterized by significantly higher relative abundance of hydroxymethyltrimethyl-alanine/trimethyl-homoserine (DGTA/DGTS) betaines, ornithine lipids and the recently discovered trimethyl ornithine (TMO) lipids. The higher relative abundance of ornithines and TMOs may relate to a higher contribution of heterotrophic bacteria relative to phytoplankton while the higher abundance of the DGTA/DGTS betaines may relate to substitution by microorganisms of these non-P lipids for PC under P-stress, as has been observed in other environments. We also detected a variety of heterocyst glycolipids (HGs) derived from N_2-fixing heterocystous Cyanobacteria in all lakes, suggesting the presence of these Cyanobacteria in the full range of trophic conditions. Correlation of HG abundance with environmental data showed that high productivity lakes have high HG abundances, while other distributional differences in HGs, which did not correlate with environmental parameters, are likely due to differences in species composition. We conclude that the significant differences in IPL composition between the eutrophic and oligo-mesotrophic lakes are either due to adaptation of the membrane composition to nutrient conditions or due to general divergences in microbial composition under the different conditions.

Polar lipids are the main building blocks of membranes of cellular microorganisms and therefore occur ubiquitously in the natural environment. They are generally composed of a glycerol backbone with ester-linked fatty acids (FAs) attached to the *sn*−1 and *sn*−2 positions and a polar head group at the *sn*−3 position (e.g., Fahy et al. 2005; Fig. 1). Some intact polar lipid (IPL) classes, or FAs derived thereof, are synthesized predominately by specific microbial groups and can, thus, potentially be used as chemotaxonomic markers (e.g., Shaw 1974; Lechevalier and Lechevalier 1989; Sturt et al. 2004). Furthermore, IPL molecules are thought to degrade rapidly on cell death and, therefore, predominately derive from living (microbial) cells (White et al. 1979; Harvey et al. 1986). Thus, IPL analysis can provide valuable information on living microbes complementary to that obtained by microbiological and molecular techniques. IPL compositions are known to be sensitive to environmental conditions such as nutrient limitation (e.g., Benning et al. 1995), thus potentially also yielding information on the physiology of microbes. For example, phytoplankton have been shown to

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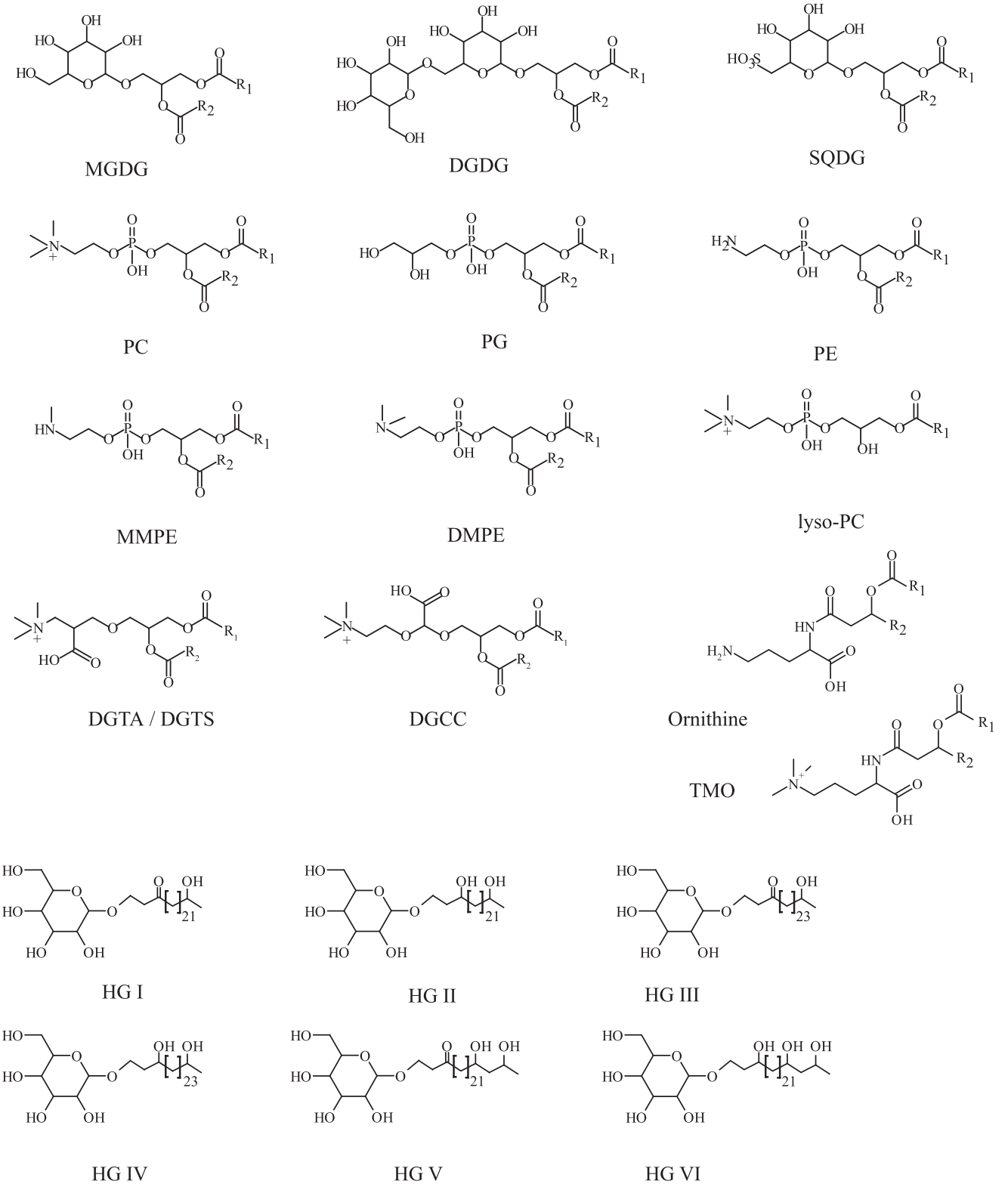

Fig. 1. Structures of IPLs investigated in this study. MGDG = monogalactosyldiacylglycerol, DGDG = digalactosyldiacylglycerol, SQDG = sulfoquinovosyldiacylglycerol, PC = phosphatidylcholine, PG = phosphatidylglycerol, PE = phosphatidylethanolamine, MMPE = methylphosphatidylethanolamine, DMPE = dimethylphosphatidylethanolamine, lyso-PC = lyso-phosphatidylcholine, DGTS = diacylglyceryl-trimethyl-homoserine, DGTA = diacylglyceryl-hydroxymethyltrimethyl-alanine, DGCC = diacylglyceryl-carboxyhydroxymethyl-choline, TMO = trimethylornithine. R_1 and R_2 = fatty acid tails. Structures of the heterocyst glycolipids detected in this study. C_6 glycolipids: I 1-(O-hexose)−3-keto-25-hexacosanol, II 1-(O-hexose)−3,25-hexacosanediol, III 1-(O-hexose)−3-keto-27-octacosanol, IV 1-(O-hexose)−3,27-octacosanediol, V 1-(O-hexose)−3-keto-25,27-octacosanediol, VI 1-(O-hexose)−3,25,27-octacosanetriol.

replace phospholipids with non-P lipids, such as nitrogen- and sulfur-containing lipids, under P-limiting or P-stress conditions (Benning et al. 1993; Geiger et al. 1999; Van Mooy et al. 2006, 2009; Martin et al. 2011).

The development of methods for the direct analysis of IPLs (e.g., Fang and Barcelona 1998; Rütters et al. 2002; Sturt et al. 2004; Popendorf et al. 2013) has allowed the rapid assessment of the full structural diversity of IPLs in environmental samples and has increased our knowledge on the sources and diversity of IPLs in natural environments, in particular in sediments (e.g., Zink et al. 2003; Lipp and Hinrichs 2009; Seidel et al. 2012), in suspended particulate matter (SPM) from marine waters (e.g., Van Mooy et al. 2006, 2009; Van Mooy and Fredricks 2010; Popendorf et al. 2011; Brandsma et al. 2012), and sediments and microbial mats from extreme environments (e.g., Bradley et al. 2009; Bühring et al. 2009; Borin et al. 2010; Rossel et al. 2011; Schubotz et al. 2013). Studies of the IPL composition of SPM in lakes have, however, been relatively limited. Ertefai et al. (2008) examined a polluted meromictic lake and found a range of IPLs with a predominance of betaine lipids in the epilimnion and phosphatidylethanolamine (PE) lipids in the hypolimnion. Bühring et al. (2009) analyzed a microbial mat from a hypersaline lake and found a predominance of ornithine and betaine lipids. Woermer et al. (2012) analyzed HGs specific for heterocystous Cyanobacteria in a number of Spanish lakes and found their distribution to relate to different taxonomic groups within the heterocystous Cyanobacteria.

We performed this study with the aim of shedding light on the potential of IPLs to differentiate between contrasting lake environments. We hypothesized that the IPL composition of the lake SPM will be different depending on the trophic states of lake, due to the difference in the lakes' microbial composition and adaptations of microbes to different environmental conditions. To test this hypothesis, we analyzed the IPL composition of SPM in surface waters from lakes from the U.S.A. states of Iowa and Minnesota: eleven eutrophic lakes and eleven oligo-mesotrophic lakes (de Kluijver 2012; de Kluijver et al. 2014). We determined the general structural diversity of diacyl- and lyso-IPLs and compared this with the trophic nature of the lakes. In addition, we performed targeted analysis of HGs derived from N_2-fixing heterocystous Cyanobacteria (Bauersachs et al. 2009b).

Materials and methods

Sampling and environmental data

The lakes were sampled between July 20th, 2009 and August 12th, 2009 (de Kluijver et al. 2014). Water samples for lipid analysis were collected from the deepest part of the lake within the mixed-zone layer (~2 m below surface) and transferred in precleaned 1 L bottles and subsequently transported on ice to the Limnology Laboratory of Iowa State University. The lake water was filtered through 0.7 μm GF/F filters (47 mm diameter, precombusted at 450°C for 12 h; Whatman) using a multivalve vacuum filtration unit to obtain SPM for lipid analysis. All filters were stored frozen at −20°C for the duration of the sampling and until extraction in the lab.

Background data are available as part of a long-term lake monitoring program and can be found at http://limnology.eeob.iastate.edu/lakereport and http://limnoweb.eeob.iastate.edu/itascalakes as well as in de Kluijver (2012) and de Kluijver et al. (2014). For details of sampling and analysis for environmental data see de Kluijver et al. (2014) and Schoon et al. (2013). For details of the collection and analysis of samples for phytoplankton biomass data, see Filstrup et al. (2014).

Intact polar lipid extraction

The GF/F filters were freeze-dried before the IPLs were extracted using a modified Bligh-Dyer procedure (Bligh and Dyer 1959). Briefly, the filters were extracted ultrasonically three times for 10 min in a solvent mixture of methanol, dichloromethane and phosphate buffer (2 : 1 : 0.8, v : v). After sonication, the combined supernatants were phase-separated by adding additional dichloromethane and buffer to a final solvent ratio of 1 : 1 : 0.9 (v : v). The organic phase containing the IPLs was collected and the aqueous phase re-extracted three times with dichloromethane. Finally, the combined extract was dried under a stream of N_2 gas. Before analysis, the extract was redissolved in a mixture of hexane:2-propanol:water (72 : 27 : 1, v : v) at a concentration of 10 mg mL^{-1}, and aliquots were filtered through 0.45 μm regenerated cellulose syringe filters (4 mm diameter; Grace Alltech, Deerfield, IL).

Intact polar lipid analysis

General IPL screening

For general IPL screening, the Bligh Dyer extracts were analyzed according to Sturt et al. (2004) with modifications as described by Sinninghe Damsté et al. (2011). High performance liquid chromatography/mass spectrometry (HPLC-MS) was performed on an Agilent 1200 series LC (Agilent, San Jose, California), equipped with thermostatted auto-injector and column oven, coupled to a LTQ XL linear ion trap with Ion Max source with electrospray ionization (ESI) probe (Thermo Scientific, Waltham, Massachusetts). Separation was achieved on a Lichrosphere diol column (250 μm × 2.1 μm, 5 μm particles; Grace Alltech, Deerfield, IL) maintained at 30°C. The following elution program was used with a flow rate of 0.2 mL min^{-1}: 100% A for 1 min, followed by a linear gradient to 66% A: 34% B in 17 min, maintained for 12 min, followed by a linear gradient to 35% A: 65% B in 15 min, where A = hexane/2-propanol/formic acid/14.8 M NH_{3aq} (79 : 20 : 0.12 : 0.04 [v : v]) and B = 2-propanol/water/formic acid/14.8 M NH_{3aq} (88 : 10 : 0.12 : 0.04 [v : v]). The lipid extract was analyzed by an MS routine where a positive ion scan (*m/z* 400–2000) was followed by a data dependent MS^2 experiment where the base peak of the mass spectrum was fragmented (normalized collision energy

Table 1. Location and background data on Iowa (IA) and Minnesota (MN) lakes (de Kluijver et al. 2014). Eu (eutrophic); O-M (oligo-mesotrophic); See text for abbreviations. nd (not detected).

Lake	Us state	Trophic state	Ph	Alkalinity (mmol L^{-1})	Temp (°C)	DOC (mg L^{-1})	DIC (mmol L^{-1})	POC (mg L^{-1})	Chl *a* (μg L^{-1})	TN (mg L^{-1})	NO_x (μg L^{-1})	NH_3 (μg L^{-1})	TP (μg L^{-1})	TN:TP
Beaver	IA	Eu	9.5	1.03	24.1	2.10	1695.7	5.07	72.6	1.69	1.00	0.30	152.8	11.10
Beeds	IA	Eu	8.5	2.28	23.8	0.97	4185.9	0.93	9.10	9.32	48.0	2.10	36.10	258.2
Big Creek	IA	Eu	8.5	1.97	25.0	1.56	3888.4	1.30	8.00	6.12	32.0	2.00	21.30	287.3
Coralville Reservoir	IA	Eu	7.8	2.37	24.2	1.16	4554.3	1.91	10.9	6.50	72.0	3.40	207.1	31.40
Lower Pine	IA	Eu	8.6	1.44	24.1	1.46	2500.2	4.00	60.2	4.15	45.0	3.40	128.3	32.30
Macbride	IA	Eu	8.8	1.18	22.0	1.71	1977.0	2.84	42.6	1.27	6.00	0.30	67.90	18.70
Meyers	IA	Eu	9.8	1.08	27.1	2.70	1491.7	9.91	86.2	2.14	3.00	1.60	208.7	10.30
Rodgers Park	IA	Eu	8.4	1.82	24.6	1.20	3337.1	0.68	5.40	6.81	101	3.50	50.60	134.6
Saylorville Reservoir	IA	Eu	8.5	2.05	25.3	1.80	4032.9	1.30	23.0	4.90	242	15.1	116.3	42.10
Three Mile	IA	Eu	9.1	0.98	21.3	1.90	1694.9	2.66	37.8	1.00	Nd	nd	44.90	22.30
Beaver	MN	O-M	6.9	0.09	19.7	4.34	88.000	1.50	3.60	0.12	71.0	0.70	16.20	7.400
Brush Shanty	MN	O-M	7.4	0.28	20.3	4.75	290.00	0.56	1.40	0.34	4.00	0.10	10.00	34.00
Hatch	MN	O-M	8.4	1.81	20.3	2.08	3037.9	0.30	0.80	0.55	14.0	1.30	1.900	289.5
Horsehead	MN	O-M	6.7	0.07	19.9	2.91	55.200	2.11	1.80	0.09	Nd	nd	6.900	13.00
Kelly	MN	O-M	6.9	0.08	20.2	2.50	51.600	1.22	2.60	0.01	6.00	0.00	8.900	1.100
Leighton	MN	O-M	8.4	1.84	19.4	2.62	2991.5	0.67	2.40	0.49	nd	nd	8.100	60.50
Little Sand	MN	O-M	8.2	0.75	22.8	3.68	1880.1	1.24	4.90	0.19	9.00	1.20	11.90	16.00
Little Split Hand	MN	Eu	8.2	1.04	19.2	3.70	1687.0	2.07	19.5	0.01	nd	nd	29.50	0.300
O'Leary	MN	O-M	6.7	0.09	19.2	3.18	80.100	2.28	2.60	0.44	19.0	0.40	13.80	31.90
Sand	MN	O-M	7.7	1.18	19.9	3.81	605.50	0.85	3.10	0.66	6.00	0.30	20.40	32.40
South Sturgeon	MN	O-M	6.1	0.22	18.6	6.71	196.60	0.55	3.60	0.03	19.0	1.10	14.40	2.100
Thirty	MN	O-M	7.1	0.13	22.0	3.65	123.30	3.02	6.30	0.10	16.0	1.20	15.70	6.400

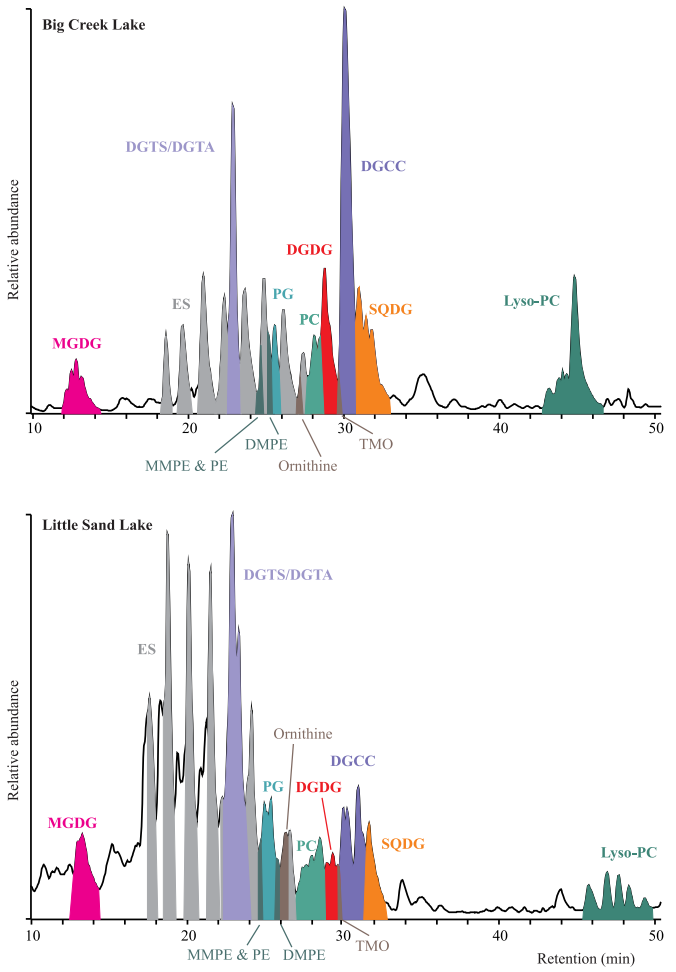

Fig. 2. Partial base peak chromatogram (Gaussian smoothed) of the HPLC-ESI/MS analysis of IPLs in the SPM from (a) Big Creek Lake, Iowa and (b) Little Sand Lake, Minnesota. For structures and acronyms of IPLs see Fig. 1. ES = ethoxylated surfactants. For interpretation of the peak labels in this figure, the reader is referred to the colors in the web version of this article. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

(NCE) 25, isolation width 5.0, activation Q 0.175). This was followed by a data-dependent MS^3 experiment where the base peak of the MS^2 spectrum was fragmented under identical fragmentation conditions. This process was repeated on the second to fourth most abundant ions of the initial mass spectrum. The IPL groups were identified through comparison with fragmentation patterns of authentic standards, as described in Brandsma et al. (2012). The chain length and number of double bond equivalents of the IPL-bound FA were determined by either the fragment ions or neutral losses diagnostic for FAs obtained in the MS^2 and MS^3 spectra (Brügger et al. 1997; Brandsma et al. 2012). However, as the individual FAs could not be identified for all IPLs, for statistical treatments the total number of carbon atoms and the total number of double bond equivalents (cf. Supporting Information Tables S1–S5) of the two FA moieties were combined.

IPL species are highly diverse (cf. Van Mooy and Fredricks 2010) and obtaining authentic standards for absolute quantification is difficult, therefore IPLs were examined in terms of their peak area response. Thus, the relative abundance of peak area does not necessarily reflect the actual relative abundance of the different IPLs, however, this method allows for some comparison between the samples analyzed in this study. The peak areas were determined from extracted ion chromatograms of the dominant ion formed for each individual IPL species (see Supporting Information Tables S1–S5 for all individual IPL species examined). The peak areas for the individual species were summed into IPL groups.

Heterocyst glycolipid analysis

HGs were analyzed using HPLC-ESI/MS^2 as previously reported by Bauersachs et al. (2009b). HPLC-MS^2 analysis of total lipid extracts was performed using an Agilent 1100 series LC (Agilent, San Jose, California) coupled to a Thermo TSQ Quantum ultra EM triple quadruple mass spectrometer with an Ion Max Source with ESI probe (Thermo Electron Corporation, Waltham, Massachusetts) operated in positive ion mode. Separation was achieved on a LiChrosphere DIOL column (250 mm × 2.1 mm i.e., 5 μm: Alltech, Deerfield, IL) maintained at 30°C. HPLC-MS^2 analysis was performed in selective reaction monitoring mode using transitions specific for HGs I-VI (Fig. 1) following Bauersachs et al. (2010). As we did not have an available HG standard, we reported the total HG abundances as peak area L^{-1} and the individual HGs as % of total HGs.

A number of indices have been suggested to express correlation between the distribution of HGs and growth temperature (Bauersachs et al. 2009a,). We examined our data using three such indices, the HG_{26} index, the HG_{28} index and the $HD_{26/28}$ index, defined as follows:

$$HG_{26} = \frac{HG\ I}{HG\ I + HG\ II}$$

$$HG_{28} = \frac{HG\ III}{HG\ III + HG\ IV}$$

$$HD_{26/28} = \frac{HG\ IV}{HG\ II + HG\ IV}$$

Statistical analysis

t-tests (two-tailed) and Pearson correlations were determined using Sigmaplot software (version 12.0) and principal component analysis (PCA) was done using XLSTAT 2014.

Results

Environmental context of the studied lakes

In total 22 lakes were examined, 10 from the U.S. state of Iowa and 12 from the state of Minnesota (Table 1). These

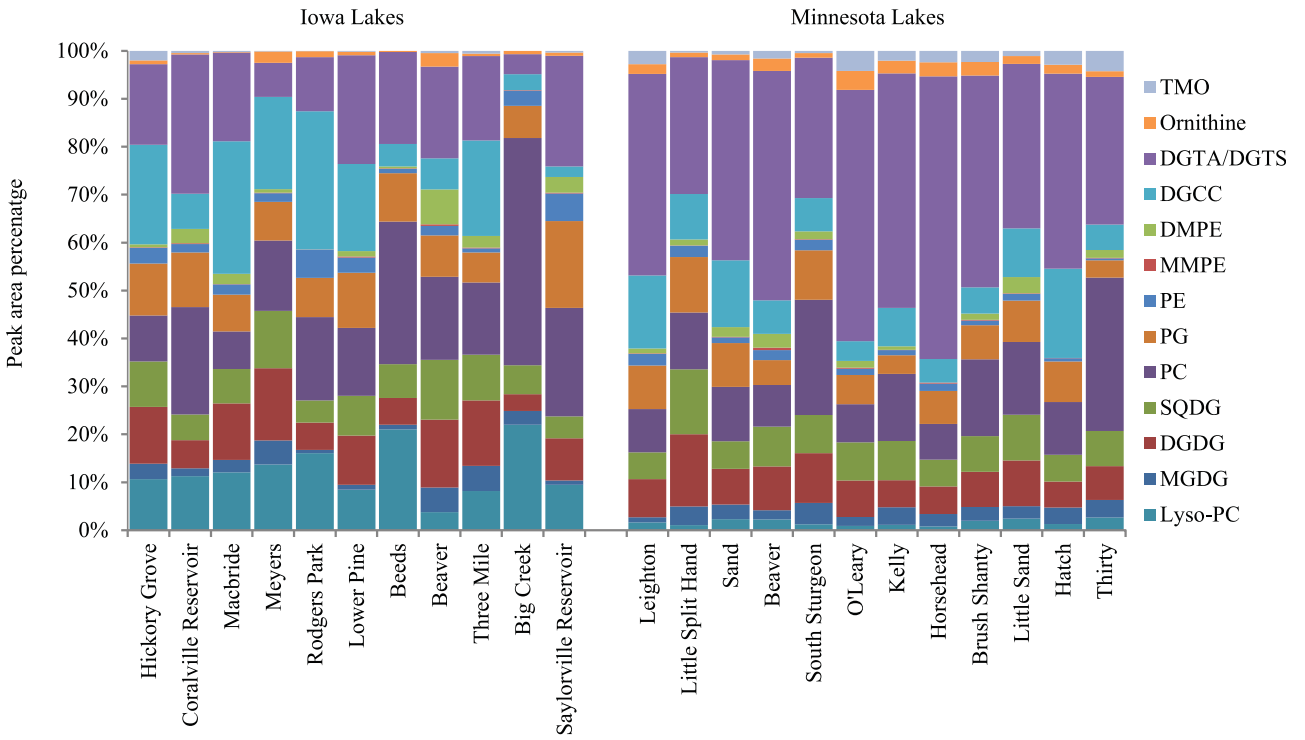

Fig. 3. Percent of diacyl- and lyso-IPLs (based on sum of peak area). See text and Fig. 1 for acronyms of IPL names. For interpretation of the figure, the reader is referred to the color figure in the web version of this article. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

lakes have previously been assigned as either eutrophic lakes (all of the Iowa lakes plus the Minnesota lake Little Splithand, $n = 11$) or oligo-mesotrophic lakes (all remaining Minnesota lakes, $n = 11$) based on their average summer total P (TP) concentration as described in de Kluijver et al. (2014). The trophic state of the eutrophic lakes is caused by high nutrient loads from agricultural run-off (Arbuckle and Downing 2001), while the oligo-mesotrophic lakes in the forested area of Minnesota are relatively pristine in nature. This leads to relatively high TP and total N (TN) concentrations, low dissolved organic carbon (DOC), and high pH for the eutrophic lakes and low TP and TN concentrations, low pH and high DOC for the oligo-mesotrophic lakes (Table 1).

IPLs diversity

The IPLs detected using HPLC/ESI-MS in the SPM of surface waters of all 22 lakes contained a large variety of head groups, including, the glycerophospholipids phosphatidylcholine (PC), phosphatidylglycerol (PG), PE, methylphosphatidylethanolamine (MMPE), and dimethylphosphatidyle thanolamine (DMPE) (e.g., Fig. 2; for structures see Fig. 1). Non-phospholipids detected included the sulfur-containing glycerolipid sulfoquinovosyldiacylglycerol (SQDG), the betaine lipids diacylglyceryl-trimethyl-homoserine (DGTS), diacylglyceryl-hydroxymethyltrimethyl-alanine (DGTA), and diacylglyceryl-carboxyhydroxymethyl-choline (DGCC), and the glycerolipids mono- and digalactosyldiacylglycerol (MGDG and DGDG) (e.g., Fig. 2). We were unable to distinguish between DGTA and DGTS betaine lipids using our analytical methods so the group is hereafter referred to as DGTA/DGTS. A range of lyso-PCs, i.e., PCs in which one of the FA chains is not present, were detected in all lake SPM. We detected, also using our general IPL LC/MS method, a range of ornithine lipids in all lake SPM and a range of trimethylornithine lipids (TMO) in the majority of lake SPM. In addition to the IPLs, we detected sometimes high abundances of ethoxylated surfactants (cf. Frömel and Knepper 2008), likely of anthropogenic origin. As these compounds were absent in some lakes and did not show up in laboratory blanks, they are likely present in the SPM of the lake water itself.

We examined the difference in the IPL distribution between the Iowa (all eutrophic) and Minnesota (of which all except one were oligo-mesotrophic) lakes. The distribution was examined in terms of the peak area sum for each IPL group, treated as a percentage of the total of the peak areas (Fig. 3; Supporting Information Table S6). Four IPL groups were significantly different (as determined by *t*-test) between the Iowa and Minnesota lakes: the DGTA/DGTSs ($p < 0.001$), the lyso-PCs ($p < 0.001$), the ornithines ($p = 0.013$) and the TMOs ($p < 0.001$). In particular the average percent of the summed peak area of the DGTA/DGTSs was substantially lower in the Iowa lakes (17.2% ± 7.6) compared to the Minnesota lakes (41.6% ± 9.6), while the

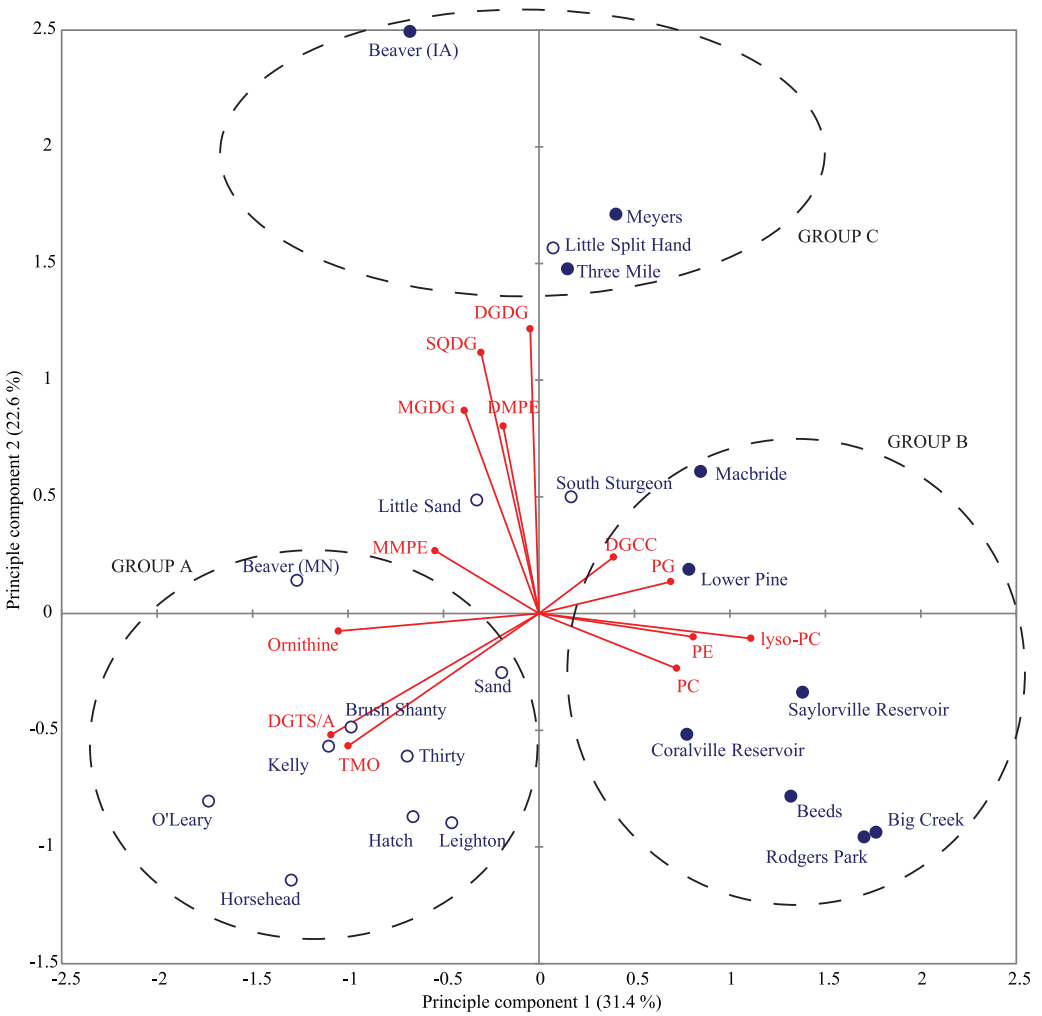

Fig. 4. Principal component analysis (PCA) plot for the percent of diacyl- and lyso-IPLs from all lakes ($n = 22$). Iowa lakes shown with filled circles and Minnesota lakes shown with open circles. See text for abbreviations of IPL names. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

percentage of the lyso-PCs was higher in the Iowa lakes (12.6% ± 5.7) than the Minnesota lakes (1.6% ± 0.6).

To further examine the differences in the IPL distribution between the lakes we performed a PCA (Fig. 4) using the peak area percentages of the thirteen lipid groups (Table S6). The two first principal components accounted for 53.9% of the variability in the IPL data with principal component one accounting for 31.4% and principal component two for 22.6% (Fig. 4). Lyso-PC was positively loaded on the first principal component, while ornithine lipids, as well as the DGTA/DGTSs and the TMOs, were negatively loaded. The glycosidic lipids, the MGDGs, DGDGs and SQDGs, were positively loaded on the second principal component. The Iowa lakes and Minnesota lakes were mostly separated along the first principal component (Groups A and B, Fig. 4). Four lakes, Beaver Lake, Meyers Lake and Three Mile Lake from Iowa and Little Split Hand Lake from Minnesota, were separated from the other lakes along principal component 2 (Group C, Fig. 4).

Each of the diacyl- and lyso-IPLs classes contained a variety of species with different FA compositions (Supporting Information Tables S1–S5). The most commonly observed FA constituents were $C_{14:0}$, $C_{16:0}$, $C_{16:1}$, $C_{18:1}$, $C_{18:2}$, and $C_{18:3}$ with more minor amounts of $C_{16:2}$, $C_{20:5}$, and $C_{22:6}$.This compares well with the phospholipid-derived FA (PLFA) composition determined separately for SPM from the same lakes (de Kluijver 2012). The PLFA showed a dominance of $C_{16:0}$ (~ 30% on average of total PLFA), $C_{16:1\omega7c}$ (14%), $C_{18:3\omega3}$ (11%), $C_{18:1\omega9c}$ (9%), $C_{20:5\omega3}$ (8%), $C_{18:2\omega6}$ (6%), and $C_{22:6\omega3}$ (5%) (de Kluijver 2012). To investigate the FA differences in IPLs between the Iowa and Minnesota lakes, each class of IPL was examined in terms of the average combined FA chain length (number of acyl carbons) and the average combined FA double bond equivalent number (Bale et al. 2015). In the case of the β-OH FAs of the ornithines and TMOs, these were also examined in terms of number of acyl carbons and number of double bond equivalents (cf. Supporting Information Table S5). Some IPL groups exhibited a significant

difference (*t*-test, $p \leq 0.05$) in average combined FA chain length between the Iowa and Minnesota lakes, i.e., the MGDG FA chain length was on average 33.6 ± 0.9 and 32.8 ± 0.7 for Iowa and Minnesota respectively ($p = 0.034$), the DGDG FAs were 34.0 ± 0.6 and 33.1 ± 0.6 ($p = 0.012$), DGTA/DGTS FAs were 33.8 ± 0.5 and 33.3 ± 0.4 ($p = 0.004$), the ornithines FAs were 30.5 ± 0.8 and 31.4 ± 0.4 ($p = 0.006$) and TMO FAs were 36.1 ± 0.2 and 36.6 ± 0.3 ($p < 0.001$) (c.f. Supporting Information Table S7 for individual lake data). Similarly, certain IPL groups also exhibited a significant difference in average combined FA double bond equivalents between the Iowa and Minnesota lakes, i.e., the MMPE FAs contained on average 2.6 ± 0.1 and 2.4 ± 0.2 double bond equivalents respectively ($p = 0.003$), the lyso-PCs 0.7 ± 0.4 and 0.1 ± 0.1 ($p < 0.001$), the ornithines 1.6 ± 0.2 and 1.7 ± 0.1 ($p = 0.067$) and the TMOs 1.2 ± 0.2 and 1.7 ± 0.1 ($p < 0.001$). PCA analysis (not shown) in the overall FA composition did not reveal distinction between Iowa and Minnesota lakes.

Heterocyst glycolipids

General screening of IPLs using HPLC-ESI/MS did not directly reveal the presence of heterocyst glycolipids (HGs). Therefore, to assess the structural diversity in HGs we performed a targeted assay using HPLC/MS^2 following Bauersachs et al. (2009b). We found all targeted HGs in all lakes although with varying abundance and distribution (Supporting Information Table S8). In general, 1-(O-hexose)−3,25-hexacosanediol (HG II; Fig. 1) dominated and comprised between 48% and 96% of the targeted HG peak areas. The other HGs varied in abundance between 1% and 29% with 1-(O-hexose)−3-keto-27-octacosanol (HG III) generally always in lowest abundance, never reaching more than 7% of total targeted HGs (Supporting Information Table S8). The total HGs abundance was not significantly different between the two sets of lakes, but the relative percent of HG I was ($7.6\% \pm 6.9$ in Iowa lakes and $10.3\% \pm 2.9$ in Minnesota lakes; *t*-test, $p = 0.023$) as was the HG_{28} index (0.18 ± 0.07 in Iowa lakes and 0.25 ± 0.06 in Minnesota lakes; *t*-test, $p = 0.032$).

Discussion

Influence of trophic state on IPL distribution

The different trophic states of the lakes sampled in this study, ranging from oligo-mesotrophic to eutrophic (Table 1), did not lead to a clear dichotomy in IPL diversity, i.e., most IPL groups were found in SPM of both the eutrophic lakes and the oligo-mesotrophic lakes. Indeed, the distribution of IPLs observed in the lake surface waters are similar to those in SPM from the marine photic zone (e.g., Van Mooy et al. 2006; Schubotz et al. 2009; Van Mooy and Fredricks 2010; Brandsma et al. 2012). However, statistical analysis revealed some distinct differences in the IPL distribution between the lakes (Fig. 4). Group A containing 9 of the 11 oligo-mesotrophic lakes was characterized by a high percentage of ornithine lipids, TMOs and the DGTA/DGTS betaine lipids.

Ornithine and TMO lipids are exclusively associated with bacteria (López-Lara et al. 2003; Moore et al. 2013; Escobedo-Hinojosa et al. 2015) and around 50% of all bacteria have the genetic capacity to synthesize ornithines (Vences-Guzmán et al. 2015). The higher relative percentage of ornithine and TMO lipids in oligo-mesotrophic lakes may thus indicate a higher amount of bacterial biomass relative to phytoplankton biomass than in the more eutrophic lakes. Indeed, oligo-mesotrophic lakes, where DOC concentrations are equal to or higher than POC concentrations, have been shown to be dominated by heterotrophic bacterial processes compared to eutrophic lakes (Biddanda et al. 2001). de Kluijver et al. (2014) reported the concentration of phytoplankton derived C (C_{phyto}, based on chlorophyll *a* concentration and summed concentration of phytoplankton derived FAs) and bacterial derived C (C_{bac}, based on the summed concentration of bacterial specific FAs) for the Iowa and Minnesota lakes. The ratio of C_{bac} to C_{phyt} was indeed higher in the Minnesota lakes (av. 0.18 ± 0.10) than in the Iowa lakes (av. 0.11 ± 0.05), although this was not a significant difference (*t*-test, $n = 22$, $p = 0.055$).

TMOs were recently described for the first time in four isolated *Planctomycetes* from ombrotrophic wetlands from north Russia (Moore et al. 2013), while the gene involved in the methylation of the ornithine lipid head group to form TMOs has also been found in the Gammaproteobacteria *Arhodomonas aquaeolei* (Escobedo-Hinojosa et al. 2015). Moore et al. (2013) postulated that the methylation of ornithines may be an adaption by the *Planctomycetes* to increase their membrane stability under acidic conditions without using P, which is scarce in ombrotrophic conditions. While the wetlands pH range (3.6–4.0) was considerably more acidic than those found in the Minnesota lakes (6.1–8.4) the TP concentration was higher (0.04–0.35 mg L^{-1}) than that in the Minnesota lakes (0.002–0.03 mg L^{-1}). The adaption to pH in low TP conditions could further explain the significantly higher percentage of TMOs in the Minnesota lakes than in the Iowa lakes, as the Iowa lakes have higher pH and higher TP concentrations (Table 1). However, it should be noted that the ratio of TMO: ornithines was not significantly different between the two sets of lakes (data not shown).

The oligo-mesotrophic Minnesota lakes, and in particular Group A of the PCA, were also characterized by a significantly higher percentage of DGTA/DGTSs. As we were unable to distinguish between DGTA and DGTS betaines it is impossible to say which of these two betaine lipids is grouping with the majority of the oligo-mesotrophic lakes. While betaine lipids have been found in a variety of eukaryotes (Dembitsky 1996), DGTS has also been identified in bacteria grown under P-limited conditions (Benning et al. 1995; Geiger et al. 1999). Culture studies have found that glycerophospholipids are substituted with non-P glycerolipids under P-limiting conditions (e.g., Minnikin et al. 1974; Benning et al. 1995). Indeed, in the oligo-mesotrophic Minnesota

Table 2. Pearson rank correlation coefficients (*r*) between the abundance and distribution of heterocyst glycolipids (HGs) with the environmental data over the whole data set ($n = 22$; $p \leq 0.01$ indicated in bold and $p \leq 0.05$ indicated in italics). DOC (dissolved organic carbon); DIC (dissolved inorganic carbon); POC (particulate organic carbon); Chl *a* (chlorophyll *a*); TN (total nitrogen); TP (total phosphorus). For the biomass of *Anabaena* $n = 10$.

	HG sum	HG I	HG III	HG IV	HG V	HG VI	$HD_{26/28}$
pH	*0.51*						
Temp	*0.49*			*0.42*			*0.44*
DOC						*0.44*	
DIC						*−0.46*	
POC	**0.89**				**0.59**		
Chl *a*	**0.71**	*−0.45*			*0.48*		*0.45*
TN							*0.43*
TP	**0.60**		*0.46*	*0.52*		*−0.43*	**0.56**
Anabaena (mg L^{-1})	**0.81**						

lakes the sum of the non-P glycerolipids (MGDGs, DGDGs, SQDGs, DGTA/DGTS and DGCC) constituted on average 69.6% ± 6.9 of the total, whereas they constituted on average 50.7% ± 14.8 in the eutrophic Iowa lakes, a significant difference (*t*-test, $p < 0.001$). To further examine this, we calculated the substitution ratios DGTA/DGTS:PC (Geiger et al. 1999), DGCC:PC (Martin et al. 2011) and SQDG:PG (Benning et al. 1993) (Supporting Information Table S9). Only the DGTA/DGTS:PC ratio was significantly different between the two lake sets (*t*-test, $p = 0.001$) with higher values of this ratio in the Minnesota lakes where TP values were lower although there was no significant relationship between this ratio and TP (Pearson rank correlation, $p > 0.050$).

The PCA grouped 7 of the 10 eutrophic lakes (Group B, Fig. 4) mainly because of the relative percentages of the PCs, PEs, DGCCs, PGs, and lyso-PCs. The eutrophic lakes would be expected to have a higher relative input of photoautotrophic biomass, but these could be eukaryotic, e.g., algae or prokaryotic, e.g., Cyanobacteria, which is difficult to distinguish based on IPL composition. Furthermore the relative abundances of prokaryotes:eukaryotes could not be inferred from the FA composition (c.f. Supporting Information Table S7) of the IPLs analyzed. Prokaryotic IPLs would be expected to bear shorter, more saturated FAs (Kenyon 1972; Bertone et al. 1996) but there was not a distinct difference in FA chain length or saturation between the two lake sets. However, it is interesting to note that, except for the DGCCs, the IPLs in Group B lakes are all phospholipids, and the lakes in this group have a significantly (*t*-test, $n = 16$, $p = 0.001$) higher average TP concentration (89.7 μg L^{-1} ± 65.2, all Iowa) than the lakes in Group A (11.3 μg L^{-1} ± 5.7, all Minnesota). This suggests that in these lakes the amount of P is sufficient to produce relatively higher amounts of phospholipids. It is unclear why there is a higher DGCC percentage in the eutrophic Iowa lakes given DGCC often acts as a substitute for PC under low P conditions.

Of the phospholipids, only the lyso-PCs was present in a significantly higher percentage in the eutrophic lakes than in the oligo-mesotrophic lakes. Lyso-lipids are intermediates in the metabolism and turnover of IPLs, but lyso-(phospho)-lipids also function as secondary messengers and appear to influence lipid-protein interactions in the lipid membrane (Meijer and Munnik 2003 and references therein; Ishii et al. 2004 and references therein; Fuller and Rand 2001). Furthermore, lyso-lipids were shown to stimulate channels that protect bacterial cells from osmotic pressure (Nomura et al. 2012). The higher percentage of lyso-lipids in the eutrophic Iowa lakes might therefore be an environmental adaptation to the higher pH or ionic strength of the lake waters or to the higher nutrient availability in the Iowa lakes. Indeed, the peak area percentage lyso-PCs exhibited a significant ($p \leq 0.05$) positive Pearson correlation with pH ($r = 0.53$), alkalinity ($r = 0.67$) and dissolved inorganic carbon (DIC; $r = 0.70$) when all 22 lakes were examined together (data not shown). The ion concentrations and nutrient availability are generally higher in Iowa lakes than in Minnesota lakes, due to a combination of geological and anthropogenic factors (Jones and Bachmann 1978; Gorham et al. 1983).

The final lake grouping (Group C, Fig. 4) constituted four eutrophic lakes, three from Iowa and one from Minnesota, all of which have relatively high percentages of the DGDGs, MGDGs, SQDGs and DMPEs. The three glycolipids (MGDG, DGDG and SQDG) are predominately associated with chloroplasts and cyanobacteria, although they can also be produced by non-photosynthetic bacteria (Hölzl and Dörmann 2007; Popendorf et al. 2011). These four lakes had Chl *a* concentrations (Table 1) above the average for all lakes (18.6 ± 25.2 μg L^{-1}) and indeed Mayer Lake had the highest Chl *a* concentration of the Iowa lakes and Little Split Hand Lake had the highest Chl *a* concentration of the Minnesota lakes. Thus, our results suggest that higher percentages of the three glycolipids is associated with more productive lakes. Indeed this may be why the ratios of SQDG: PG, thought to be indicative of P-stress (see above), was not significantly different between the eutrophic Iowa lakes and oligo-mesotrophic Minnesota lakes. The reason for the higher percentage of DMPE in group C is not evident, although it is possible that some algae may synthesize DMPE. Another factor that might be a cause of the different IPL composition of Group C is that the four lakes have significantly (*t*-test, $n = 11$, $p = 0.041$) higher pH values (av. pH 9.2 ± 0.7) relative to the other eutrophic lakes of Group B (av. pH 8.4 ± 0.3).

In summary, there were some significant differences in IPL composition between the eutrophic and oligo-

mesotrophic lakes, which we surmise is either due to adaptation of the membrane composition to nutrient conditions or due to general divergences in microbial composition. However, none of these IPLs can be unambiguously associated with specific microbes, i.e., at the genera/family level. Therefore, we performed a targeted screening of HGs which are associated exclusively with heterocystous Cyanobacteria (Bauersachs et al. 2010).

Influence of N and P on heterocyst glycolipid distribution and abundance

HGs are unique tracers for heterocystous Cyanobacteria and are only found when these Cyanobacteria form heterocyst cells and perform N_2 fixation (Walsby 1985; Murry and Wolk 1989; Bauersachs et al. 2009a). Thus, the presence of HGs unambiguously indicates the presence of N_2-fixing heterocystous Cyanobacteria. Many studies, mainly using microscopy, have been performed examining the controlling factors on the distribution of Cyanobacteria in lakes. A frequently cited hypothesis is that they particularly dominate at low N: P ratios due to their ability to fix N_2 (Schindler 1977; Smith 1983). Other studies have shown, however, that this hypothesis cannot fully explain their occurrence and that this is correlated to other factors such as total nutrient concentrations, CO_2 concentrations, light, temperature, and stratification conditions (Downing et al. 2001; Wagner and Adrian 2009). Furthermore, these studies rarely considered whether the Cyanobacteria were actually performing N_2-fixation or were mainly using available nutrients (Ferber et al. 2004).

The fact that we find HGs in all lakes strongly suggests that cyanobacterial N_2-fixation is ongoing in both the oligo-mesotrophic Minnesota lakes as well as the eutrophic Iowa lakes. To investigate the factors controlling their abundance, we compared the abundance of total HGs with the environmental data. Significant Pearson correlations ($p \leq 0.05$, Table 2) were found with pH ($r = 0.51$), temperature ($r = 0.49$), POC ($r = 0.89$), the Chl *a* concentration ($r = 0.71$) and TP ($r = 0.60$) but not with TN or TN : TP. Ferber et al. (2004) also noted a correlation of heterocyst cell concentrations with Chl *a* concentrations for a hypertrophic lake. We should, however, note that most environmental data in our lake data set are also significantly correlated with each other (data not shown) making it difficult to constrain the relation of individual environmental data with HG abundance.

There is some variability between the lakes in the distribution of the 6 HGs examined (Supporting Information Table S8). One possible cause is that different HGs occur in different genera, i.e., HGs I-IV exclusively occur in genera of the family Nostocaceae (Bauersachs et al. 2009a). Microscopical analysis of the phytoplankton composition of the Iowa lakes from the same time period as our sampling identified the presence of two heterocystous Cyanobacteria, *Anabaena* and *Aphanizomenon* (Supporting Information Table S8), both belonging to the family Nostocaceae and known producers of HGs I-IV (Bauersachs et al. 2009a; Woermer et al. 2012). While neither the percent of the individual HGs nor the summed percent of HGs I-IV correlated with the biomass of *Anabaena* or *Aphanizomenon*, the abundance of total HGs did exhibit a significant correlation with the biomass of *Anabaena* ($r = 0.81$, $p \leq 0.01$, Table 2). HGs V-VI occur in *Calothrix* species of the family Rivulariaceae (Bauersachs et al. 2009a). *Calothrix* species were not detected at the time of sampling and have rarely been reported at any time in the Iowa lakes. It might be possible that undetected heterocystous cyanobacterial species other than *Calothrix* were the source for HGs V-VI. Nevertheless, if we assume that HG V-VI are derived from species other than those belonging to the family Nostocaceae, then their relative abundance among N_2-fixing heterocystous Cyanobacteria varies between 1% and 40%, assuming that the HG abundance (Supporting Information Table S8) reflects the abundance of heterocystous cyanobacterial biomass.

Bauersachs et al. (2009a) found a correlation between incubation temperature and the distribution of HGs I and II in cultivated heterocystous Cyanobacteria and quantified this in the so-called HG_{26} index. Indeed, Woermer et al. (2012) found a strong sigmoidal relation of the HG_{26} with lake water temperature in their Spanish lakes. Other indices which correlated with growth temperature suggested by Bauersachs et al. (2014) include the HG_{28} index and $HD_{26/28}$ index. While no significant correlation of the HG_{26} or HG_{28} indices were found with temperature, nor with other environmental parameters, the $HD_{26/28}$ index correlated significantly (Table 2, $p \leq 0.05$) with temperature ($r = 0.44$), Chl *a* ($r = 0.45$), TN ($r = 0.43$) and TP ($r = 0.56$). There were also a number of significant ($p \leq 0.05$) Pearson correlations between the percentage of the individual HGs and the environmental data (see Table 2 for *r* values), but it is unclear if this is coincidental or not. Overall, it is uncertain what the mechanism is behind the diversity in the HG distributions of the heterocystous Cyanobacteria in lakes.

Conclusion

Through our characterization of the IPL composition of SPM in the surface waters of 22 lakes from Minnesota and Iowa (U.S.A.) we found a number of significant differences in the IPL distribution. The eutrophic Iowa lakes contained a substantially higher percentage of lyso-PC than the oligo-mesotrophic Minnesota lakes, possibly as a consequence of the high ion content or high nutrient availability in the Iowa lakes. We found that the oligo-mesotrophic lakes of Minnesota were characterized by higher percentages of ornithines and the TMOs, which we suggest relates to the higher relative contribution of heterotrophic bacteria that would be expected in the oligo-mesotrophic lakes, as well as the lower availability of P and their lower pH levels. The

oligo-mesotrophic lakes also contained a higher percentage of the DGTA/DGTS betaines, which we surmise relates to substitution by microorganisms of these non-P lipids for PC under P-stress. Conversely, we did not find a difference in the SQDG:PG ratio, a ratio also found to change under P-stress, between the two lake sets. Instead, there was a high proportion of SQDGs and the galactosyldiacylglycerol lipids in a group of lakes with above average Chl *a* concentrations.

We detected a variety of HG lipids derived from N_2-fixing heterocystous Cyanobacteria in all lakes, suggesting their presence in the full range of trophic conditions. Correlation of HG abundance with environmental data did show that high productivity lakes have high HG abundances. We also observed distributional differences in HGs, likely due to differences in the cyanobacterial species composition. Thus, our study shows that IPL composition in lakes are different depending on the trophic states of lakes due to changes adaptation of membranes to environmental conditions and differences in the microbial composition.

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