

1 Dear Dr. Niemann,

2

3 We thank you for your comments on our manuscript. Below we respond to the comments (*in*
4 *italic*) and indicate how we have modified the manuscript. As you will see, we have followed
5 most of your suggestions.

6

7 We hope that you find the revised manuscript suitable for publication in *Biogeosciences*.

8

9 Yours sincerely,

10 Thorsten Bauersachs

11

1 **Editor's comments:**

2

3 P8, L10 add that MBT/CBT is often complicated because allochthonous brGDGT signals may be
4 overprinted by autochthonous brGDGTs (eg. Tierney and Russell, org. Geochem 40, 2009; Schouten
5 et al., Org. Geochem. 54, 2013; Weber et al., GCA 154, 2015). This strengthens your argument for the
6 need of additional T-proxies.

7

8 *As requested by the editor, we now extended our discussion on MBT/CBT-based temperature*
9 *reconstructions by adding information on the autochthonous production of branched GDGT in aquatic*
10 *systems (p. 3, l.4-14).*

11

12 Fig 4 panel indications are missing (a-i).

13

14 *Panel indicators were present in the lower right corner of each figure but we double-checked that*
15 *they are indeed present in the final pdf-version of the manuscript.*

16

17 I leave this up to you as it is more of personal preference. Consider to use more contrasting colours
18 for Fig. 3 and Supl. Fig. 1: ie., red, green, blue, black, grey, white. The ones you used, brown, red,
19 yellow, olive (I guess earth tones would be the appropriate description) do not have lots of contrast
20 and are hard to distinguish for anyone with a color vision deficiency - ~9% of the male- and ~1% of
21 the female population.

22

23 *We now used more contrasting colors in Figure 3 and Supplement Figure 1. To keep the colour-coding*
24 *consistent throughout the manuscript, we also changed colours in Supplement Figures 2 and 4.*

25

Seasonal lake surface water temperature trends reflected by heterocyst glycolipid based molecular thermometers

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Abstract

It has been demonstrated that the relative distribution of heterocyst glycolipids (HGs) in cultures of N₂-fixing heterocystous cyanobacteria is largely controlled by growth temperature, suggesting a potential use of these components in paleoenvironmental studies. Here, we investigated the effect of environmental parameters (e.g. surface water temperatures, oxygen concentrations and pH) on the distribution of HGs in a natural system using water column filtrates collected from Lake Schreventeich (Kiel, Germany) from late July to the end of October 2013. HPLC-ESI/MS analysis revealed a dominance of 1-(O-hexose)-3,25-hexacosanediols (HG₂₆ diols) and 1-(O-hexose)-3-keto-25-hexacosanol (HG₂₆ keto-ol) in the solvent extracted water column filtrates, which were accompanied by minor abundances of 1-(O-hexose)-3,27-octacosanediol (HG₂₈ diol) and 1-(O-hexose)-3-keto-27-octacosanol (HG₂₈ keto-ol) as well as 1-(O-hexose)-3,25,27-octacosanetriol (HG₂₈ triol) and 1-(O-hexose)-3-keto-25,27-octacosanediol (HG₂₈ keto-diol). Fractional abundances of alcoholic and ketonic HGs generally showed strong linear correlations with surface water temperatures and no or only weak linear correlations with both oxygen concentrations and pH. Changes in the distribution of the most abundant diol and keto-ol (e.g., HG₂₆ diol and HG₂₆ keto-ol) were quantitatively expressed as the HDI₂₆ (heterocyst diol index of 26 carbon atoms) with values of this index ranging from 0.89 in mid-August to 0.66 in mid-October. An average HDI₂₆ value of 0.79, which translates into a calculated surface water temperature of 15.8±0.3°C, was obtained from surface sediments collected from Lake

Schreventeich. This temperature - and temperatures obtained from other HG indices (e.g., HDI₂₈ and HTI₂₈) - is similar to the one measured during maximum cyanobacterial productivity in early to mid-September and suggests that HGs preserved in the sediment record of Lake Schreventeich reflect summer surface water temperatures. As N₂-fixing heterocystous cyanobacteria are widespread in present-day freshwater and brackish environments, we conclude that the distribution of HGs in sediments may allow the reconstruction of surface water temperatures of modern and potentially ancient lacustrine settings.

1 Introduction

Lipid paleothermometers have become an indispensable tool in paleoenvironmental studies as they allow the reconstruction of oceanic surface water temperatures over geological time scales and thus provide essential information on past climate changes. The two most commonly employed lipid paleothermometers are the U^K₃₇ (Brassell et al., 1986) and the TEX₈₆ (Schouten et al., 2002), which use the distribution of long chain alkenones or glycerol dialkyl glycerol tetraether (GDGT) preserved in marine sediments to reconstruct oceanic surface water temperatures. The more recently introduced long chain diol index (LDI), which is based on the distribution of C₂₈ 1,13-, C₃₀ 1,13-, and C₃₀ 1,15-diols produced by eustigmatophyte algae (Rampen et al., 2012), provides an additional mean to determine past changes in sea surface temperatures (SST) and has successfully been applied in a number of paleoceanographic studies (Smith et al., 2013; Rodrigo-Gámiz et al., 2014).

The TEX₈₆ proxy has previously been applied to a number of freshwater environments but seems to reliably predict surface water temperatures only in some large lakes, such as the North American Great Lakes and the African Rift Valley lakes, where the contribution of isoprenoid GDGTs of a terrestrial origin is only negligible (Powers et al., 2010). Likewise, long chain alkenones have been reported from some modern lake systems (Volkman et al., 1988; Thiel et al., 1997; Theroux et al., 2012) and were employed to reconstruct past changes in surface water temperatures in Lake Steisslingen, SW Germany (Zink et al., 2001). However, due to our incomplete knowledge on the biological sources of long chain alkenones and their comparatively limited distribution in freshwater environments, temperature estimates based on long chain alkenones in lacustrine sediments are comparatively few.

Another lipid paleothermometer that has attracted considerable attention over the recent past is the MBT (methylation index of branched tetraethers)/CBT (cyclisation ratio of branched tetraethers) index. This proxy, based on the distribution of branched GDGTs that are ubiquitously distributed in soils, peats as well as lacustrine and coastal marine sediments (see Schouten et al., 2013 and references therein), has been shown to correlate well with mean annual air temperature (MAAT) and soil pH (Weijers et al., 2007). ~~Consequently, the MBT/CBT lipid paleothermometer~~ It has since also been applied to ~~a number of various~~ lakes and coastal marine environments, containing a large proportion of terrestrial organic matter, to infer past changes in continental climate (Zink et al., 2010; Niemann et al., 2012; Berke et al., 2014). ~~However, a number of more recent studies have demonstrated that branched GDGTs may also be produced in aquatic systems, possibly complicating the application of the MBT/CBT index as a tool for reconstructing MAAT (De Jonge et al., 2014; Weber et al., 2015). Nonetheless, the MBT/CBT index and other~~ Hence while the MBT/CBT index as well as lipid paleothermometers in general have proven most valuable in determining detrends in deciphering climate evolution both on regional and global scales. ~~trends in paleoenvironmental studies.~~ A lipid-based proxy that allows deciphering ~~a number of lipid paleothermometers allow the reconstruction of SST and continental MAAT, Yet, no such proxy is currently available to reliably decipher determine~~ past changes in surface water temperatures in lacustrine environments on the contrary is currently missing (Castañeda and Schouten, 2011).

Heterocystous cyanobacteria are oxygenic photoautotrophs that are known to be an abundant component of the phytoplankton community of many present-day freshwater lakes of polar to tropical latitudes (Whitton, 2012). They are also known to form massive blooms in river deltas and semi-enclosed basins such as the Baltic Sea (Stal et al., 1999; Larsson et al., 2001). Their dominant role in the primary production of freshwater and brackish environments is related to their unique ability to simultaneously perform oxygenic photosynthesis and nitrogen fixation, enabling them to outcompete eukaryotic algae under nitrogen limiting conditions (Levine and Schindler, 1999). For this, heterocystous cyanobacteria confine the fixation of N₂ to heterocysts, which host the oxygen-sensitive enzyme nitrogenase that catalyzes the reduction of dinitrogen gas to ammonia. These specialized cells are enveloped in a set of unique glycolipids, so-called heterocyst glycolipids (HGs), which are exclusively present in N₂-fixing heterocystous cyanobacteria (Nichols and Wood, 1968; Gambacorta et al., 1999; Bauersachs et al., 2009a) and

are considered to act as a gas diffusion barrier that limits the entry of oxygen into the heterocyst (Wolk, 1982). These components are composed of sugar head groups that are glycosidically bound to long chain diols, triols, keto-ols or keto-diols with an even carbon chain ranging from C₂₆ to C₃₂ carbon atoms (Fig. 1). The distribution of HG diols and keto-ols has previously been shown to strongly correlate with growth temperature in cultures of the heterocystous cyanobacteria *Anabaena* CCY9613 and *Nostoc* CCY9926 (Bauersachs et al., 2009a; 2014). These authors demonstrated that in both types of cyanobacteria the relative proportion of HG diols significantly increased compared to their corresponding HG keto-ols with increasing growth temperature and introduced the HG₂₆ (heterocyst glycolipid index of 26 carbon atoms) and HG₂₈ (heterocyst glycolipid index of 28 carbon atoms) as means to quantify structural changes in the HG composition of the heterocyst cell envelope. It should be pointed out though that the overall change in the structural composition of the heterocyst cell envelope varied significantly between both cyanobacteria with HG₂₆ values varying from 0.10 to 0.18 in *Anabaena* CCY9613 and from 0.12 to 0.30 in *Nostoc* CCY9926 (Bauersachs et al. 2014), indicating that individual species of heterocystous cyanobacteria may tune the properties of the gas diffusion barrier in a slightly different fashion. Nonetheless, the finding of temperature induced changes in the heterocyst glycolipid composition of N₂-fixing heterocystous cyanobacteria may offer the exciting possibility to reconstruct surface water temperatures of modern and possibly also fossil lacustrine environments given that (1) heterocystous cyanobacteria are a common component of the phytoplankton community in many contemporary and fossil freshwater environments (Whitton, 2012) and (2) HGs have been shown to preserve well in the geological record (Bauersachs et al., 2010). Here, we investigated temporal variations in the distribution of heterocyst glycolipids in water column filtrates of Lake Schreventeich (Kiel, Germany). We also analyzed the distribution of HGs in surface sediments of this small holomictic lake and discuss the potential use of HGs in the reconstruction of surface water temperatures in modern and fossil freshwater environments.

2 Material and methods

2.1 Study site and sampling

Lake Schreventeich is a small holomictic lake situated in northern Germany (54°19'36.79"N, 10°07'17.57"E). Its surface area covers approximately 0.38 km² and it has an average depth of

1.4-1.6 m (maximum depth of 3.4 m). The lake has no tributaries and is solely fed by precipitation and ground water inflow.

Surface water samples for the analysis of HGs were taken from late July to the end of October 2013. Oxygen concentrations and surface water temperatures were measured at time of sampling using the portable oxygen measuring instrument “Oxi 1970i” coupled to a “CellOx325” oxygen probe (WTW, Germany). The pH of all water samples was determined using a “FG2-FiveGo” (Mettler-Toledo, Germany) using a two-point calibration on certified reference solutions obtained from Hanna Instruments. Surface sediments (0-1 cm) from two locations within Lake Schreventeich were obtained in March 2014 using an Uwitech gravity corer (Uwitech, Switzerland). All sediments were freeze-dried and ground to a homogenous powder using pestle and mortar.

2.2 Determination of algal biomass

100 mL of surface water were collected during each sampling and filtered over a preweighed Whatman filter GF/C (1.2 μm , diameter 47 mm). After filtration, filters were manually inspected and non-phytoplankton biomass was removed using a pair of tweezers. All filters were subsequently dried in an oven at 105 °C for 24 hours. Phytoplankton biomass was calculated as the weight difference between the preweighed and the oven-dried filters.

2.3 Bligh and Dyer extraction of water column filtrates and core top sediments

Measured volumes (e.g., 3-4 L) of surface water were filtered through a MN 85/70 BF glass fiber filter with a pore size of 0.45 μm (Macherey-Nagel, Germany). All filters were freeze-dried and extracted following a modified Bligh and Dyer procedure as described by Rütters et al. (2002). Briefly, filters were cut into fine pieces with a solvent-cleaned scissor and ultrasonically extracted using a solvent mixture of methanol (MeOH), dichloromethane (DCM) and phosphate buffer (2/1/0.8; v/v/v). After centrifugation, the supernatant was collected and the residue extracted twice with the solvent mixture specified above. DCM and phosphate buffer were added to the pooled supernatants to achieve a ratio of MeOH/DCM/phosphate buffer of 1:1:0.9 (v/v/v), allowing separation of two phases. The bottom layer, containing the organic fraction, was transferred to a glass vial and the remaining aqueous phase was extracted twice with DCM. The combined extracts were reduced under rotary vacuum, transferred to preweighed vials and dried

under a gentle stream of N₂. All Bligh and Dyer extracts were subsequently dissolved in DCM:MeOH (9:1; v/v) to a concentration of 2 to 4 mg mL⁻¹ and filtered through a 0.45- μ m-pore-size regenerated cellulose filter (13 mm; LLG Labware, Germany) prior to analysis. In addition to water column filtrates, 0.5 gram of freeze-dried core top sediments obtained from Lake Schreventeich were extracted using the procedure outlined above.

2.4 Analysis of heterocyst glycolipids

Heterocyst glycolipids were analyzed following the procedure described by Bauersachs et al. (2014) with some brief modifications. Separation of the target compounds was achieved using an Alliance 2690 HPLC system (Waters, UK) fitted with a Luna Hilic 200A column (150 mm x 2 mm i.d.; 3 μ m; Phenomenex, Germany) maintained at 30 °C. The following linear gradient was used with a flow rate of 0.2 mL min⁻¹: 95% eluent A/5% eluent B to 70% A/30% B in 10 min (held 20 min), followed by 70% A/30% B to 35% A/65% B in 15 min (held 15 min), then back to 95% A/5% B in 1 min (held 20 min) to re-equilibrate the column. Eluent A was hexane-isopropanol-formic acid-14.8 M aqueous NH₃ (79:20:0.12:0.04; v/v/v/v) and eluent B was isopropanol-water-formic acid-14.8 M aqueous NH₃ (88:10:0.12:0.4; v/v/v/v).

Detection of heterocyst glycolipids was accomplished using a Quattro LC triple quadrupole mass spectrometer (Micromass, UK). The positive electrospray ionization (ESI) conditions were as follows: capillary voltage, 3.2 kV; cone voltage, 25 V; source temperature 120 °C; desolvation temperature, 200 °C; cone gas flow, 1 L min⁻¹ and desolvation gas flow, 4 L min⁻¹. To qualitatively determine the distribution of HGs in water column filtrates of Lake Schreventeich, all Bligh and Dyer extracts were analyzed in data dependent mode with two scan events, where a positive ion scan (m/z 300-1000) was followed by a product ion scan of the base peak of the mass spectrum of the first scan event. Identification of HGs was based on comparison with published mass spectra (Bauersachs et al., 2009b). To improve the sensitivity of the measurement and therewith increase reproducibility, HGs were also detected via single ion recording (SIR) of their protonated molecules [M+H]⁺ (dwell time 234 ms) with m/z 575.5 (HG₂₆ keto-ol), m/z 577.5 (HG₂₆ diol), m/z 603.5 (HG₂₈ keto-ol), m/z 605.5 (HG₂₈ diol), m/z 619.5 (HG₂₈ keto-diol) and m/z 621.5 (HG₂₈ triol). Selected samples were analyzed in duplicate and fractional abundances of HGs as well as calculated HG ratios (e.g., HDI₂₆, HDI₂₈, HTI₂₈) given in the text represent

average values of these measurements. Quantification was done by integration of the peak area using the QuanLynx application manager.

3 Results

3.1 Variation of environmental parameters and algal biomass in Lake Schreventeich

Physical and biological data of Lake Schreventeich collected from late July to the end of October 2013 are summarized in Figure 2. All investigated physical parameters (i.e., temperature, oxygen concentration and pH) show maxima in late July or at the beginning of August and gradually decline to yield minima in late October. Surface water temperatures ranged from 10.5 to 24.0°C and were highest in late July (Fig. 2a). Oxygen concentrations in the surface waters ranged from 2.5 to 7.6 mg L⁻¹ with highest values occurring in late July and they subsequently declined over the investigated time interval to yield minimum values in late October (Fig. 2b). pH values ranged from 7.18 to 7.79 and were comparatively high during the first half of the sampling campaign with values averaging 7.56 in August (Fig 2c). In contrast, the pH showed a significant drop by almost 0.2 units at the beginning of September and stayed around 7.32 throughout the first half of September before increasing again to values of ca. 7.50 at the beginning of October. Lake productivity was determined by measuring the amount of biomass present at time of sampling. Comparatively low amounts of biomass were found in late July with values of 11.6 mg L⁻¹ that almost doubled in August with an average value of 20.7 mg L⁻¹ (Fig. 2d). After a pronounced peak in the first half of September (maximum 50.1 mg L⁻¹; average 35.3 mg L⁻¹), biomass concentrations declined to an average value of 22.5 mg L⁻¹ in October.

3.2 Distribution and fractional abundances of heterocyst glycolipids in water column filtrates of Lake Schreventeich

Heterocyst glycolipids were below detection limit in late July and early August. They were first identified in mid-August in low relative abundances, gradually increased in late August to reach peak abundances in early to mid-September (Fig. 3). In late September, the relative abundance of HGs declined to reach comparatively low but constant values from mid- to late October. As shown in Fig. S1 in the Supplement, two structural isomers of 1-(O-hexose)-3,25-hexacosanediol

(HG₂₆ diol) and 1-(O-hexose)-3-keto-25-hexacosanol (HG₂₆ keto-ol) generally dominated the HG pool and together they constituted 71 to 100% (average $82.7 \pm 7.2\%$) of all heterocyst glycolipids over the investigated time interval. The early eluting HG₂₆ diol, however, generally constituted only a minute fraction of all HGs (on average $<0.5\%$). The heterocyst glycolipids 1-(O-hexose)-3,25,27-octacosanetriol (HG₂₈ triol) and 1-(O-hexose)-3-keto-25,27-octacosanediol (HG₂₈ keto-diol) were particularly abundant in late August with fractional abundances of up to 25% but in general they contributed 6 to 17% (average $12.3 \pm 6.2\%$) to the heterocyst glycolipid content of Lake Schreventeich. 1-(O-hexose)-3,27-octacosanediol (HG₂₈ diol) and 1-(O-hexose)-3-keto-27-octacosanol (HG₂₈ keto-ol) were below detection limit in water column filtrates taken before early September (Fig. 3) and they usually constituted a minor component of the total HG pool with fractional abundances of both compounds ranging from 0 to 13% (average $4.9 \pm 3.8\%$).

It is interesting to note that the fractional abundance of all HG diols and triols declined over the investigated time interval, while the fractional abundance of their corresponding keto-ol and keto-diol varieties showed a concomitant increase (Fig. 3). For example, the fractional abundance of the HG₂₆ diol was highest ($>70\%$) in late August to early September and thereafter declined gradually to yield values around 50% at the end of October. Over the same time period, the fractional abundance of the HG₂₆ keto-ol significantly increased from 9% at the end of August to 25% in late October. Overall similar trends were also observed for the HG₂₈ triol and HG₂₈ keto-diol as well as for the HG₂₈ diol and HG₂₈ keto-ol. It should be pointed out though that for the HG₂₈ diol and the HG₂₈ keto-ol this trend was less apparent, which may be due to the low analytical response and the resulting uncertainties in determining the contribution of both components to the total HG pool.

3.3 Distribution and fractional abundances of heterocyst glycolipids in surface sediments of Lake Schreventeich

The distribution of HGs in surface sediments of Lake Schreventeich largely resembled those observed in the water column filtrates with the HG₂₆ diol and HG₂₆ keto-ol being most abundant (Fig. 3). Both components constituted ca. 81% of the total HG pool with HG₂₆ diol and HG₂₆ keto-ol accounting for 64% and 17% of all HGs, respectively. HG₂₈ triol and HG₂₈ keto-diol were the second most abundant types of HGs in Lake Schreventeich sediments contributing 7% and 4% of all HGs. Similar to the distribution of HGs in the water column filtrates, HG₂₈ diol and

HG₂₈ keto-ol constituted only a minor component of the HG pool with 5% and 3%, respectively. Fractional abundances of HGs found in the core top sediments of Lake Schreventeich are thus well in line with those observed in water column filtrates; in particular with those obtained in mid-September.

4 Discussion

4.1 Sources and environmental controls on the HG distribution in water column filtrates of Lake Schreventeich

Heterocyst glycolipids were below detection limit in water column filtrates collected throughout July to early August and were first observed in mid-August. In general, the distribution of heterocyst glycolipids in Lake Schreventeich is well in line with those previously reported from nostocalean cyanobacteria (Bauersachs et al., 2009a; Wörmer et al. 2012) and likely suggests that members of the genera *Anabaena* and/or *Aphanizomenon* were part of the phytoplankton community of Lake Schreventeich in late summer 2013. This agrees well with microbiological studies of the phytoplankton community of other North German lakes, for which representatives of both genera have indeed been reported in abundance (Arp et al., 2013). The simultaneous increase in total HG abundances and aquatic biomass in early to mid-September (Figs. 2 and 3) may also suggest that heterocystous cyanobacteria constituted a significant component of the lake's phytoplankton.

We observed systematic changes in the distribution of heterocyst glycolipids in water column filtrates of Lake Schreventeich over the time interval investigated. The most apparent was a systematic decline in the fractional abundances of HG diols and the HG triol from mid-August to late October, which was significantly positively correlated with surface water temperature (Table 1). On the contrary, fractional abundances of HG keto-ols and keto-diols gradually increased from late August to the end of the sampling campaign. This increase in fractional abundances was significantly negatively correlated with changes in surface water temperatures (Table 1). Similar changes in the fractional abundances of HG₂₆ and HG₂₈ diols and keto-ols with growth temperature have previously been described from cultures of the N₂-fixing heterocystous cyanobacteria *Anabaena* CCY9613 and *Nostoc* CCY9926 (Bauersachs et al., 2009a; 2014) and been explained as a physiological adaptation to compensate for greater gas diffusion rates of O₂

at higher temperatures in order to keep the entry of atmospheric gases into the heterocyst at a minimum, which is considered a prerequisite for optimum N₂ fixation. To quantitatively express these structural changes of the heterocyst cell envelope, Bauersachs et al. (2009a) introduced the HG₂₆-index (*heterocyst glycolipid index of 26 carbon atoms*), which is defined as:

$$\text{HG}_{26} = \text{HG}_{26} \text{ keto-ol} / (\text{HG}_{26} \text{ diol} + \text{HG}_{26} \text{ keto-ol}). \quad (1)$$

This notation, however, is somewhat counterintuitive as values of the HG₂₆-index decline with increasing growth temperature. Therefore, we here used the HDI₂₆ (*heterocyst diol index of 26 carbon atoms*), which in contrast to the HG₂₆-index is positively correlated with temperature and defined as given below. It should be pointed out though that the HG₂₆-index and the HDI₂₆ have the same statistical significance.

$$\text{HDI}_{26} = \text{HG}_{26} \text{ diol} / (\text{HG}_{26} \text{ keto-ol} + \text{HG}_{26} \text{ diol}), \quad (2)$$

$$\text{HDI}_{26} = 0.0224 \times \text{SWT} + 0.4381; r^2 = 0.93. \quad (3)$$

In Lake Schreventeich, HDI₂₆ values ranged from 0.89 in mid-August to 0.66 in late October (Fig. 4) and closely followed variations in surface water temperatures (Fig. S2 in the Supplement). For example, HDI₂₆ values gradually declined over the investigated time period until mid-October and afterwards slightly increased again in agreement with a rise in measured surface water temperature in late October. Least squares analysis of the data showed that variations in HDI₂₆ values are strongly linearly correlated with surface water temperatures. As the HG₂₈ diol and keto-ol as well as the HG₂₈ triol and keto-diol showed similar changes in fractional abundances compared to the HG₂₆ diol and the HG₂₆ keto-ol, we also employed the HDI₂₈ (*heterocyst diol index of 28 carbon atoms*) and the HTI₂₈ (*heterocyst triol index of 28 carbon atoms*) in order to quantitatively determine changes in HG distributions with environmental parameters. Both indices were calculated as given in the following equations:

$$\text{HDI}_{28} = \text{HG}_{28} \text{ diol} / (\text{HG}_{28} \text{ keto-ol} + \text{HG}_{28} \text{ diol}), \quad (4)$$

$$\text{HDI}_{28} = 0.0405 \times \text{SWT} + 0.0401; r^2 = 0.70, \quad (5)$$

$$\text{HTI}_{28} = \text{HG}_{28} \text{ triol} / (\text{HG}_{28} \text{ keto-diol} + \text{HG}_{28} \text{ triol}), \quad (6)$$

$$\text{HTI}_{28} = 0.0288 \times \text{SWT} + 0.2292; r^2 = 0.78. \quad (7)$$

Similar to the HDI_{26} , the HDI_{28} and the HTI_{28} closely followed measured surface water temperatures with absolute values of these indices gradually declining over the investigated time period from 0.82 to 0.42 and from 0.81 to 0.49, respectively (Fig. 4). Least squares analysis of the data demonstrates that both indices are significantly correlated with surface water temperatures, although correlations are generally less strong as compared to the HDI_{26} . All three HG indices, however, seem to track temperature changes in the lake's surface waters in a similar fashion, albeit with slight differences in absolute values and trends between the individual indices (see Fig. S2 in the Supplement). One explanation for the slight offsets between the individual indices may be the contribution of heterocyst glycolipids from different cyanobacterial sources. Bauersachs et al. (2009a; 2014) as well as Wörmer et al. (2012) noticed that fractional abundances of heterocyst glycolipids may vary between different genera of heterocystous cyanobacteria and even within heterocystous cyanobacteria belonging to the same genus. Moreover, Bauersachs et al. (2014) observed that fractional abundances of HG_{26} and HG_{28} diols and keto-ols changed differently in *Anabaena* CCY9613 and *Nostoc* CCY9926, resulting in slightly different HGI_{26} and HGI_{28} values for each of the investigated species. As multiple members of heterocystous cyanobacteria (e.g., *Anabaena* and *Aphanizomenon*), adapting the composition of the heterocyst cell envelope in slightly different fashions, likely contributed to the total pool of HGs in Lake Schreventeich, absolute values of the different HG indices may have varied depending on the amount of heterocyst glycolipids contributed by each individual cyanobacterium. In this context it is interesting to note that the different HG indices show a similar trend with surface water temperatures but that HDI_{26} values are generally higher compared to HDI_{28} and HTI_{28} values, resulting in a deviation from the 1:1 line as shown in Fig. S3. HDI_{28} and HTI_{28} values on the contrary are very similar to each other and fall close to the 1:1 line, indicating that they may have the same biological origin.

When the different HG indices are plotted against environmental parameters other than surface water temperatures (Fig. 4), it is apparent that the HDI₂₆ ($p < 0.001$; $r^2 = 0.64$) and the HTI₂₈ ($p < 0.05$; $r^2 = 0.42$) are positively correlated with decreasing oxygen concentrations and that the HDI₂₆ ($p < 0.05$; $r^2 = 0.35$) and the HDI₂₈ ($p < 0.05$; $r^2 = 0.35$) also show a weak positive correlation with pH. However, these correlations are generally less significant and not as strong as observed for the correlation with surface water temperatures. It should also be noted that oxygen concentrations and pH are strongly correlated with surface water temperatures and that both parameters show a positive correlation with each other (Table 1). Therefore, the observed correlations between the different HG indices and oxygen concentrations as well as pH are likely indirect rather than indicating a statistically significant relationship between the individual environmental parameters and changes in the heterocyst glycolipid distribution. However, Kangatharalingham et al. (1992) reported that the heterocyst cell envelope of *Anabaena flos-aquae* increased in thickness when this cyanobacterium was grown under increased levels of oxygen stress and it can therefore not be excluded that environmental factors other than growth temperature may affect the distribution of heterocyst glycolipids in heterocystous cyanobacteria (although these authors did not analyze changes in the chemical structure of the heterocyst cell envelope). Additional investigations employing culture-dependent approaches and studying the effect of environmental parameters other than growth temperature will be needed to elucidate whether and to which extent oxygen concentrations and pH exert a control on the structural composition of the heterocyst cell envelope of heterocystous cyanobacteria.

4.2 Accuracy of surface water reconstructions based on HG indices

The accuracy with which surface water temperatures of a given aquatic environment can be reconstructed is essential for any novel lipid thermometer. Based on replicate analysis of individual water column filtrates and surface sediments, the average analytical precision with which the HDI₂₆ can be determined is ± 0.006 . Using the respective temperature calibration (see Eq. 3), this equals a standard error in temperature estimates of $\pm 0.27^\circ\text{C}$. The determination of HDI₂₈ (± 0.012) and HTI₂₈ (± 0.010) values is slightly less accurate than for the HDI₂₆, which may be due to the lower abundance of HG₂₈ diols, triols, keto-ols and keto-diols in the analyzed water column filtrates, with the standard error in temperature estimates being $\pm 0.30^\circ\text{C}$ for the HDI₂₈ and $\pm 0.34^\circ\text{C}$ for the HTI₂₈. However, the overall analytical precision in the analysis of the different HG indices is in the same order of magnitude or even slightly better when compared to

other well-established temperature proxies, such as the TEX_{86} and $U^{K'}_{37}$, and indicates that reconstructions of surface water temperatures using the HDI_{26} and other HG indices may be achieved in a relatively high accuracy. This is also suggested by analysis of the residual errors of the HG-estimated SWTs (calculated SWTs – measured SWTs), which are generally $<2^\circ\text{C}$ with a mean standard error of 0.97°C , 1.62°C and 1.69°C for HDI_{26} -, HDI_{28} - and HTI_{28} -reconstructed SWTs, respectively, and without following a clear trend with SWT (see Fig. S4 in the Supplement).

4.3 Distribution of heterocyst glycolipids in Lake Schreventeich surface sediments

In order to determine if the heterocyst glycolipid signal observed in the water column filtrates is transferred to the sedimentary realm, we also analyzed two surface sediments collected from Lake Schreventeich for their HG content. Sedimentary HG distributions were indeed very similar to those observed in water column filtrates with HG_{26} diol and HG_{26} keto-ol dominating over smaller quantities of HG_{28} triol and HG_{28} keto-diol as well as HG_{28} diol and HG_{28} keto-ol. It is interesting to note that the distribution of HGs in the two surface sediment samples most closely resembled the one observed during the period of maximum lake productivity and peak abundances of HGs in early to mid-September (Figs. 2 and 3), suggesting that the preserved HGs were mainly produced during maximum activity of heterocystous cyanobacteria in Lake Schreventeich. HDI_{26} values of surface sediments from Lake Schreventeich averaged 0.791 ± 0.008 . Using the temperature calibration obtained from the analysis of the water column filtrates, the HDI_{26} value translates into an average surface water temperature of $15.8\pm 0.3^\circ\text{C}$. Considering the current accuracy of the HPLC/MS method for the HDI_{26} analysis, the HDI_{26} -based temperature reconstructed for Lake Schreventeich largely agrees with surface water temperatures measured from early to mid-September and thus during the time period of highest productivity of heterocystous cyanobacteria. Likewise, reconstructed surface water temperatures based on HDI_{28} (0.575 ± 0.018) and HTI_{28} (0.637 ± 0.012) values obtained from the analysis of surface sediments of Lake Schreventeich and using their respective temperature calibrations are $13.1\pm 0.4^\circ\text{C}$ and $14.1\pm 0.3^\circ\text{C}$, respectively. Although slightly lower than the HDI_{26} -based SWT estimates, both values again agree well with surface water temperatures measured during mid-September. Together these observations suggest that the analysis of sedimentary HGs may allow reconstructing summer surface water temperatures in Lake Schreventeich and possibly also other

lacustrine environments with sufficient export and incorporation of cyanobacterial-derived organic matter into the sediment.

Despite the good agreement between measured and reconstructed surface water temperatures, it should be pointed out that the recovered surface sediments most likely not only contained HGs produced during the investigated time interval but HG distributions probably reflect a time-integrated signal that covers several years. In addition, surface water temperatures of Lake Schreventeich are expected to vary over the time-course of a day and the obtained temperatures (though always recorded at the same time of the day) provide only a snap shot of the actual temperature variance of the lake. Parts of the uncertainties in the correlation of HG indices and surface water temperatures may in fact be related to the low number of diurnal temperature measurements but may be improved by continuous temperature logging of the lake's surface waters in future studies. As discussed above, contributions of HGs from heterocystous cyanobacteria with slightly different HG distribution patterns and absolute abundances of HGs may also result in the observed offsets between the HG-based SWT calculations. Nonetheless, the overall good agreement of HG distributions in surface sediments and water column filtrates seems to indicate that HGs in Lake Schreventeich are largely produced in late summer, coinciding with blooms of heterocystous cyanobacteria, and that HG-reconstructed surface water temperatures primarily reflect a summer signal in this temperate lake.

4.4 Geochemical implications

As mentioned previously, N₂-fixing heterocystous cyanobacteria are a common component of the phytoplankton community in contemporary freshwater and brackish environments of polar to tropical latitudes, where they may form massive blooms during summer (Whitton, 2012). Likewise, HGs seem to be widely distributed in modern freshwater and brackish environments. They have been reported from surface sediments of several European and African lakes including Lake Ohrid, Lake Malawi and Lake Challa (Bauersachs et al., 2010) as well as in phytoplankton collected from a number of Spanish freshwater reservoirs (Wörmer et al., 2012). HG distributions dominated by HG₂₆ and HG₂₈ diols have been reported from core top sediments recovered from the Landsort Deep, Baltic Sea (Bauersachs et al., 2010). They have also been described in several microbial mats growing along the coast of the southern North Sea (Bauersachs et al., 2011; Bühring et al., 2014) and western Spitsbergen (Rethemeyer et al., 2010) as well as in an Icelandic

hot spring (Bauersachs et al., 2013). A suite of HG₂₆ to HG₂₈ diols, triols, keto-ols and keto-diols was detected in suspended particulate matter in the surface waters of 23 oligotrophic and eutrophic lakes in Minnesota and Iowa, USA (Schoon, 2013), while HG₂₆ to HG₂₈ diols and keto-ols were present in variable abundances and distributions in microbial mats recovered from Shark Bay, Western-Australia (Bauersachs et al., unpublished data).

The remarkable strong linear correlations found for the distribution of HGs in water column filtrates of Lake Schreventeich and surface water temperatures indicates that HG distributions, in form of the HDI₂₆ and other HG indices, may be well suited to track changes in water temperatures of the photic zone in freshwater environments. The generally good agreement of HG indices obtained from core top sediments of Lake Schreventeich with summer surface water temperatures furthermore suggests that the distribution of sedimentary HGs may also record surface water temperatures of lacustrine settings over time. In addition, it may suggest that no or only little selective degradation of HGs (e.g., diols vs. keto-ols) upon sinking and transport through the water column as well as during the incorporation into the sediment record occurred in this shallow lake system. At this point, however, it cannot be ruled out that microbial reworking may bias the initially-synthesized HG signal in deeper lakes. Hence, additional studies determining degradation rates of individual HGs as well as changes in the overall HG distribution patterns with water depth will be necessary in order to elucidate whether and to which extent the HG inventory of lakes experiences early diagenetic alteration. Likewise, only limited information on the preservation potential of HGs over geological time scales exists. These components have been reported from Pleistocene Mediterranean sapropels as well as lacustrine deposits from the Oligocene Lake Enspel and the Eocene Messel oil shale (Bauersachs et al., 2010), indicating that they may readily preserve in the sediment record. However, detailed studies investigating the preservation of HGs in sedimentary sequences and their stability under varying environmental conditions are currently missing but will be essential to determine the robustness of HGs as lipid paleothermometers.

It has previously been demonstrated that changes in the distribution of HGs as a function of growth temperature can vary significantly between different cyanobacterial species as reported for *Anabaena* CCY9613 and *Nostoc* CCY9928 (Bauersachs et al., 2014). A finding that we confirmed for other nostocalean cyanobacteria such as *Aphanizomenon* sp. and *Nodularia* sp. in recent culture experiments (Bauersachs et al., unpublished data). The application of HDI₂₆ and

other HG-based indices may thus potentially be biased in lakes that are characterized by simultaneous growth of multiple species of heterocystous cyanobacteria, each modifying the composition of the heterocyst cell envelope in a slightly different fashion. It should also be pointed out that core top calibrations (such as those obtained from Lake Schreventeich) may not be applicable to accurately determine surface water temperatures in lake environments, in which the cyanobacterial community gradually changed over time.

5 Conclusion

The presence of heterocyst glycolipids in core top sediments of Lake Schreventeich, the overall good agreement of HG-based temperature estimates with measured surface water temperatures and the ubiquitous distribution of heterocystous cyanobacteria in modern freshwater and brackish environments, suggests that the HDI₂₆ and other HG-based indices may hold great promise as proxies for the reconstruction of surface water temperatures in modern and possibly also fossil lacustrine environments, something that is currently not achieved by any other organic geochemical proxy. As heterocyst glycolipids constitute highly specific biological markers for diazotrophic heterocystous cyanobacteria, they also allow a direct study of the overall impact of surface water temperature changes on the cyanobacterial community structure of a given lake system. However, additional analyses of HG distributions in freshwater environments in combination with environmental parameters (such as water temperatures, oxygen concentrations, pH, light intensities etc.) and molecular studies are clearly needed to evaluate the potential use of HG-based proxies in the determination of lacustrine surface water temperatures on a larger scale.

Author contribution

T.B. and L.S. designed the experiments. J.R. was involved in sample collection, the determination of the physical properties of the lake's surface waters and quantification of phytoplankton biomass. T. B. analyzed the water column filtrates for their HG content and prepared the manuscript with contributions from all co-authors.

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Table 1. Correlations of fractional abundances (F) of individual heterocyst glycolipids and heterocyst glycolipids indices with surface water temperatures (SWT), oxygen concentrations, pH and biomass. Significant correlations were, among others, observed between fractional abundances of heterocyst glycolipids and SWT as well as between the different HG indices and SWT. Note that certain environmental parameters were also positively correlated with each other. Significant correlations are indicated in bold. r = Correlation coefficient; p = p -value.

Parameter		SWT (°C)	Oxygen Con. (mg l ⁻¹)	pH	Biomass (mg l ⁻¹)
$F_{\text{HG26 diol}}$	r	0.807	0.746	0.424	0.216
	p	0.000	0.001	0.115	0.439
$F_{\text{HG26 keto-ol}}$	r	-0.954	-0.777	-0.671	0.027
	p	0.000	0.001	0.006	0.925
$F_{\text{HG28 diol}}$	r	-0.714	-0.621	0.494	-0.444
	p	0.009	0.031	0.103	0.148
$F_{\text{HG28 keto-ol}}$	r	-0.715	-0.467	0.624	-0.571
	p	0.009	0.126	0.030	0.052
$F_{\text{HG28 triol}}$	r	0.680	0.445	0.856	-0.257
	p	0.007	0.111	0.000	0.374
$F_{\text{HG28 keto-diol}}$	r	-0.288	-0.251	0.550	-0.574
	p	0.318	0.387	0.042	0.032
HDI ₂₆	r	0.962	0.803	0.591	0.070
	p	0.000	0.000	0.020	0.805
HDI ₂₈	r	0.835	0.530	-0.590	0.624
	p	0.001	0.077	0.044	0.030
HTI ₂₈	r	0.884	0.646	0.109	0.451
	p	0.000	0.013	0.711	0.105
SWT (°C)	r		0.866	0.335	-0.316
	p		0.000	0.101	0.124
Oxygen Con. (mg L ⁻¹)	r			0.430	-0.232
	p			0.036	0.275
pH	r				-0.415
	p				0.039

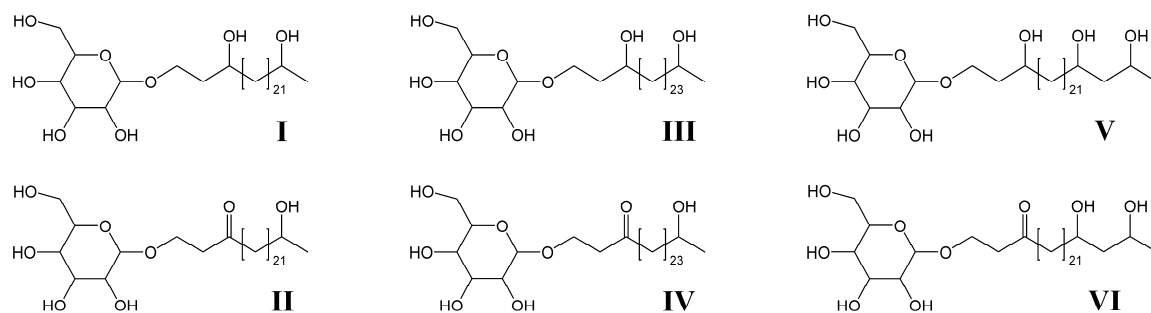


Figure 1. Structures of heterocyst glycolipids detected in water column filtrates and surface sediments of Lake Schreventeich. 1-(O-hexose)-3,25-hexacosanediol (**I**), 1-(O-hexose)-3-keto-25-hexacosanol (**II**), 1-(O-hexose)-3,27-octacosanediol (**III**), 1-(O-hexose)-3-keto-27-octacosanol (**IV**), 1-(O-hexose)-3,25,27-octacosanetriol (**V**) and 1-(O-hexose)-3-keto-25,27-octacosanediol (**VI**).

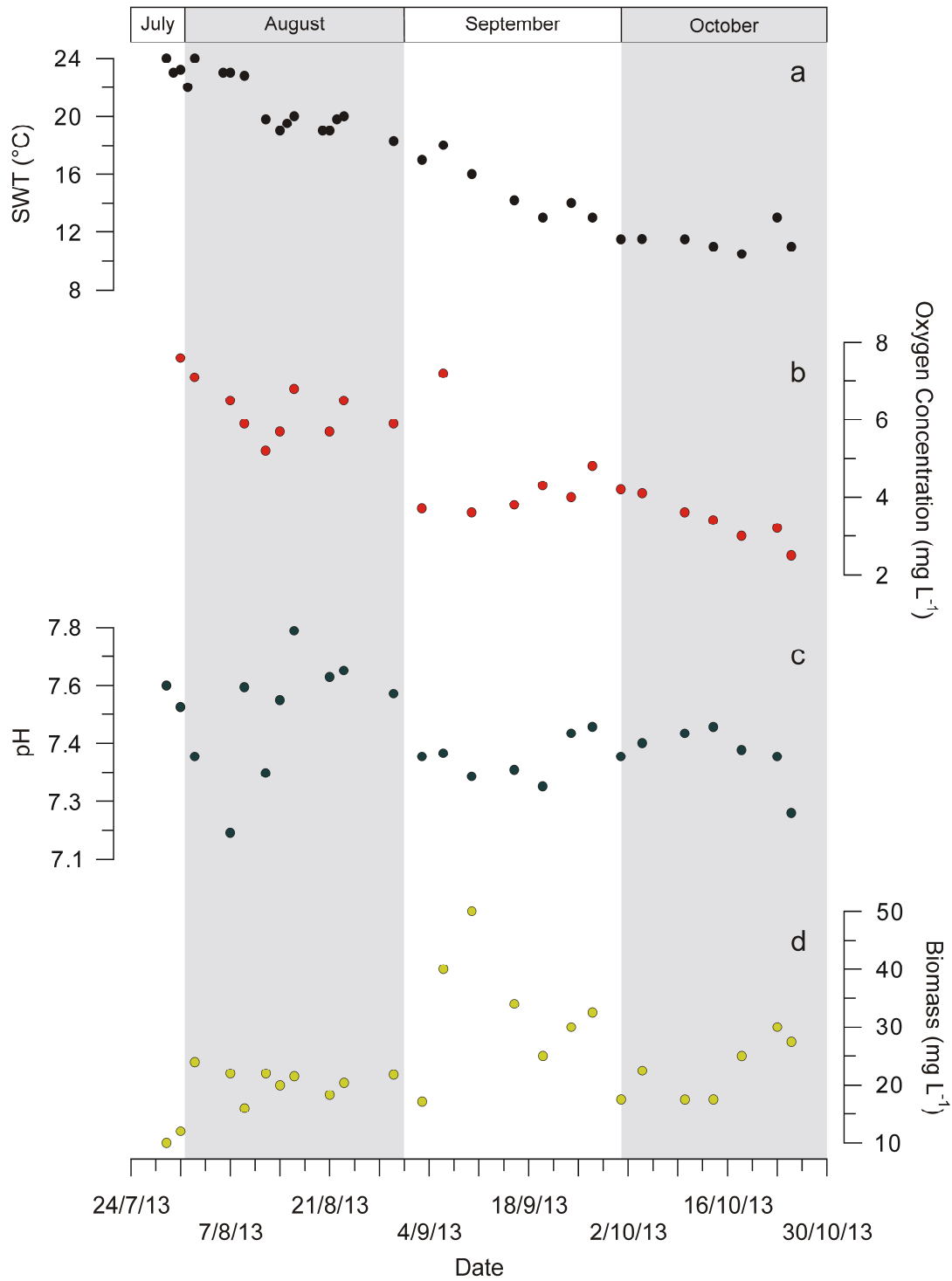


Figure 2. (a) Surface water temperatures (SWT), (b) oxygen concentrations, (c) pH and (d) amount of phytoplankton biomass measured in Lake Schreventeich from late July until the end of October 2013.

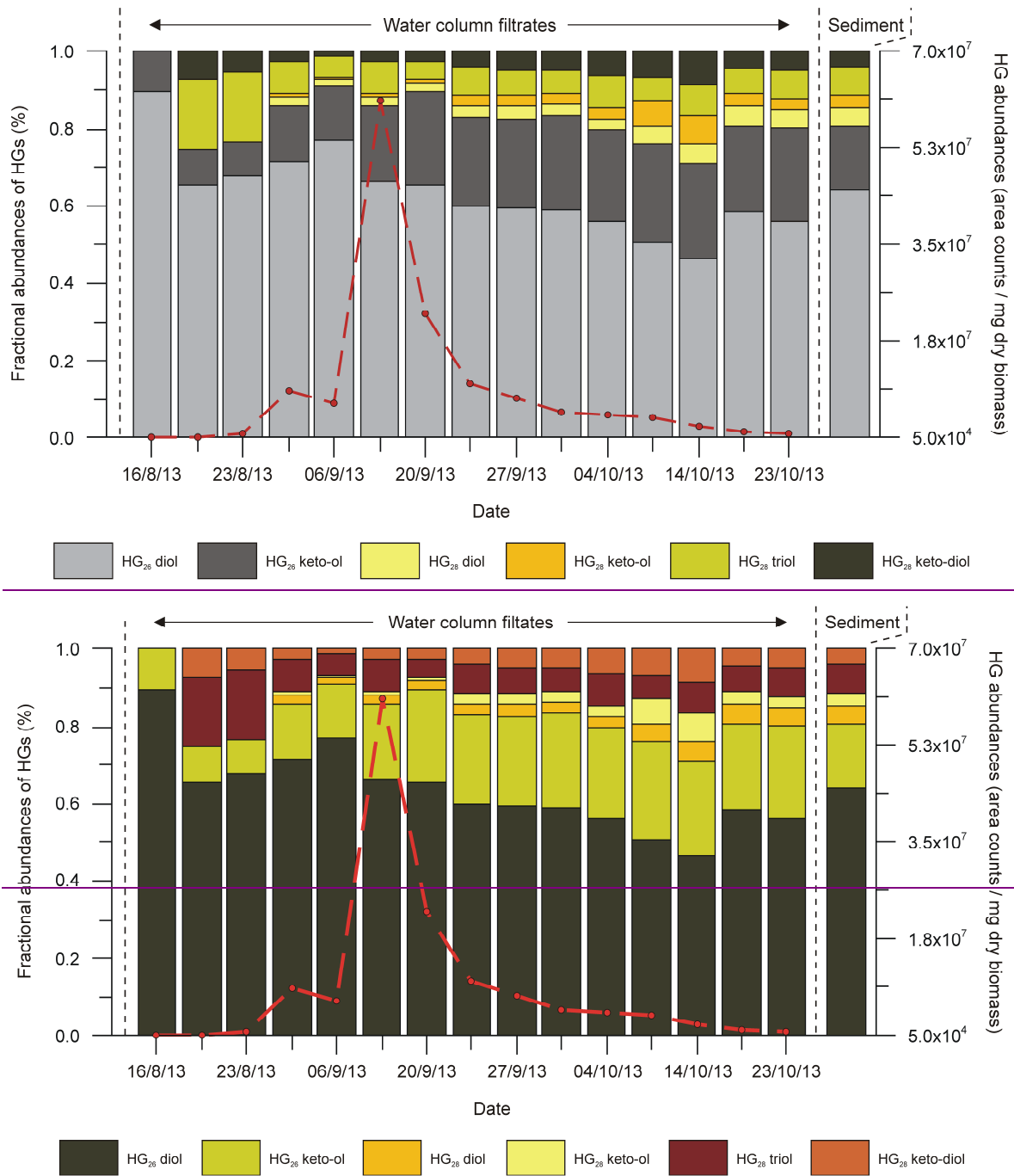


Figure 3. Fractional abundances of heterocyst glycolipids (HG₂₈) in surface waters of Lake Schreventeich. Dashed line indicates relative abundances of the sum of all heterocyst glycolipids over the investigated time interval. Note that heterocyst glycolipids were not detected in water

column filtrates taken before mid-August. Fractional abundances of HGs in the sediment of Lake Schreventeich represent average values obtained from the analysis of two core top samples.

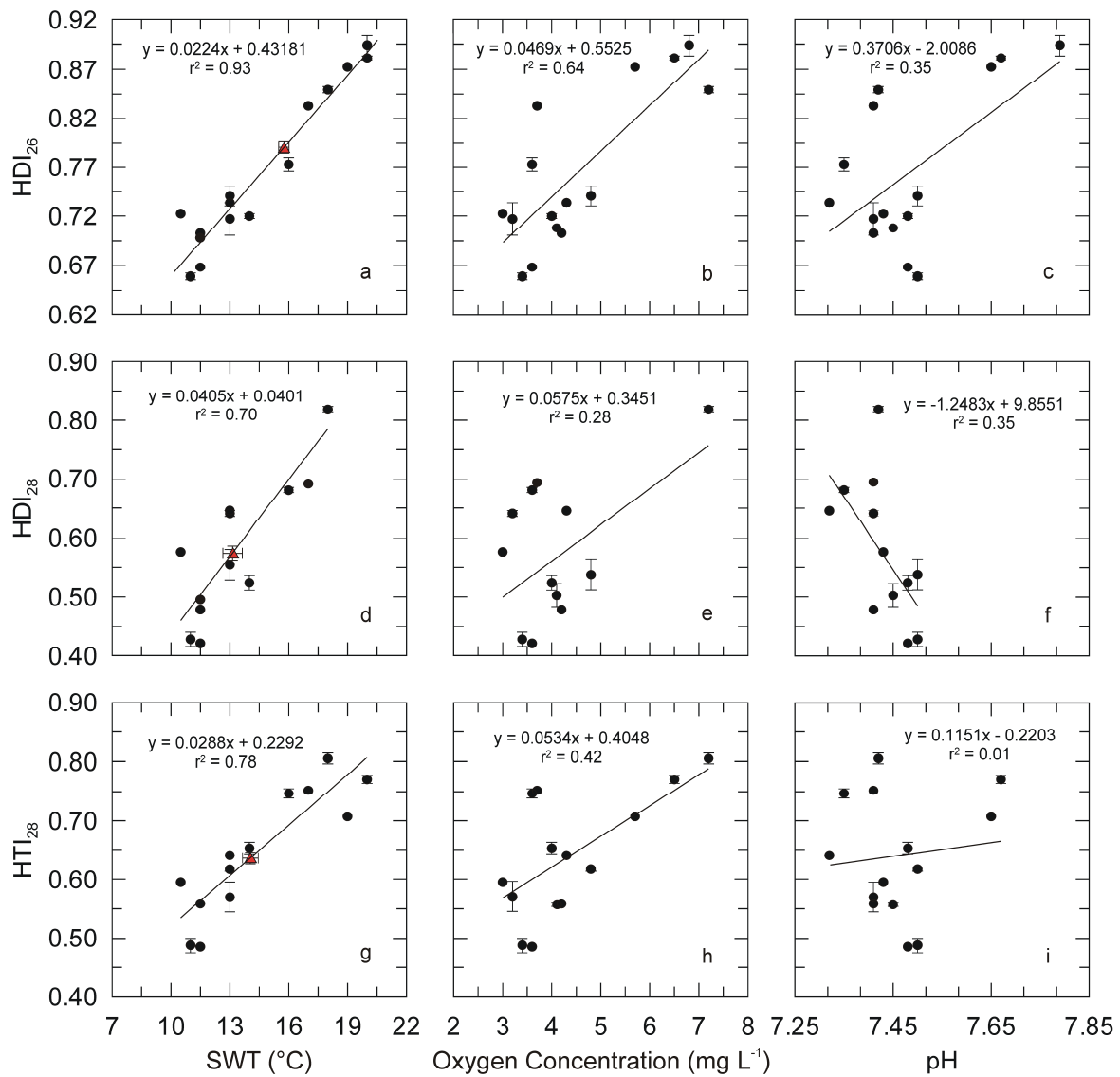


Figure 4. Cross plots of the HDI₂₆ (a-c), HDI₂₈ (d-f) and HTI₂₈ (g-i) obtained from water column filtrates with measured surface water temperatures (SWT), oxygen concentrations and pH of Lake Schreventeich's surface waters. Red triangles represent HDI₂₆-, HDI₂₈- and HTI₂₈-reconstructed SWT obtained from the analysis of surface sediments of Lake Schreventeich.

Supplementary Table 1. Physical and biological data obtained from surface waters of Lake Schreventeich at time of sampling. n.d. = not determined

Date	SWT (°C)	Oxygen Concentration (mg L ⁻¹)	pH	Biomass (mg L ⁻¹)
29/07/2013	24.0	n.d.	7.62	10.0
30/07/2013	23.0	n.d.	n.d.	n.d.
31/07/2013	23.2	7.6	7.56	12.0
01/08/2013	22.0	n.d.	n.d.	n.d.
02/08/2013	24.0	7.1	7.41	24.0
06/08/2013	23.0	n.d.	n.d.	n.d.
07/08/2013	23.0	6.5	7.18	22.0
09/08/2013	22.8	5.9	7.62	16.0
12/08/2013	19.8	5.2	7.36	22.0
14/08/2013	19.0	5.7	7.58	20.0
15/08/2013	19.5	n.d.	n.d.	n.d.
16/08/2013	20.0	6.8	7.79	21.5
20/08/2013	19.0	n.d.	n.d.	n.d.
21/08/2013	19.0	5.7	7.65	18.3
22/08/2013	19.8	n.d.	n.d.	n.d.
23/08/2013	20.0	6.5	7.67	20.4
30/08/2013	18.3	5.9	7.60	21.8
03/09/2013	17.0	3.7	7.41	17.1
06/09/2013	18.0	7.2	7.42	40.0
10/09/2013	16.0	3.6	7.35	50.0
16/09/2013	14.2	3.8	7.37	34.0
20/09/2013	13.0	4.3	7.32	25.5
24/09/2013	14.0	4.0	7.48	30.0
27/09/2013	13.0	4.8	7.50	32.5
01/10/2013	11.5	4.2	7.41	17.5
04/10/2013	11.5	4.1	7.45	22.5
10/10/2013	11.5	3.6	7.48	17.5
14/10/2013	11.0	3.4	7.50	17.5
18/10/2013	10.5	3.0	7.43	25.0
23/10/2013	13.0	3.2	7.41	30.0
25/10/2013	11.0	2.5	7.24	27.5

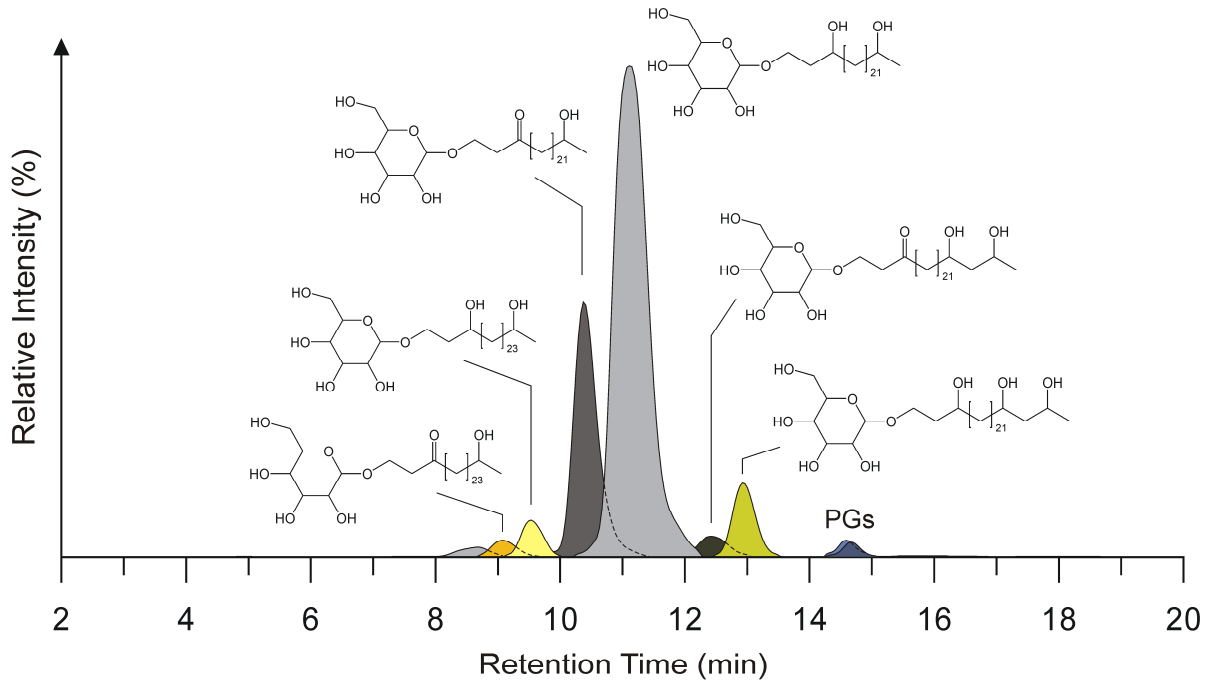
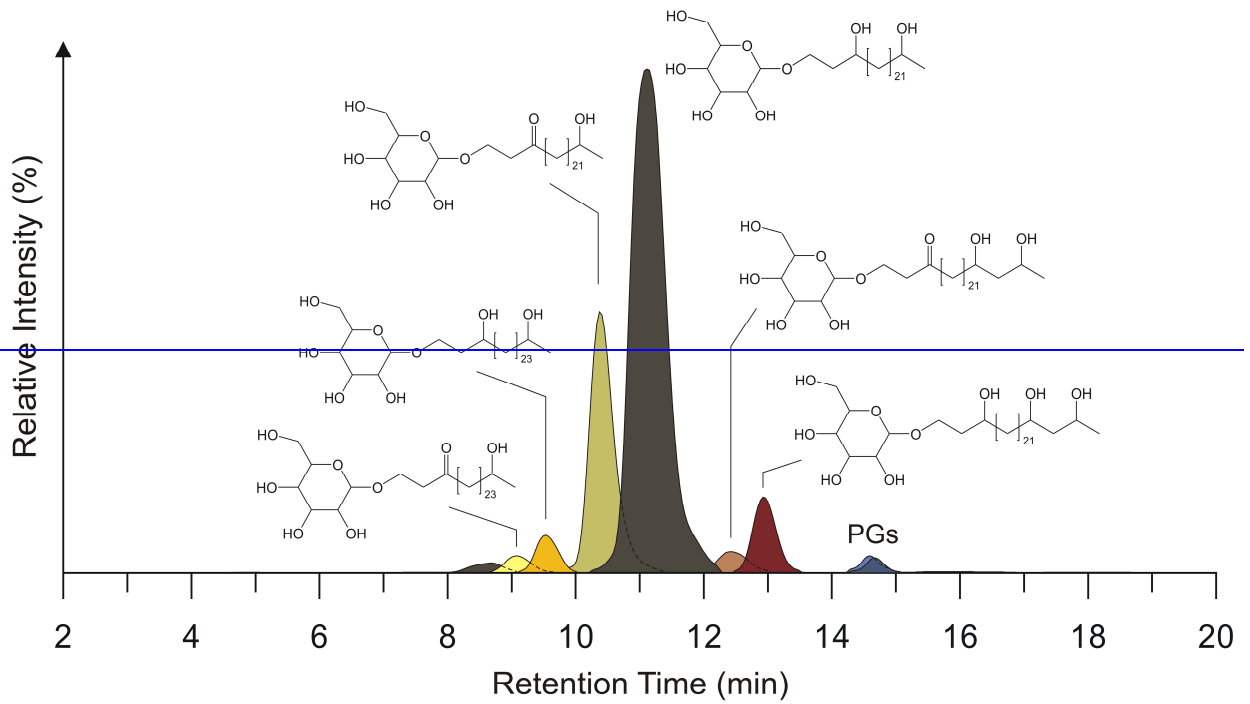
Supplementary Table 2. Relative concentrations and fractional abundances (*f*) of heterocyst glycolipids detected in water column filtrates and surface sediments of Lake Schreventeich. Note that HGs were below detection limit in water column samples collected before mid-August a.c. = area counts; n.d. = not detected

Date	HG concentration (a.c./ mg dry biomass)	<i>f</i> HG ₂₆ diol	<i>f</i> HG ₂₆ keto-ol	<i>f</i> HG ₂₈ diol	<i>f</i> HG ₂₈ keto-ol	<i>f</i> HG ₂₈ triol	<i>f</i> HG ₂₈ keto-ol
16/08/2013	6.21E+04	0.894	0.106	n.d.	n.d.	n.d.	n.d.
21/08/2013	8.31E+04	0.652	0.095	n.d.	n.d.	0.178	0.074
23/08/2013	9.18E+05	0.677	0.091	n.d.	n.d.	0.178	0.055
03/09/2013	8.65E+06	0.716	0.144	0.022	0.010	0.082	0.027
06/09/2013	6.27E+06	0.772	0.137	0.017	0.004	0.056	0.013
10/09/2013	6.10E+07	0.662	0.195	0.023	0.011	0.082	0.028
20/09/2013	2.31E+07	0.657	0.238	0.022	0.012	0.046	0.026
24/09/2013	9.84E+06	0.597	0.232	0.030	0.027	0.074	0.040
27/09/2013	7.12+E06	0.630	0.212	0.025	0.020	0.070	0.044
01/10/2013	4.72E+06	0.588	0.249	0.026	0.028	0.061	0.048
04/10/2013	4.02E+06	0.563	0.232	0.029	0.029	0.082	0.065
10/10/2013	3.88E+06	0.508	0.253	0.046	0.064	0.062	0.066
14/10/2013	2.22E+06	0.466	0.242	0.054	0.072	0.081	0.085
18/10/2013	1.20E+05	0.584	0.225	0.048	0.035	0.064	0.044
23/10/2013	7.34E+05	0.563	0.222	0.040	0.022	0.087	0.066
Average	8.92E+06	0.635	0.192	0.025	0.022	0.080	0.045
Surface sediment		0.589	0.156	0.087	0.064	0.067	0.038

Supplementary Table 3. Variation of the HDI₂₆, HDI₂₈ and HTI₂₈ in water column filtrates and surface sediments of Lake Schreventeich together with surface water temperatures (SWT) calculated from the different HG indices as well as residual SWT (reconstructed SWT – measured SWT). Note that HGs were below detection limit in water column samples collected before mid-August.

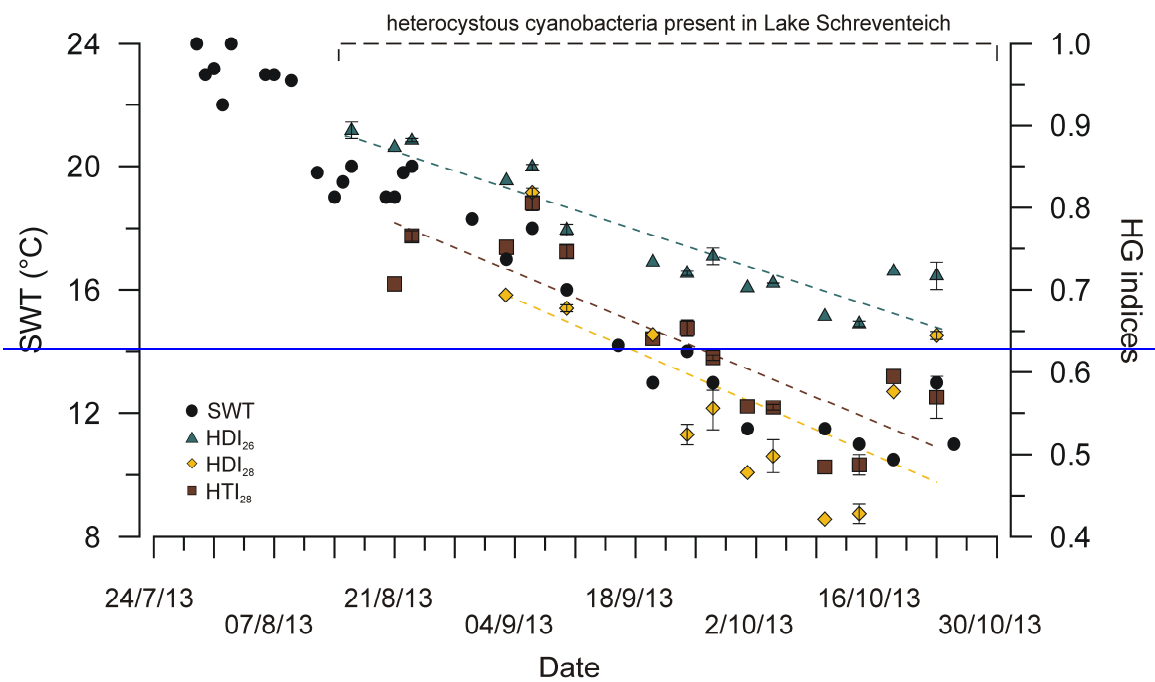
Date	HDI ₂₆	SWT ^a (°C)	Residual SWT (°C)	HDI ₂₈	SWT ^b (°C)	Residual SWT (°C)	HTI ₂₈	SWT ^c (°C)	Residual SWT (°C)
16/08/2013	0.894	20.35	0.35	-	-	-	-	-	-
21/08/2013	0.873	19.40	0.40	-	-	-	0.707	16.60	-2.40
23/08/2013	0.882	19.80	-0.20	-	-	-	0.765	18.59	-1.41
03/09/2013	0.833	17.61	0.61	0.691	16.08	-0.92	0.752	18.15	1.15
06/09/2013	0.849	18.35	0.35	0.818	19.22	1.22	0.805	20.01	2.01
10/09/2013	0.773	14.93	-1.07	0.678	15.75	-0.25	0.748	18.02	2.02
20/09/2013	0.734	13.20	0.20	0.646	14.95	1.95	0.641	14.29	1.29
24/09/2013	0.720	12.59	-1.41	0.524	11.95	-2.05	0.653	14.72	0.72
27/09/2013	0.748	13.83	0.83	0.556	12.75	-0.25	0.615	13.41	0.41
01/10/2013	0.703	11.80	0.30	0.478	10.82	-0.68	0.559	11.43	-0.07
04/10/2013	0.708	12.05	0.55	0.499	11.33	-0.17	0.558	11.41	-0.09
10/10/2013	0.668	10.25	-1.25	0.421	9.41	-2.09	0.485	8.88	-2.62
14/10/2013	0.659	9.85	-1.15	0.427	9.55	-1.45	0.486	8.92	-2.08
18/10/2013	0.722	12.69	2.19	0.577	13.25	2.75	0.595	12.70	2.20
23/10/2013	0.717	12.45	-0.55	0.648	15.01	2.01	0.570	11.85	-1.15
Average	0.765	14.61	-	0.58	13.34	-	0.639	14.21	-
Surface sediment	0.791	15.75	-	0.575	13.21	-	0.637	14.16	-

^a HDI₂₆ = 0.0224 × SWT + 0.4381; ^b HDI₂₈ = 0.0405 × SWT + 0.0401; ^c HTI₂₈ = 0.0288 × SWT + 0.2292

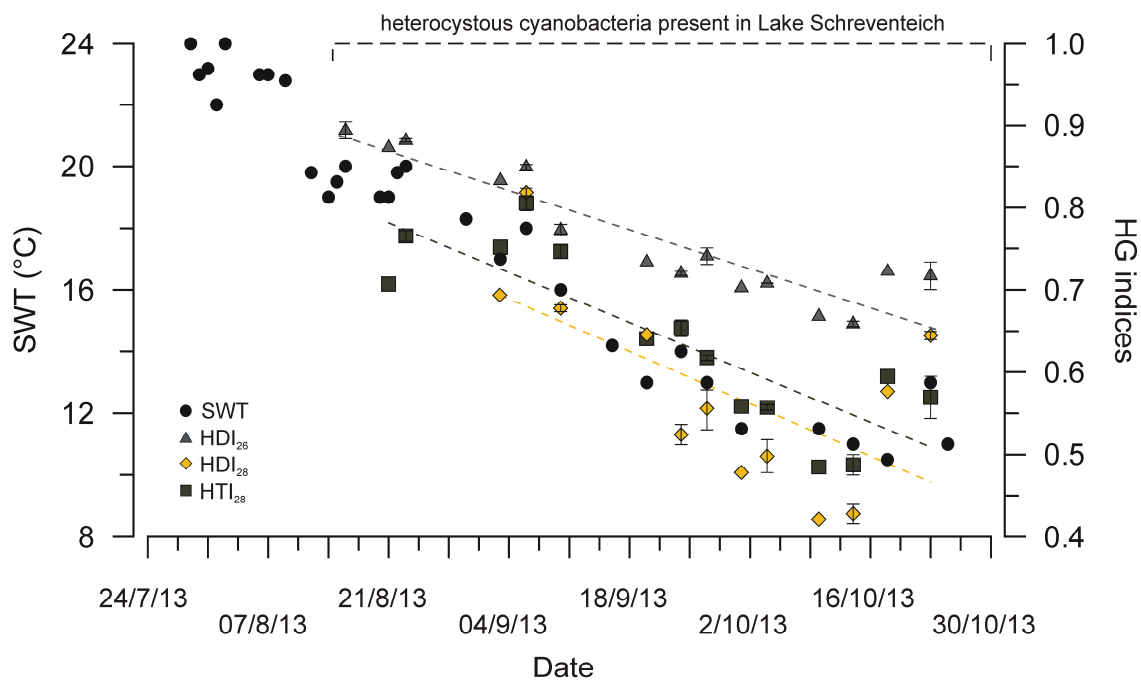


3 Supplementary Figure 1. Composite HPLC/MS chromatogram showing the distribution of
 4 heterocyst glycolipids in a water column filtrate of Lake Schreventeich collected in early
 5 September. Note that mass traces of the HG₂₆ diol and HG₂₆ keto-ol contained additional
 6 signals at around 14.7 min, which resulted from the in-source fragmentation of
 7 phosphatidylglycerols (PGs).

8



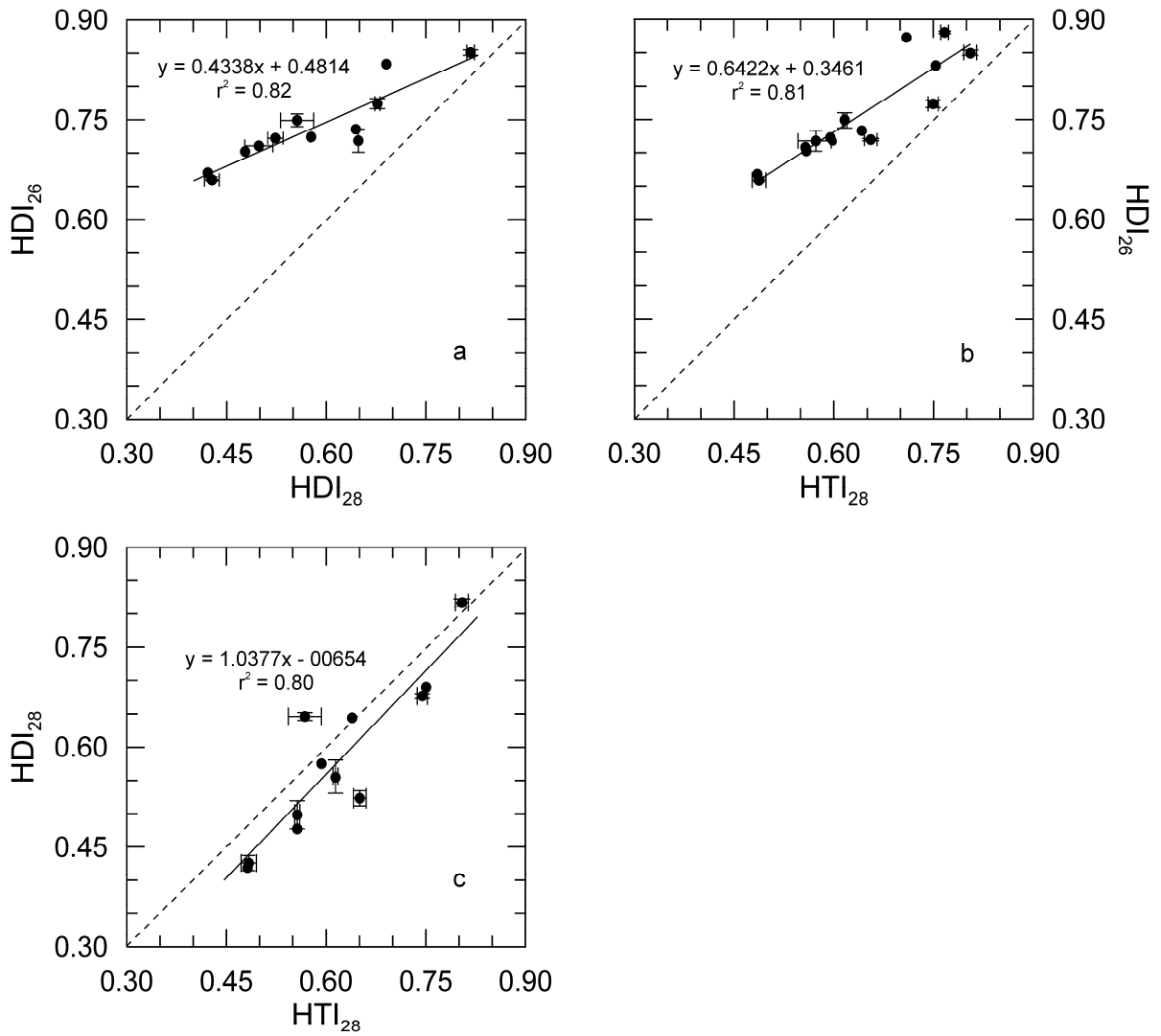
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3 Supplementary Figure 2. Comparison of measured surface water temperatures (SWT) with
 4 variation of calculated HG indices (e.g. HDI₂₆, HDI₂₈ and HTI₂₈) in Lake Schreventeich over
 5 time.

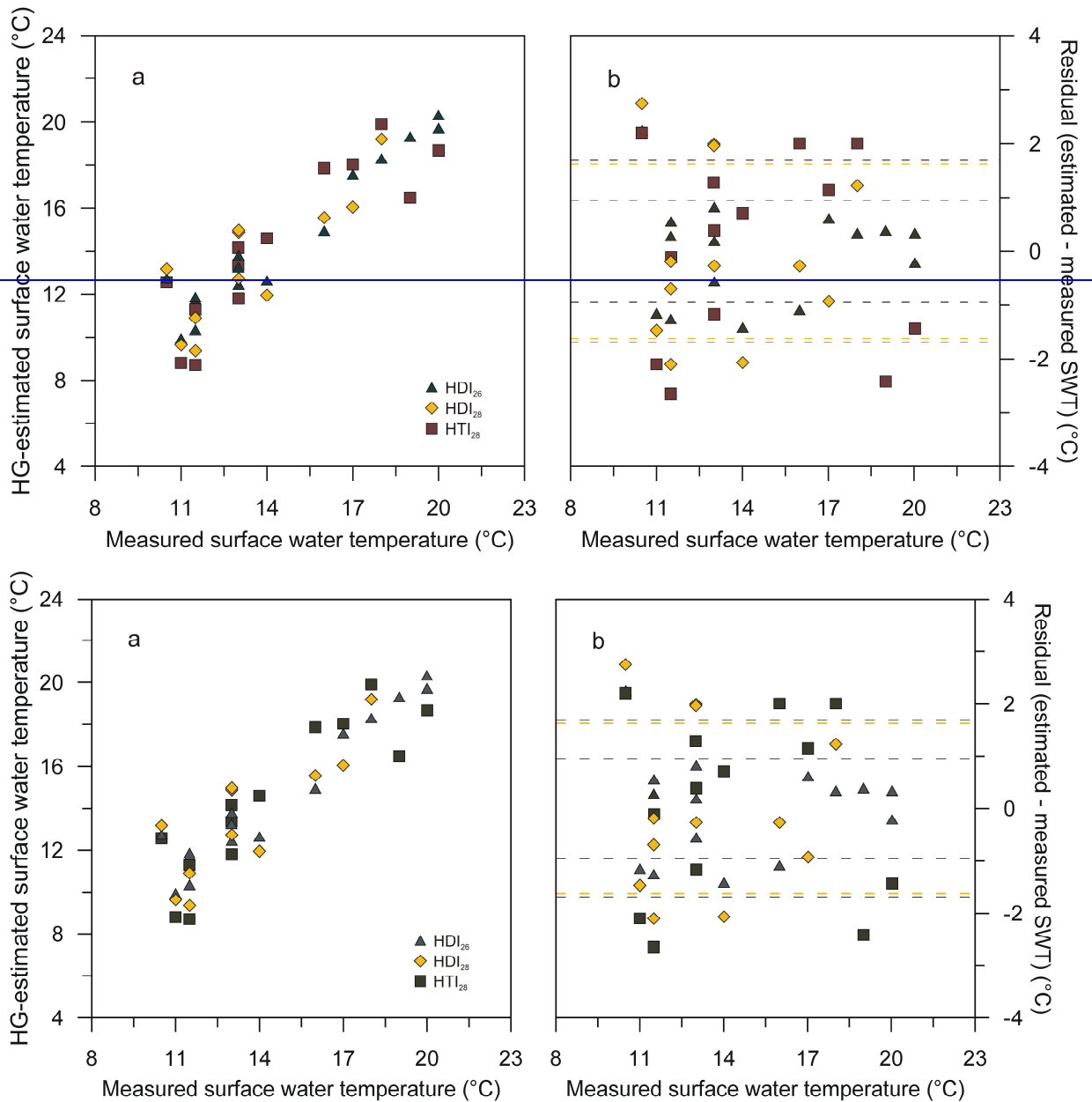
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2 Supplementary Figure 3. Correlations between the different HG indices determined in this
 3 study. (a) HDI₂₆ vs. HDI₂₈, (b) HDI₂₆ vs. HTI₂₈ and (c) HDI₂₈ vs. HTI₂₈. Dashed lines
 4 represent 1:1 lines. Note that all correlations are statistically significant with p -values <0.001 .

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3 Supplementary Figure 4. (a) HG-calculated surface water temperatures vs. measured surface
 4 water temperatures (SWT). (b) Residual SWT (estimated SWT using the respective HG
 5 temperature calibrations – measured SWT). Coloured dashed lines denote the standard
 6 deviations of the residuals of each HG index (grey = HDI₂₆, yellow = HDI₂₈; brown =
 7 HTI₂₈).

8