


Incomplete recovery of intact polar glycerol dialkyl glycerol tetraethers from lacustrine suspended biomass

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Abstract

Branched and isoprenoid glycerol dialkyl glycerol tetraethers (GDGTs) are membrane lipids of bacteria and archaea, respectively, and their core lipid distributions are used as proxy indicators in paleolimnological studies. In addition, the amount and composition of intact polar lipid (IPL) GDGTs yield information on the presence and abundance of GDGT-producing microbes within the water column. GDGTs are, however, not always easily recovered from cultured microbial cells by commonly applied extraction methods, and this may also apply to suspended particulate matter (SPM) in aquatic systems. In order to investigate potential biases induced by incomplete GDGT recovery, we analyzed both core- and IPL-GDGTs in SPM from Lake Lugano (Switzerland) using (1) ultrasonic solvent extraction with mixtures of methanol (MeOH) and dichloromethane (DCM), and (2) two modifications of the Bligh–Dyer (BD) protocol. Acid hydrolysis of the post-extraction residues revealed that particularly *branched* GDGTs were poorly recovered from SPM by the MeOH/DCM mixtures (25–62%). Much better extraction yields (> 85%) were achieved with the BD method, however, during subsequent phase separation, up to 75% of the extracted *branched* IPL-GDGTs partitioned into the aqueous phosphate buffer that is usually discarded. In contrast, when this buffer was substituted with 5% trichloroacetic acid, only 13% were lost into the aqueous phase. Depending on the protocol used, the distribution of the IPL-derived GDGTs varied substantially. Our results indicate that both bacterial- and archaeal IPL-GDGTs can be difficult to extract from lacustrine microbial communities, and caution is advised when targeting the “viable” GDGT pool.

Glycerol dialkyl glycerol tetraethers (GDGTs) are organic compounds synthesized by microorganisms that thrive ubiquitously in both aquatic and terrestrial environments. They are cellular membrane lipids of many *Archaea* and certain *Bacteria*, and contain isoprenoid (isoGDGTs) or branched (brGDGTs) hydrocarbon chains, respectively, as their central structural building blocks (Supporting Information Figs. S1, S2). It has been shown that the relative abundances of specific GDGTs correlate with environmental parameters, such as sea or lake surface temperature (Schouten et al. 2002; Powers et al. 2010), mean annual air temperature, and soil pH (Weijers et al. 2007; Peterse et al. 2012; De Jonge et al.

2014a; Ding et al. 2015), attesting to their potential as paleo-environmental indicators in aquatic sediments (see Schouten et al. 2013 for a review).

Despite the widespread application of GDGTs in paleolimnological studies (e.g., Fawcett et al. 2011; Niemann et al. 2012; Woltering et al. 2014; Buckles et al. 2015; Loomis et al. 2015; Keisling et al. 2017), the identity and ecology of the source organisms (in particular those of bacterial, i.e., branched, GDGTs) is not well constrained. One way to gain insight into GDGT biosynthesis in the environment is to specifically quantify the intact polar lipid (IPL) -derived GDGT fraction (Schoon et al. 2013; Buckles et al. 2014; De Jonge et al. 2014b). IPL-GDGTs represent the functional form of GDGTs within the membranes of living cells, and contain polar head groups (e.g., phosphohexose and [poly] hexose) that are linked to the core lipid (CL) via ester- and

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glycosidic bonds (Biddle et al. 2006; Schouten et al. 2008; Schubotz et al. 2009; Liu et al. 2010; Peterse et al. 2011). Because these head group moieties are comparably labile after cell death (Harvey et al., 1986; Logemann et al., 2011), higher relative amounts of IPL-GDGTs in the environment are commonly attributed to the presence of living GDGT-producing organisms (Biddle et al. 2006; Schouten et al. 2012; Buckles et al. 2013). Nevertheless, recent studies suggest that IPLs, at least to some extent, may be preserved in sediments over geological timescales (Lengger et al. 2013; Xie et al. 2013). GDGTs without polar head groups (i.e., CL-GDGTs), on the other hand, are only minor components in the lipidomes of living microbes ($\leq 10\%$, Huguet et al. 2010a; Elling et al. 2014). Most of the CL-GDGTs present in environmental samples are therefore believed to derive from the corresponding IPL precursors after loss of their polar head group(s). IPL-GDGTs in lipid extracts are commonly analyzed in their intact form using electro spray ionization mass spectrometry (ESI-MS) when information on the type of head groups is primarily desired (e.g., Peterse et al., 2011; Zhu et al. 2013), whereas for quantification and for the determination of paleoenvironmental proxy indices, head groups are typically removed by acid hydrolysis, and the “IPL-derived CL-GDGTs” are then analyzed via atmospheric-pressure chemical-ionization (APCI) -MS (e.g., De Jonge et al., 2014b).

Extraction of IPL-GDGTs from environmental samples commonly follows a modified Bligh–Dyer (BD) protocol (Bligh and Dyer 1959), which was specifically designed for the assessment of intact eukaryotic and bacterial phospholipids (e.g., White et al. 1979). Currently, two basic variants of the original protocol are widely applied, in which the solvent mixture contains either phosphate buffer (White et al. 1979), an aqueous solution of trichloroacetic acid (TCA) (Nishihara and Koga 1987), or a combination of both (Sturt et al. 2004). It has been reported, however, that during extraction of archaeal cultures, only a minor fraction of the membrane IPLs is recovered in the total lipid extract (TLE) (Huguet et al. 2010a; Cario et al. 2015). Similarly, branched GDGTs and their putative precursor molecules produced by acidobacterial isolates were almost completely inaccessible by common extraction techniques, including the phosphate buffer-based BD method (Sinninghe Damsté et al. 2011, 2014). In the above-mentioned culture studies, highest (near-complete) recovery of branched- and isoprenoid GDGTs was achieved by means of acid hydrolysis of the cells. Since hydrolysis of samples and/or the extracted residues is not commonly applied to environmental samples, total GDGT concentrations, and particularly that of the IPL-fraction, may thus be substantially underestimated depending on the extraction protocol. Moreover, the incomplete recovery of IPL-GDGTs derived from living microbes may lead to biases in the measured GDGT distribution,

potentially affecting GDGT-based proxy indices calculated from the IPL pool.

In order to further examine the effect of, and the possible reasons for, incomplete lipid recovery, we determined and compared yields of IPL- and CL-GDGTs in lacustrine suspended particles (1) after ultrasonic extraction using methanol (MeOH) and dichloromethane (DCM) as solvents (Huguet et al. 2010b; Ingalls et al. 2012), and (2) after extraction following modifications of the BD protocol. In addition, we quantified the fraction of GDGTs not recovered in the TLE by acid hydrolysis of (1) the extracted sample residues, and (2) the aqueous phase of the BD solvent mixture, both of which are normally discarded.

Materials and procedures

Materials

Suspended particulate matter (SPM) was collected from the north basin of Lake Lugano (Switzerland; 46°00'12"N, 9°00'14"E; max. water depth 286 m) by in situ filtration of lake water. Filtering was performed at two distinct depths in September 2014 (Experiment#1; 70 m and 275 m) and June 2016 (Experiment#2; 275 m) at a position close to the deepest point of the lake. Hydrographic water column characteristics (i.e., position of the thermocline and oxycline) were similar during both sampling campaigns. Also, there were no extreme weather conditions (e.g., heavy rainfalls) prior (< 5 d) to sampling that may have caused unusually high concentrations of settling terrestrial organic matter in the water column. In order to prevent rapid clogging, and to increase loading capacity, particles were collected on a double layer of glass fiber filters, consisting of a 0.7 μm base- and a 2.7 μm pre-filter (nominal pore size; Whatman GF/F and GF/D, respectively), which were subsequently processed together. The filters were frozen immediately on dry ice after sampling, stored at -80°C , and lyophilized prior to lipid extraction.

Procedures

Lipid extraction

Filters were split into equal parts and cut into small pieces. For the MeOH/DCM extraction (modified from Ingalls et al. 2012) the lyophilized filter material was placed in a PTFE tube and repeatedly extracted in an ultrasonic bath (six times; 5 min each), twice using MeOH, twice with DCM/MeOH (1 : 1, v : v), and twice with DCM (30 mL each). The tube was centrifuged after each extraction, and the supernatants were combined. The resulting TLE was concentrated by rotary evaporation (27°C), filtered through a Pasteur pipet plugged with pre-cleaned quartz wool, and completely dried under a gentle stream of N_2 . In order to estimate the reproducibility of this extraction procedure, the filter material from 70 m was extracted in triplicate.

Another part of lyophilized filter material was placed in glass centrifuge tubes and extracted using a modified BD protocol (e.g., Pitcher et al. 2009). Briefly, the filter pieces were

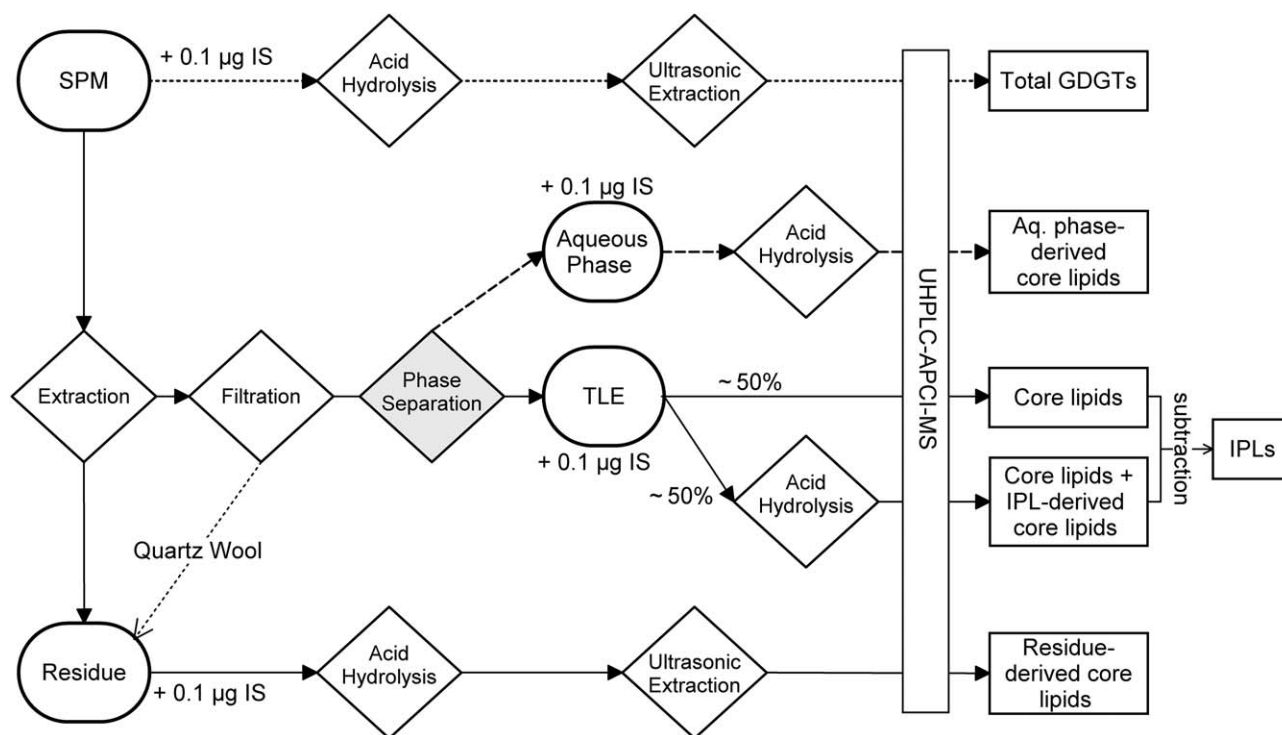


Fig. 1. Flow chart illustrating the major sample processing steps (rhombuses) for the quantification of GDGTs. An IS was added to the TLEs, sample residues, and aqueous phases, respectively. Note that the “phase separation” step (grey) only applies to the BD extractions. For Experiment#2, the aqueous BD solvent phases were acid-hydrolyzed and analyzed separately (dashed arrows). Direct acid hydrolysis of the sample (dotted arrows) was used to independently determine the total amount of recoverable GDGTs (Experiment#2 only).

sonicated in an ultrasonic bath (three times, 10 min each) in 20 mL of a solvent mixture consisting of MeOH:DCM:phosphate buffer (2 : 1 : 0.8, v : v : v), centrifuged, and all supernatants were combined. Phase separation was achieved by altering the solvent composition to MeOH:DCM:phosphate buffer (1 : 1 : 0.9, v : v : v) and subsequent centrifugation. The lipid-containing DCM phase was transferred to a round-bottom flask, and the aqueous phase was extracted twice more with DCM. The DCM phases were combined, and the resulting TLE was concentrated by rotary evaporation (27°C), filtered through pre-cleaned quartz wool and dried under N₂. For SPM from 275 m (in Experiment#2), a second part of the filter material was subjected to a modified BD extraction as described above, however, the phosphate buffer was substituted with 5% TCA (Nishihara and Koga 1987). All glassware involved in subsequent transfers of the extracts obtained by either extraction protocol was rinsed at least three times with MeOH and subjected to sonication in order to minimize the loss of IPL-GDGTs with sugar head groups (i.e., glycolipids), as the latter have been shown to adsorb on glass surfaces (Pitcher et al. 2009).

Sample processing and GDGT analysis

The concentration and composition of IPL-GDGTs were determined indirectly following the “subtraction method”

described by Huguet et al. (2010a). Briefly, CL-GDGTs were quantified in the TLE before and after acid hydrolysis (Fig. 1) and the excess of GDGTs detected in the hydrolyzed aliquot is assumed to represent the IPL-GDGT pool. To this end, the TLEs obtained by either extraction method were re-dissolved in DCM:MeOH (1 : 1, v : v) using an ultrasonic bath, and 0.1 µg of an internal standard (IS; a C₄₆ tetraether; Huguet et al. 2006) was added. The TLE was then split in two halves, one of which was subsequently processed for CL-GDGT analysis (see below). The other half was transferred to a glass reaction vial, and hydrolyzed in 2 mL 1.5 N methanolic HCl (4 h, 70°C). After cooling, 2 mL MilliQ water were added to the vial. Subsequently, the hydrolyzed lipids (i.e., CL- plus IPL-derived CL-GDGTs) were extracted twice with 2 mL DCM and dried under a stream of N₂.

To quantify the GDGTs still remaining in the SPM samples, the residual filter material from both extraction methods, together with the quartz wool used for filtration of the respective TLE, was placed into a capped glass flask containing 0.1 µg IS. The residue was then hydrolyzed with 25 mL 1.5 N methanolic HCl (5 h, 70°C). After cooling, the reaction mixture was transferred into a PTFE tube, placed in an ultrasonic bath for 5 min, and centrifuged. The supernatant was decanted into a separatory funnel, and the residue was subsequently extracted by ultrasonication, twice with 25 mL

DCM:MeOH (1 : 1, v : v), and once with 25 mL DCM (5 min each). After centrifugation, all supernatants were combined in the separatory funnel. MilliQ water (60 mL) was added to the mixture to achieve phase separation, and the DCM phase (containing the CL-GDGTs released from the residue) was concentrated by rotary evaporation, and dried completely under N₂. To independently approximate the total amount of recoverable GDGTs, another aliquot of the lyophilized filter material from 275 m (Experiment#2), together with 0.1 µg IS, was directly subjected to acid hydrolysis and subsequently extracted, as described above for the residual material.

We also probed for the presence of GDGTs in the aqueous phases of the BD solvent (Experiment#2 only) by subjecting the P-buffer- and the TCA phase (~ 50 mL each), respectively, to acid hydrolysis (5 h, 70°C), by adding 10 mL concentrated HCl (32%) and 0.1 µg IS. After cooling, 50 mL MeOH and 25 mL DCM were added and the mixture was sonicated (20 min). Phase separation was achieved in a separatory funnel by addition of DCM and MilliQ water (50 mL each), and the organic phase was concentrated and dried under a stream of N₂.

Prior to analysis, all TLE aliquots and hydrolysates were separated over activated Al₂O₃ using DCM and DCM:MeOH (1 : 1, v : v) as eluents. The second, GDGT-containing fraction was dried under N₂, re-dissolved in hexane:isopropanol (99 : 1, v : v), and passed through a 0.45 µm PTFE filter, prior to analysis by ultra-high-performance liquid chromatography positive-ion APCI MS (UHPLC-APCI-MS). Analytical separation of GDGTs was achieved on an Agilent 1260 UHPLC device equipped with two UHPLC silica columns (BEH HILIC columns, 2.1 × 150 mm, 1.7 µm; Waters) in series, and quantified on an Agilent 6130 quadrupole MSD in selected ion monitoring mode under conditions described in Hopmans et al. (2016). A 1 : 1 mixture of the IS (m/z 744) and Crenarchaeol (m/z 1292) was analyzed before each sequence of samples.

Data treatment and statistical analysis

The GDGT concentrations in the individual fractions were calculated using the added IS (Fig. 1) and its MSD response factor relative to Crenarchaeol. The CL-GDGT content measured in the non-hydrolyzed aliquot of the TLE was then subtracted from the content determined in the hydrolyzed one, yielding the amount of IPL-GDGTs that were present in the TLE prior to hydrolysis (i.e., IPL-derived CLs; Huguet et al. 2010a).

Statistical analyses of GDGT distributions were performed using the software “R” (“vegan” package, Oksanen et al. 2015). To this end, GDGTs that were below the methodological detection limit in any of the analyzed fractions were removed, and the concentrations of the remaining branched- and isoprenoid GDGTs, respectively, were normalized separately. The fractional abundances of each compound were then scaled through division by the standard

deviation and subjected to non-metric multi-dimensional scaling (NMDS), using the Canberra distance metric (Lance and Williams 1966).

Assessment

GDGT yields

Core-lipid GDGTs (CL-GDGT) accounted for 20–70% of all recoverable GDGTs (i.e., IPL + CL + residue + aqueous phase), and showed remarkably consistent extraction yields across all three tested protocols (Table 1; Fig. 2). In the MeOH/DCM-extracted residues, we found substantial amounts of branched GDGTs after acid hydrolysis (38–75%), whereas the residue yields of isoprenoid GDGTs were much lower (14–36%). All BD-extracted residues (P-buffer and TCA), in contrast, contained comparably minor amounts of either GDGT class (4–13%). In turn, substantially more IPL-GDGTs were observed in the TLEs obtained by the BD method as compared to MeOH/DCM extractions. Yet, while absolute isoprenoid IPL-GDGT yields were similar for either modification of the BD protocol (± 4%), approximately five times more branched IPL-GDGTs were detected in the TLE when the phosphate buffer was substituted by 5% TCA, as revealed by Experiment#2 (SPM from 275 m). In other words, when the BD solvent contained phosphate-buffer (P-BD), more than 50% of the brGDGTs were not recovered in the TLE (neither as CLs nor as IPLs) (Table 1; Fig. 2).

In order to examine this apparent loss of GDGTs during sample processing, we investigated the aqueous phases of the BD-extracts obtained from Experiment#2 (i.e., P-buffer and 5% TCA) by subjecting them to acid hydrolysis. We found that almost 60% of the total brGDGTs were present in the P-buffer after P-BD extraction. However, when 5% TCA was used instead (TCA-BD), the brGDGTs were almost entirely recovered in the TLE (78%), and only a minor fraction (9%) was lost into the aqueous TCA phase. In stark contrast to the brGDGTs, much less isoGDGTs were present in the aqueous phases, accounting for ~ 1% and 9% of total recovery in the P-BD and TCA-BD extractions, respectively (Experiment#2; Table 1).

In Experiment#2, the sum of GDGTs in all recovered fractions ranged between 77% and 109% relative to the amounts released by direct hydrolysis of the SPM (Table 1). The totaled yields of both branched- and isoprenoid GDGTs were highest for the TCA-BD method, while reasonable recovery of brGDGTs (94%) was still achieved with the MeOH/DCM protocol.

GDGT distributions

In order to assess potential method-dependent compositional biases, we examined the CL distribution of br- and isoGDGT in the individual fractions by multivariate statistics. Ordination by NMDS revealed that the GDGT composition in the CL pools were consistent between all extraction methods applied in this study (Fig. 3, Supporting

Table 1. Concentrations of GDGTs in SPM.

Sample	Method	Fraction	brGDGTs			isoGDGTs		
			ng/L	%	SD	ng/L	%	SD
70 m (#1)	MeOH/DCM	CL*	9.9	58	0.4	99	72	1.2
		IPL [†]	0.7	4.3	0.2	19.4	14	3.6
		R [‡]	6.5	38	0.1	18.6	14	1.1
		SUM	17.2	100	0.6	137	100	3.2
70 m (#1)	Bligh–Dyer (P-buffer)	CL*	10.8	63	—	93	68	—
		IPL [†]	1.3	7.3	—	36	26	—
		R [‡]	0.7	4	—	8.0	6	—
		SUM	12.7	74	—	137	100	—
275 m (#1)	MeOH/DCM	CL*	6.1	25	—	13.0	33	—
		IPL [†]	1.7	7	—	11.7	30	—
		R [‡]	16.9	68	—	14.1	36	—
		SUM	24.7	100	—	38.8	100	—
275 m (#1)	Bligh–Dyer (P-buffer)	CL*	5.2	21	—	12.2	31	—
		IPL [†]	4.4	18	—	22.8	58	—
		R [‡]	1.4	6	—	4.3	11	—
		SUM	11.1	45	—	39.2	100	—
275 m (#2)	MeOH/DCM	CL*	1.7	19	—	7.2	39	—
		IPL [†]	0.5	5.9	—	6.7	36	—
		R [‡]	6.6	75	—	4.7	25	—
		SUM	8.8	100	—	18.6	100	—
275 m (#2)	Bligh–Dyer (P-buffer)	CL*	1.3	18	—	6.3	32	—
		IPL [†]	1.3	18	—	12.3	62	—
		R [‡]	0.3	4	—	1.0	5.0	—
		AQ [§]	4.3	59	—	0.1	0.7	—
		SUM	7.2	100	—	19.7	100	—
275 m (#2)	Bligh–Dyer (TCA)	CL*	1.7	17	—	7.3	31	—
		IPL [†]	6.2	61	—	12.8	54	—
		R [‡]	1.4	13	—	1.3	5.7	—
		AQ [§]	0.9	9.1	—	2.1	8.8	—
		SUM	10.2	100	—	23.6	100	—
275 m (#2)	Direct hydrolysis	Bulk	9.4	—	—	24.3	—	—

Relative abundances are given with respect to the sum of all recovered fractions (boldface). Because the aqueous phase of the Bligh–Dyer solvent (containing brGDGTs) was not analyzed in Experiment#1, concentrations were normalized to the total amount recovered by the MeOH/DCM method (italic face). Standard deviations (SD) are given for the triplicate extraction.

*Core lipids.

[†]Intact polar lipids.

[‡]Released by hydrolysis of the extraction residue.

[§]Released by hydrolysis of the aqueous phase.

^{||}Sum of all analyzed fractions.

Information Fig. S3A), in agreement with the concentration data. However, the type of extraction protocol strongly affected the CL distribution in the IPL-, the residue-, and the aqueous phase-derived GDGT fractions (Fig. 3, Supporting Information Fig. S3B–D). Remarkably, brGDGT distributions in (1) the aqueous P-buffer, (2) the IPL fraction obtained by the TCA-BD method, and (3) the residue pool after MeOH/DCM extraction were almost identical (Fig. 3C). The isoGDGT compositions in these three fractions, however,

were markedly distinct (Fig. 3F). In addition, these compositional offsets among the recovered fractions varied between the two sampled depths (70 m and 275 m), and to a lesser extent, between the two sampling campaigns (#1 and #2), for both GDGT classes (Fig. 3). When the GDGT abundances in all recovered fractions from Experiment#2 were totaled and subsequently normalized, the resulting “cumulative” branched GDGT distributions were fairly similar to the composition in the direct sample hydrolysate

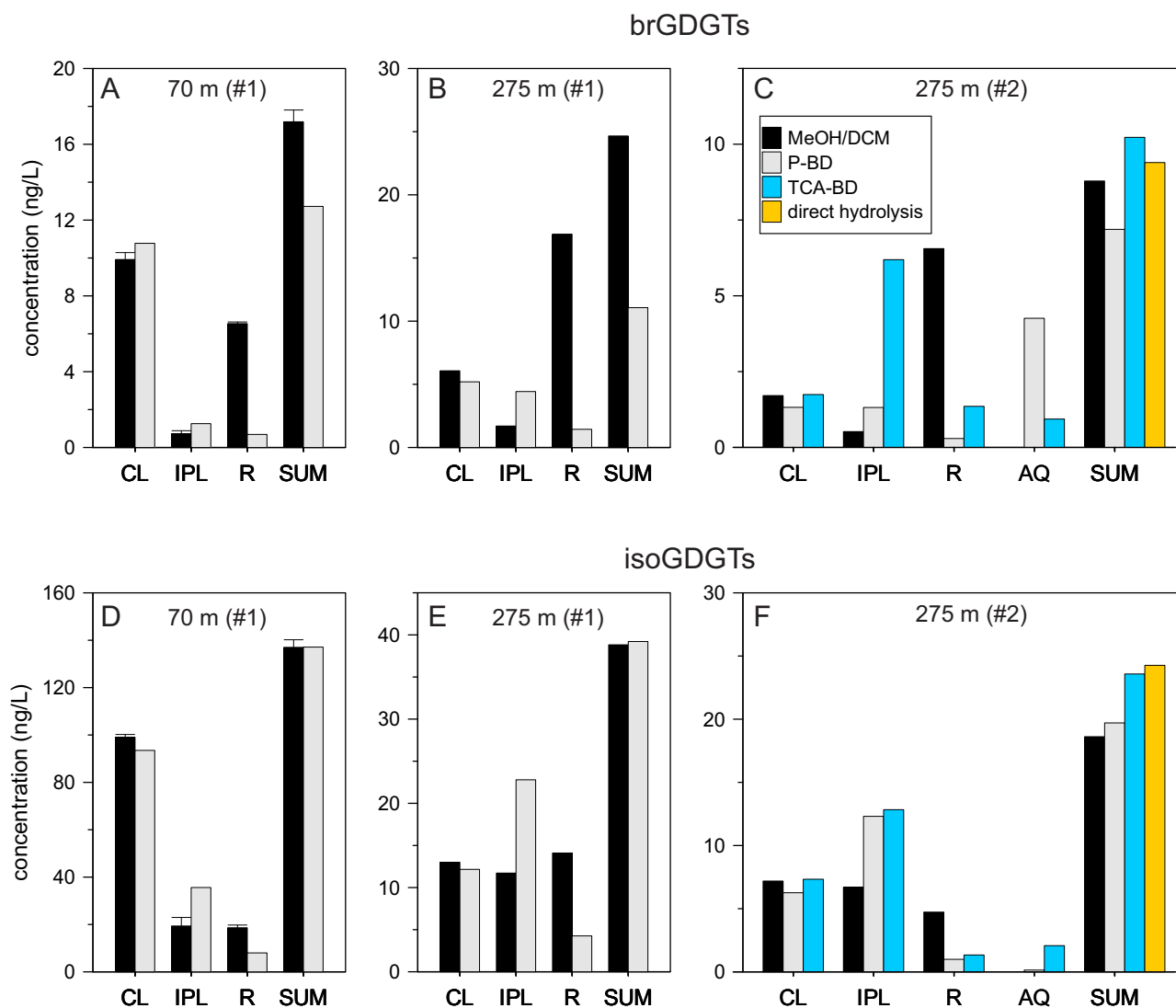


Fig. 2. Concentrations of branched (A–C) and isoprenoid (D–F) GDGTs in the CL, IPL, and residue (R) fractions, as well as in the aqueous phase of the BD solvent (AQ) for SPM collected from two depths in Lake Lugano. Cumulative concentrations are given for each experiment (SUM). Note that the increase in IPL-brGDGT yields in the TCA-BD- vs. MeOH/DCM extraction (Experiment#2; Panel C) is accompanied by a corresponding decrease in residual brGDGTs (see Table 1). For Experiment#1, the aqueous phase of the Bligh–Dyer extracts were not tested for the presence of GDGTs. Error bars indicate the standard deviation for SPM from 70 m depth (Experiment#1), where MeOH/DCM extractions were performed in triplicate.

(obtained by extraction *after* acid hydrolysis), for both branched and isoprenoid GDGTs (Fig. 3C,F, Supporting Information Fig. S3E).

Discussion

Differential extractability of branched and isoprenoid IPL-GDGTs

The consistent yields and distributions of branched and isoprenoid CL-GDGTs obtained by any of the three extraction protocols indicate that CLs represent a well-defined and readily extractable lipid pool in SPM from Lake Lugano. In contrast, the remaining fraction of the GDGT pool (i.e., the

“non-CL” pool) displayed a highly differential behavior depending on the type of extraction method used. Most obviously, near-quantitative GDGT extraction from SPM (in particular of IPL-brGDGTs) could only be achieved with the BD protocol that includes an aqueous phase (P-buffer or 5% TCA) as part of the solvent mix, demonstrating the importance of water in enhancing IPL recovery (see Fig. 2C,F). The majority of these “recalcitrant” brGDGTs extracted with the P-BD method showed a strong affinity to the aqueous phase, attesting to a pronounced hydrophilicity of the IPL-brGDGTs present in the extract prior to phase separation. This suggests that the bacterial IPL-GDGTs possess extraordinarily large and/or polar head groups, which may still be attached to

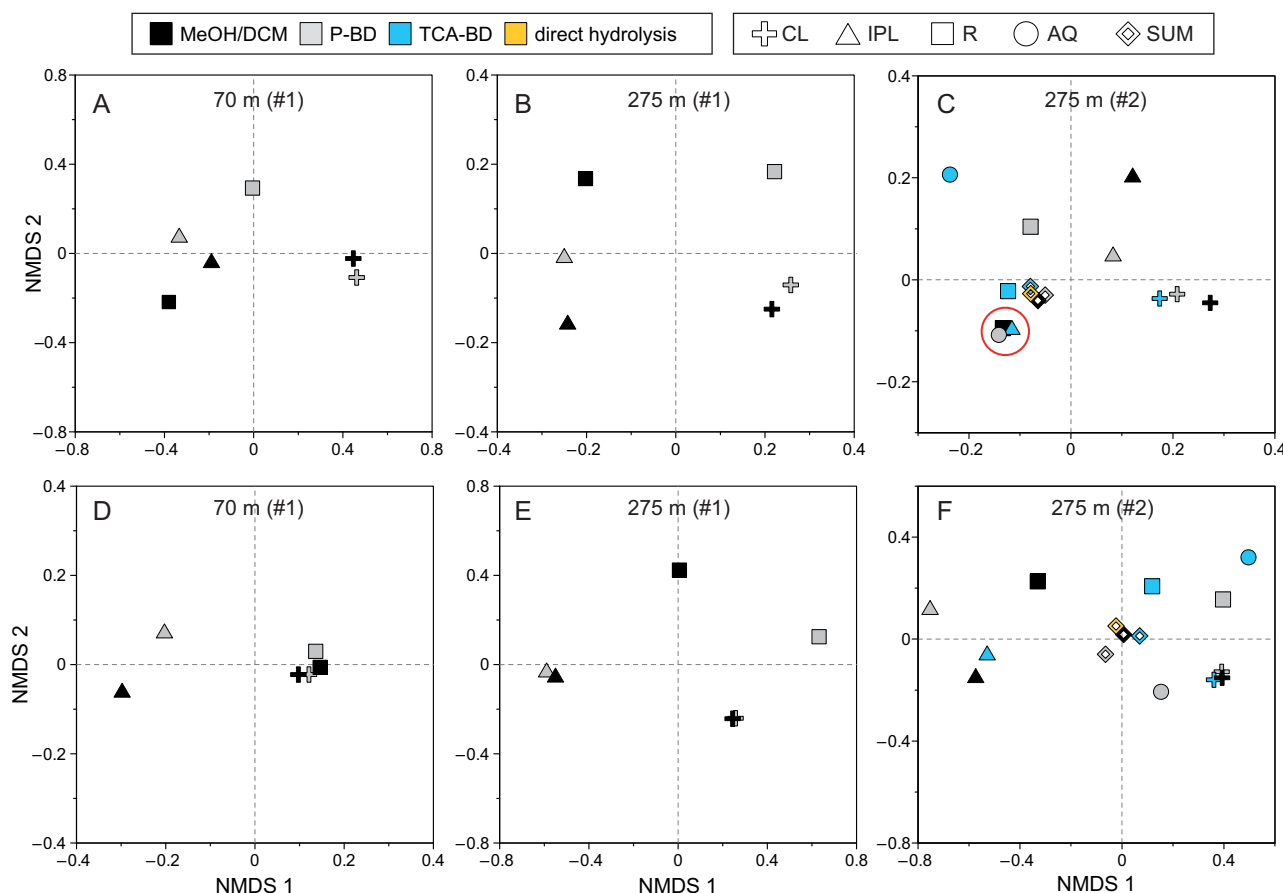


Fig. 3. NMDS plots showing method-dependent differences in branched (A–C) and isoprenoid GDGT (D–F) compositions in the core lipid- (crosses), IPL, (triangles), and residual fractions (squares), as well as in the aqueous phases of BD extracts (circles). Bulk GDGT compositions (open diamonds) are based on the sum of all recovered fractions, as well as on direct hydrolysis (Experiment#2 only). The distance between symbols reflects the dissimilarity in GDGT distribution. The almost identical GDGT compositions in the fractions “IPL-TCA-BD”, “R-MeOH/DCM”, and “AQ-P-BD” (highlighted with a red circle; panel C) demonstrate that the brGDGTs released from the MeOH/DCM residue consist of IPLs. Note that the aqueous phases were only investigated in Experiment#2. Data of the sample analyzed in triplicate (70 m) were averaged before statistical analysis.

(polar) macromolecular components of the cell wall. The use of 5% TCA, however, seems to strongly reduce these hydrophilic properties, indicating either (1) pH-controlled polarity changes within the IPLs’ head groups, e.g., by protonation of ionic moieties such as carboxylic acids or phosphate groups, or (2) partial hydrolysis of the (macromolecular) polar moieties originally attached to the brGDGTs.

In stark contrast to the bacterial tetraethers, the archaeal *iso*GDGTs did not show any considerable affinity to the aqueous P-buffer (< 1% of total recovery) but were, however, markedly more hydrophilic in the presence of TCA (9% of total recovery). Although the longer archaeal alkyl chains (see Supporting Information Figs. S1, S2) make *iso*GDGTs slightly less polar than the bacterial ones, we argue that structural differences within the lipid cores cannot explain such contrasting physicochemical properties and their alteration by TCA. Instead, we reason that considerable differences must exist between the two lipid classes with regard to the type (and/or size) of the polar moieties bound to the glycerol backbone.

Thus far, IPL head group chemistry was established for *br*GDGTs extracted from peat (Liu et al. 2010; Peterse et al. 2011), and for *iso*GDGTs derived from marine SPM and sediments (e.g., Schubotz et al. 2009; Meador et al. 2015), as well as from archaeal cultures of the mesophilic *Thaumarchaeota* (Schouten et al. 2008; Pitcher et al. 2010, 2011; Sinninghe Damsté et al. 2012). Interestingly, only the *branched* IPL-GDGTs contained glucuronic acid (GlcA), while (poly-) hexoses, phospho-hexoses and phospho-glycerol were common head groups in both GDGT classes. Given the acid equilibrium of GlcA ($R-COOH \rightleftharpoons R-COO^- + H^+$), we further hypothesize that GlcA-IPL-brGDGTs are more polar/hydrophilic under neutral- (P-buffer) than acidic pH (TCA), which may contribute to the much larger amounts of branched- vs. isoprenoid GDGTs in the aqueous P-buffer. Moreover, our findings suggest that previous studies analyzing the brGDGTs’ head groups in P-BD extracts were likely biased towards IPL classes that are dominantly hydrophobic. That is to say, because the aqueous P-buffer phase (potentially containing hydrophilic IPLs) is

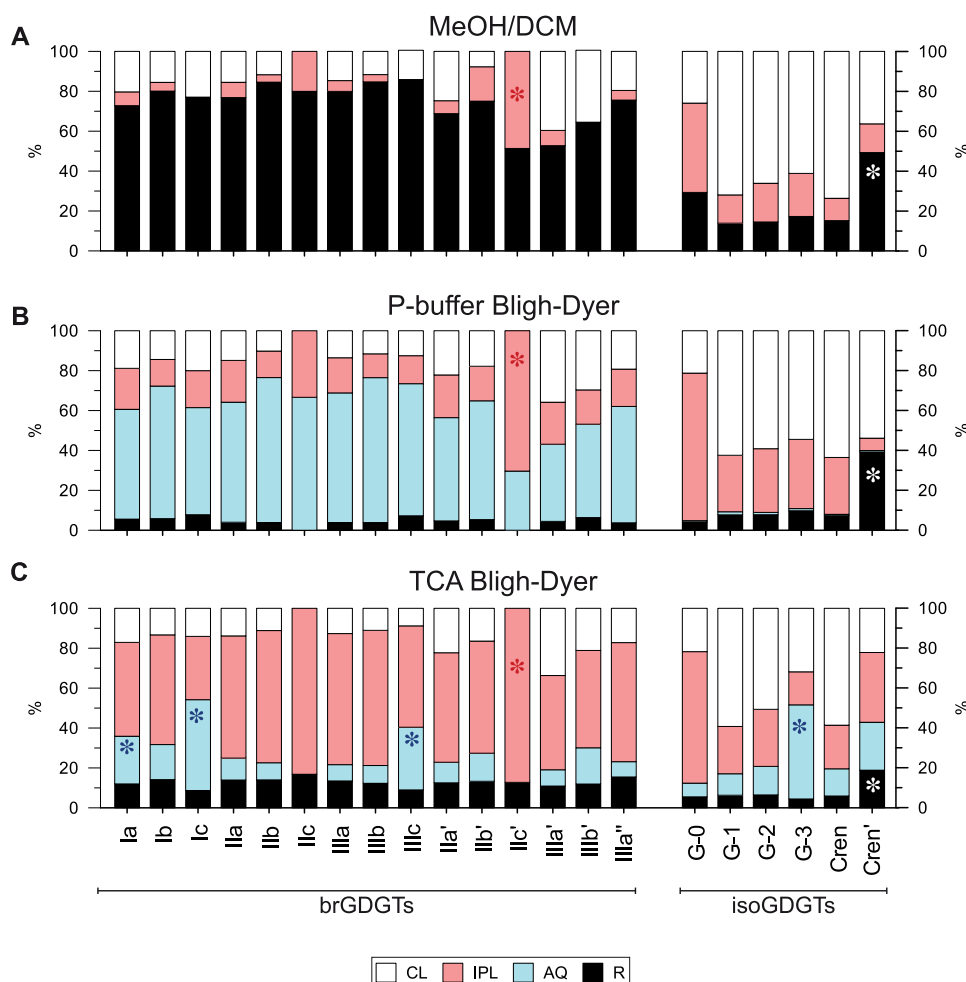


Fig. 4. Relative partitioning of individual branched- and isoprenoid core GDGT types between the recovered fractions in Experiment#2 (275 m SPM). Note that abundances were normalized for each compound separately. Fractions are as follows: CL- (white), IPL- (pink), residue- (black), aqueous fractions (light blue). Shown is the percentage relative to the totaled amount of the respective GDGT in all recovered fractions (i.e., CL + IPL + residue + aqueous phase). Compound names refer to structures in Supporting Information Figs. S1, S2. Above average contributions mentioned in the main text are indicated with an asterisk. brGDGT IIIc' was below detection in some fractions and is not shown. The extraction methods applied were (A) water-free ultrasonic MeOH/DCM extraction, (B) modified Bligh–Dyer protocol with P-buffer, and (C) modified Bligh–Dyer protocol with trichloroacetic acid.

commonly not further analyzed, IPL-brGDGTs comprising larger and/or highly polar moieties may have remained undetected. We consequently suggest that differences in the head group chemistry between branched- and isoprenoid GDGTs may be the reason for their differential extraction behavior. Future studies should attempt to obtain a more complete image of the polar lipidomes of GDGT-producing bacteria and archaea, i.e., by extending the analytical window towards hydrophilic- and higher molecular weight IPLs.

Recalcitrance of intact polar brGDGTs towards organic solvent extraction

It has previously been reported that not all GDGTs present in lake sediments, soils, and/or peatlands are accessible through common extraction techniques (i.e., P-BD, Soxhlet-,

and accelerated solvent extraction) (Huguet et al. 2010c,d; Tierney et al. 2012; Chaves Torres and Pancost 2016). In all of these studies, considerable amounts of GDGTs could still be released from the post-extraction residues by acid hydrolysis, giving rise to the hypothesis that parts of the GDGT pool had been “insolubilized” (i.e., by encapsulation in organic macromolecules, in organo-mineral complexes, or in soil- or sediment aggregates; see Lützow et al. 2006 for review). In Lake Lugano SPM, the striking similarity between the MeOH/DCM-insoluble fraction and the IPL-brGDGTs obtained by the TCA method, both in terms of composition (Fig. 3C) and absolute concentration (Table 1), demonstrates that predominantly brGDGTs with intact polar head groups are resilient to ultrasonic MeOH/DCM extraction. This “recalcitrant” IPL fraction may derive either from (1)

allochthonous organic matter from catchment soils and/or re-suspended slope sediments that has undergone “insolubilization” through pedogenic/diagenetic reactions (Lützow et al. 2006), or (2) from intact microbial cells that possess an “intrinsic recalcitrance” towards organic solvent extraction (Huguet et al. 2010c; Sinninghe Damsté et al. 2011, 2014; Cario et al. 2015). Here, we cannot further constrain the nature of this non-MeOH/DCM-extractable IPL-brGDGT pool, as this would require an in-depth investigation of the vertical distribution of GDGT in the water column of Lake Lugano.

The cell walls of bacteria are chemically heterogeneous structures dominantly consisting of hydrophilic polymers such as carbohydrates (peptidoglycan, lipopolysaccharides, teichoic acids), and “surface layer” (S-layer) (glyco-) proteins (Silhavy et al. 2010). In addition, some species can surround their cell wall with a polysaccharide capsule (Kasper 1986). It is therefore reasonable to assume that the water contained in the BD solvent mixture facilitated the permeabilization of these hydrophilic cell wall components, which could not be achieved with non-aqueous MeOH/DCM. On the other hand, it also seems possible that molecular interactions between the polar glass fiber filters, and the IPLs’ head group moieties prevented their effective extraction in the absence of an aqueous solvent phase. Nonetheless, since the source organisms of most brGDGT CL types have not yet been identified, we cannot draw definite conclusion with regards to the physicochemical- and/or biological mechanisms behind the recalcitrance that we observed in this study.

Fractionation of individual CL GDGT types during extraction

Depending on the utilized extraction method, we found marked differences in the size of the IPL- and residual GDGT pools (Fig. 2C,F). Similarly, the relative partitioning of individual CL types showed considerable variability between each of the three extraction protocols tested (Fig. 4). For instance, brGDGT IIc’ showed a much weaker “recalcitrance” and hydrophilicity than the average of all bacterial tetraethers, whereas the affinity to the TCA phase was noticeably increased for Ia, Ic, and IIc. As for the isoGDGTs, the crenarchaeol regio-isomer (Cren’) was extracted much less effectively than the remaining archaeal lipids, and GDGT-3 (G-3) was disproportionally more abundant in the TCA fraction (Fig. 4C, Supporting Information Fig. S3D). Assuming that head group chemistry dominantly determines the physicochemical properties of IPLs during solvent extraction (see above), the differential fractionation “behavior” may reflect the preferential association of certain types of head group moieties with specific CL structures, as it has been shown for ammonia oxidizing archaea of the *Thaumarchaeota* (Sinninghe Damsté et al. 2012; Elling et al. 2014). Nevertheless, solvent-impermeable cell wall structures may be present at least in some of the GDGT-producing organisms (see section

4.2). We can thus not rule out that differences in the GDGT-producing microbial community additionally contribute to compositional shifts associated with incomplete extraction.

In order to assess the effect of the method-dependent compositional variations on GDGT-based paleoenvironmental proxies, we calculated the most common GDGT indices as well as pH- and T estimates derived by available transfer functions (Supporting Information Table S1). We observed considerable method-dependent offsets only in the IPL-derived fraction, which is in line with the largely consistent composition in the CL pools (see Fig. 3). For example, offsets of up to $\pm 4.6^\circ\text{C}$ for TEX₈₆-derived lake surface temperatures, and ± 0.5 pH units based on the cyclization ratio (CBT_{Sme}; De Jonge et al. 2014a) were evident between the IPL fractions obtained by different methods. We therefore conclude that the choice of extraction protocol is of substantial importance in studies investigating proxy indices in the IPL-derived fraction (e.g., in microbial cultures or within aquatic environments). Such procedural biases are less pertinent to paleo applications, in which only CL- (and not IPL-) GDGTs are the analytical target.

Conclusions

We showed that up to 75% of the bacterial IPL-GDGTs present in lacustrine SPM may be lost into the aqueous P-buffer phase during BD extraction, suggesting that previous studies have substantially underestimated the supposedly “viable” fraction of brGDGTs in aquatic environments. Furthermore, the choice of the extraction protocol has a marked effect on the IPL-derived CL distributions of both branched and isoprenoid GDGTs, which may affect environmental proxy indices calculated from the IPL fraction. Particularly IPL brGDGTs in the water column of lakes may be inaccessible by extraction techniques that solely rely on organic solvents. For near-quantitative recovery of both bacterial and archaeal IPL-GDGTs, we therefore recommend using a modified BD extraction with 5% TCA (instead of P-buffer). If only the CL- (and not the head group-) composition of GDGTs is of interest, residue hydrolysis after MeOH/DCM extraction may be employed instead to access the recalcitrant GDGT pool. Finally, for quick determination of the bulk GDGT composition, direct acid hydrolysis prior to extraction represents a valid alternative.

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Conflict of Interest

None declared.

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