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Critical Assessment of Glyco- and Phospholipid Separation by Using Silica Chromatography

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Phospholipid-derived fatty acids (PLFAs) are commonly used to characterize microbial communities *in situ* and the phylogenetic positions of newly isolated microorganisms. PLFAs are obtained through separation of phospholipids from glycolipids and neutral lipids using silica column chromatography. We evaluated the performance of this separation method for the first time using direct detection of intact polar lipids (IPLs) with high-performance liquid chromatography–mass spectrometry (HPLC-MS). We show that under either standard or modified conditions, the phospholipid fraction contains not only phospholipids but also other lipid classes such as glycolipids, betaine lipids, and sulfoquinovosyldiacylglycerols. Thus, commonly reported PLFA compositions likely are not derived purely from phospholipids and perhaps may not be representative of fatty acids present in living microbes.

Examining microbial communities *in situ* is one of the major challenges in microbial ecology. Traditionally, isolation and cultivation techniques were used, in addition to microscopic observations, to characterize microorganisms in environmental samples. This traditional approach has its limitations, as it was estimated that, depending on the habitat, only about 0.001 to 1% of all microorganisms are cultivable by standard techniques (1). Over the last 2 decades, a number of cultivation-independent techniques for the examination of microbial community composition have been established, using in particular genomic techniques.

One of the earliest alternative approaches to study microbial communities independent of cultivation was the analysis of the compositions and abundances of fatty acids in environmental samples and comparison to the fatty acid compositions of pure cultures (2–4). Fatty acids do not occur as such in living biomass but instead occur as intact polar lipids (IPLs) with the fatty acids esterified via either a glycerol or an amide moiety to a polar head group. These fatty acids are released after cell death and can persist in natural environments. Therefore, fatty acids derived from living biomass have to be distinguished from those derived from dead and fossil biomass when analyzing microbial communities *in situ*. One way to do this is to study phospholipid-derived fatty acids (PLFAs), as phospholipids are thought to be relatively labile and fall apart shortly after cell death (5, 6). Furthermore, they are the major lipids in cell membranes and are rarely used as storage products. In contrast, glycolipids are generally classified as storage products (7), although it was recently shown that they can also be major membrane lipid components in chloroplasts of plants and algae and in cyanobacteria (8). PLFAs are thus commonly used as indicators for living microbes. The interpretation of the PLFA patterns of environmental samples is done by comparison with the PLFA patterns of microorganisms grown in pure cultures (9–11). Furthermore, they are used to investigate metabolic activity *in situ* by stable-isotope analysis in combination with labeling experiments (12, 13). PLFA analysis is also often used as chemotaxonomic information to characterize the phylogenetic positions of new microorganisms (14).

In order to obtain PLFAs rather than free fatty acids or fatty acids contained in storage lipids, Vorbeck and Marinetti (15) proposed a method to separate bacterial lipids into "neutral lipid," "glycolipid," and "phospholipid" fractions by applying a silicic acid column and eluting the fractions with different mixtures of chloroform, acetone, and methanol (MeOH). The obtained phospholipid fraction was subsequently hydrolyzed to obtain phospholipid-derived fatty acids which could be analyzed by gas chromatography (GC). The efficacy of the silicic acid column separation was verified by measuring the phosphorus and carbohydrate contents in the different fractions using colorimetric methods (carbohydrates by the anthrone reaction described by Radin et al. [16] and phosphorus content by the molybdenum blue method described by Harris and Popat [17] and modified by Marinetti et al. [18]). The separation method has subsequently been modified over the years by decreasing the volumes of eluents used, replacing chloroform with the less toxic dichloromethane (DCM), and using pure acetone and methanol, to obtain the neutral, glycolipid, and phospholipid fractions (9, 19–21). This modified separation method is now routinely used in environmental studies as well as for chemotaxonomy of microbes belonging to the bacteria and eukaryotes (10, 22–24). However, since the initial studies, the performance of the separations has been rarely reevaluated, particularly whether the PLFAs are truly derived from phospholipids only. Additionally, recent studies have shown that both betaine lipids and sulfoquinovosyldiacylglycerols (SQDGs) are abundant IPLs in the marine environment (25–29). The fate of these IPLs after separation on a silica column is, to the best of our knowledge, unknown, and thus it is unclear to what degree the fatty acids contained in these membrane lipids are accounted for in PLFA analysis.

In the last 15 years, analytical techniques were developed that made it possible to directly analyze IPLs, including phospholipids, using high-performance liquid chromatography–mass spectrom-

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etry (HPLC-MS). Typically, IPLs are separated according to the polarity of the head group and identified by multistage mass spectrometry (11, 30, 31). However, this type of analysis does not allow detailed identification of the various fatty acids contained in the IPLs. For detailed identification, separation and hydrolysis of the lipid extract and subsequent GC(-MS) analysis is still required. Additionally, for stable-isotope probing, which requires GC-amenable compounds, PLFAs are still needed. Therefore, PLFA analysis is still an important method within microbial ecology.

In this study, we have reassessed the compositions of the different chromatographic fractions isolated by silica chromatography, using lipid extracts from different environmental samples. Through direct analysis of IPLs using HPLC-MS, we accurately studied the fates of several environmentally important types of IPLs during one of the most used chromatographic fractionations and assessed whether PLFAs are truly representative of membrane lipids.

MATERIALS AND METHODS

Sampling. A marine sediment was collected in January 2012 during low tide in the Mokbaai on the Dutch Wadden Island Texel. A microbial mat sediment was collected in August 2010 on the Dutch Wadden Island Schiermonnikoog. Similar microbial mats from the same location were described by Bolhuis and Stal (32). The microbial mat was sampled from the top of the sediment and immediately sealed. Sediment cores with a diameter of 7 cm were taken by hand and sliced on location, and the first centimeter was collected. All samples were freeze-dried, homogenized, and stored at −40°C until extraction.

Extraction of intact polar lipids. The freeze-dried samples were extracted using a modified Bligh-Dyer method (11, 33). In short, the samples were extracted with MeOH-DCM-phosphate buffer (2:1:0.8, vol/vol/vol) ultrasonically three times for 10 min. The supernatants were collected, and DCM and phosphate buffer were added to achieve a phase separation. The DCM fraction was transferred to a round-bottom flask, and the aqueous phase was washed three times with DCM. All DCM fractions were combined and dried using a rotary evaporator. The resulting Bligh-Dyer extract (BDE) was transferred into a vial with DCM-MeOH (9:1), further dried under a nitrogen flow, and stored dry at −20°C.

Separation of different IPL classes. The BDE was split into three equal (by volume) aliquots. All subsequent treatments were performed in triplicate. In order to obtain the so-called neutral, glycolipid, and phospholipid fractions, the BDE was separated on a DCM-prerinsed silica column (0.5 g; activated for 3 h at 150°C) by eluting with 7 ml DCM, 7 ml acetone, and 15 ml MeOH, respectively (9). The resulting fractions were dried under nitrogen and stored at −20°C.

In order to test the effects of slightly different solvent mixtures, the BDE was also separated on a prewashed silica column (0.5 g) by eluting with 7 ml of DCM, 7 ml of acetone-MeOH (99:1) and 15 ml MeOH, respectively. These fractions were also dried under nitrogen and stored at −20°C.

IPL analysis. For the IPL analysis, the original BDE and the different chromatographic fractions were dissolved and filtered in 250 μl of injection solvent (hexane-2-propanol-H_2O, 718:271:10). IPLs were analyzed directly afterwards by high-performance liquid chromatography–electrospray ionization–tandem mass spectrometry (HPLC/ESI-MS^2) as described by Sturt et al. (30) with some modifications. We used an Agilent 1200 series LC (Agilent, San Jose, CA), which was equipped with a thermostatted autoinjector and a column oven and coupled to a Thermo LTQ XL linear ion trap with an Ion Max source with an electrospray ionization (ESI) probe (Thermo Scientific, Waltham, MA). To each sample, 5 μg/ml of 1-*O*-hexadecyl-2-acetoyl-*sn*-glycero-3-phosphocholine (C16 PAF) standard was added in order to monitor the performance of the machine. Separation was achieved on a Lichrosphere diol column (250 by 2.1 mm, 5-μm particles; Alltech Associates Inc., Deerfield, IL) maintained at 30°C. The following elution program was used with a flow rate of 0.2 ml/min: 100% A for 1 min, followed by a linear gradient to 66% A–34% B in 17 min, which was maintained for 12 min, followed by a linear gradient to 35% A–65% B in 15 min (where A is hexane–2-propanol–formic acid–14.8 M aqueous NH_3 [79:20:0.12:0.04, vol/vol/vol/vol] and B is 2-propanol–H_2O–formic acid–14.8 M aqueous NH_3 [88:10:0.12:0.04, vol/vol/vol/vol]). The total run time was 60 min, with a reequilibration period of 20 min in between runs. The settings for the ESI were as follows: capillary temperature, 275°C; sheath gas (N_2) pressure, 25 arbitrary units (AU); auxiliary gas (N_2) pressure, 15 AU; sweep gas (N_2) pressure, 20 AU; and spray voltage, 4.5 kV. The lipid extract was analyzed by positive-ion scanning (*m/z* 400 to 2000), which was followed by a data-dependent MS^2 experiment where the four most abundant masses in the mass spectrum were fragmented (normalized collision energy, 25; isolation width, 5.0; activation Q, 0.175).

For each IPL class, the individual IPL species were identified in the total BDE by their fragmentation pattern in MS^2 (34). In order to evaluate the chromatographic behaviors of the different IPL classes during silica chromatography, the three to five most abundant IPL species within each IPL class were selected for quantification. The peak areas of these IPL species were integrated in their MS^1 mass chromatograms. Since the same equivalent amount of extract was consistently injected, the peak areas of the total extract and the different fractions could be directly compared. To calculate the distribution of the IPLs over the fractions, the peak areas of the selected species within an IPL class in each fraction were summed and the percentage relative to the total amount in both the acetone and the methanol fractions was calculated. The final percentage is the average of the percentage in each of the triplicates. To determine the recovery, the peak areas of the selected species within an IPL class in each fraction was summed, and the percentage relative to the amount in the BDE could be calculated since identical injection volumes and concentrations were used for the fractions and BDE, respectively. The final value is an average of the triplicates. Due to different ionization efficiencies of different IPL classes, the different treatments for each IPL class were compared and not the amounts of IPL classes with each other within a single fraction.

RESULTS AND DISCUSSION

IPL composition. To evaluate the commonly used separation method in PLFA analysis, we subjected extracts from a microbial mat (Schiermonnikoog, The Netherlands) and a marine sediment (Mokbaai, The Netherlands) to the various silica chromatography protocols.

We first studied the IPL composition of the whole extracts of both samples prior to chromatographic separation (Fig. 1). This showed that the microbial mat sediment contained two types of glycolipids, monogalactosyldiacylglycerols (MGDGs) and digalactosyldiacylglycerols (DGDGs) (Fig. 2). For both of these IPL classes, the main sugar moiety has been recognized as galactose, which is why they are in general referred to as galactolipids (27). Both of these glycolipids mainly contained C_{16} and C_{18} fatty acids with 0 to 3 double bonds in different combinations (Table 1). In addition to glycolipids, the microbial mat sediment also contained three different phospholipid classes, phosphatidylglyceride (PG), phosphatidylcholine (PC), and phosphatidylethanolamine (PE) (Fig. 1 and 2). The different phospholipid classes contained mainly two fatty acids with a combined total number of carbon atoms of 30 to 40 and a total number of double bonds of 0 to 6 (Table 1). Due to the lack of specific fragments, we were not able to assess the compositions of individual fatty acids in these lipid classes. Sulfoquinovosyldiacylglycerols (SQDGs) (Fig. 2), which structurally resemble glycolipids but contain a sulfate group, were also found in the extract. The SQDGs contained mainly C_{16} and

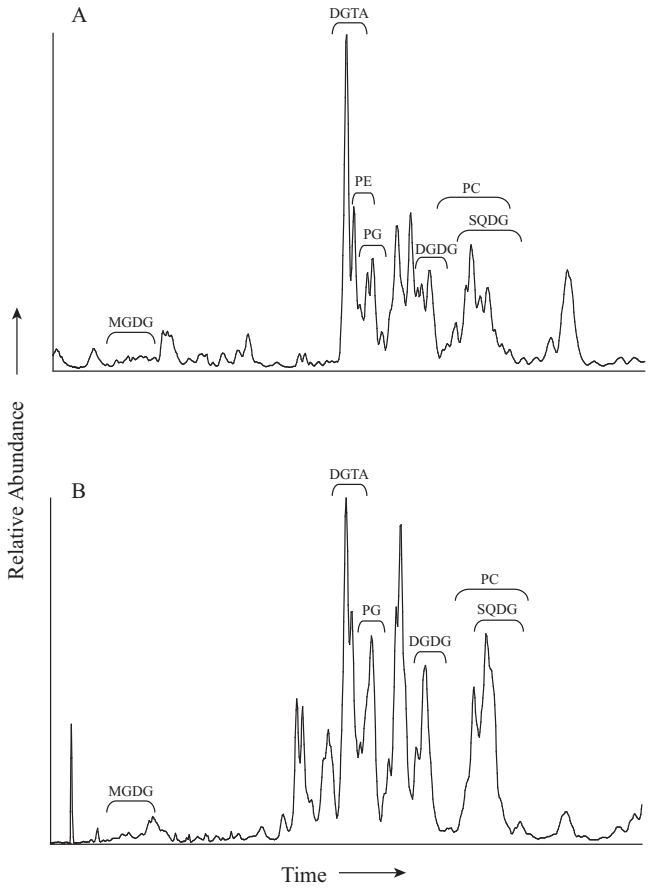

FIG 1 HPLC/ESI-MS base peak chromatogram of the Bligh-Dyer extracts of the Schiermonnikoog microbial mat (A) and the Mokbaai sediment (B). Monogalactosyldiacylglycerol (MGDG), digalactosyldiacylgylcerol (DGDG), phosphatidylglycerol (PG), phosphatidylcholine (PC), phosphatidylethanolamine (PE), sulfoquinovosyldiacylglycerol (SQDG); and diacylglyceryl-hydroxymethyl-trimethylalanine (DGTA) are indicated. For structures of IPLs, see Fig. 2.

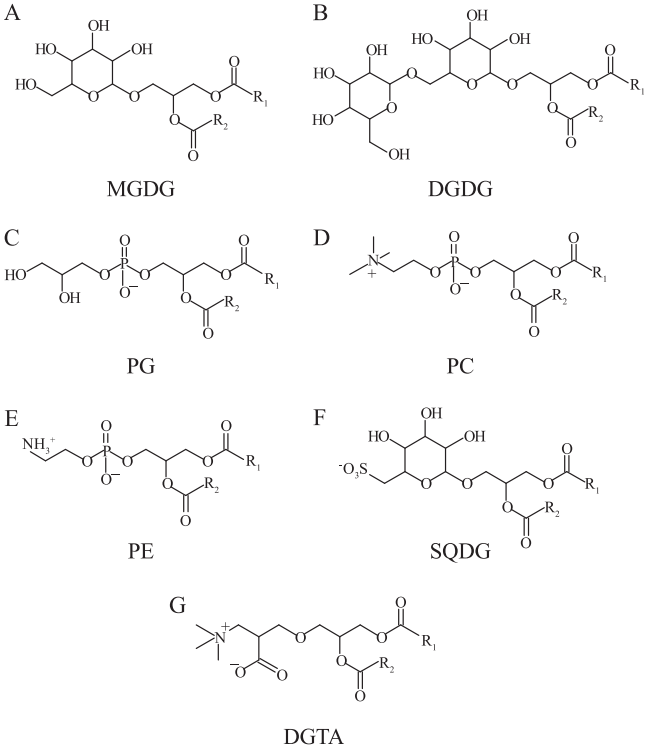

FIG 2 Overview of head groups of analyzed intact polar lipids (IPLs). (A) MGDG; (B) DGDG; (C) PG; (D) PC; (E) PE; (F) SQDG; (G) DGTA. R_1 and R_2 represent different fatty acid moieties. For definitions of IPL abbreviations, see the legend to Fig. 1.

C_{18} fatty acids with 0 to 3 double bonds. Lipids with a diacylglyceryl-hydroxymethyl-trimethylalanine (DGTA) head group (Fig. 2), belonging to the class of betaine lipids, were also detected in the extract. The DGTAs contained mainly C_{16} and C_{18} fatty acids with 0 to 2 double bonds (Fig. 1A).

The BDE extract of the Mokbaai sediment also contained MGDGs and DGDGs, both containing mainly C_{16} and C_{20} fatty acids with 0 to 5 double bonds (Table 1). Of the different phospholipid classes, both PGs and a large variety of different PCs could be identified, containing two fatty acids with a combined total carbon number of 31 to 42 and a total number of double bonds of 0 to 12. SQDGs were much less diverse in their fatty acid composition, containing mainly C_{16} fatty acids with 0 to 2 double bonds (Table 1). We also found DGTAs in this sample, consisting mainly of C_{16} and C_{18} fatty acids with no or one double bond (Fig. 1B).

Thus, both the microbial mat and the marine sediment contain a variety of IPLs, including glycolipids (MGDG and DGDG) as well as phospholipids (PG, PC, and PE), making them suitable for evaluation of the commonly used silica separation method. In addition, they contained other IPLs such as SQDG and betaine lipids (DGTA), for which the fraction in which they elute is not known.

Standard separation method. Analysis of the different chromatographic fractions obtained from the most commonly used silica gel separation method showed for the microbial mat that MGDGs were distributed evenly between the acetone ("glycolipid") and the methanol ("phospholipid") fractions (54% ± 5% and 46% ± 5%, respectively) (Table 2). In contrast, all of the DGDGs were detected in the methanol fraction. Of the phospholipids, only PCs eluted exclusively in the methanol fraction. Both PGs and PEs were also present in minor amounts in the acetone fraction, i.e., 9% ± 1% and 11% ± 1% in the acetone fraction and 91% ± 1% and 89% ± 1% in the methanol fraction, respectively. Of the "nonphospholipids," the majority (68 ± 3) of the SQDGs eluted in the methanol fraction, although a relatively high percentage (32% ± 1%) eluted in the acetone fraction. DGTAs were found exclusively in the methanol fraction (Table 2). We also assessed the overall recovery of the different IPLs by normalizing on the original amounts in the extract. Of the DGDGs, SQDGs, DGTAs, and PCs, 75 to 98% could be recovered in the different fractions. All PGs could be recovered in the different fractions. Of all studied IPL classes, the recovery of the PEs was the lowest, with 30% not recovered from the column (Fig. 3A). The recovery of the MGDGs was higher than 100%, which at first may be surprising. However, this could be due to ion suppression; i.e., the ionization of the early-eluting MGDGs might be suppressed due to matrix effects in the complex total BDE extract. This ion suppression may be much less in the cleaner chromatographic fractions, leading to enhanced ionization and thus an increase in peak areas.

TABLE 1 Fatty acid compositions of different IPL classes in the BDE

Lipid class[a]	Fatty acid composition in: Microbial mat	Marine sediment
MGDG	$C_{14:0}$, $C_{16:0\text{-}16:3}$, $C_{18:0\text{-}18:4}$, $C_{20:3\text{-}20:5}$	$C_{14:0}$, $C_{16:0\text{-}16:4}$, $C_{17:0\text{-}17:1}$, $C_{17:3}$, $C_{18:1}$, $C_{18:4}$, $C_{20:4\text{-}20:5}$
DGDG	$C_{14:0}$, $C_{16:0\text{-}16:3}$, $C_{18:3\text{-}18:4}$	$C_{16:0\text{-}16:3}$, $C_{20:4\text{-}20:5}$
PG[b]	$C_{30:0\text{-}30:1}$, $C_{31:1}$, $C_{32:1\text{-}32:3}$, $C_{33:1}$, $C_{34:1\text{-}34:3}$, $C_{36:2}$	$C_{32:1\text{-}32:2}$, $C_{33:1}$, $C_{34:1\text{-}34:2}$, $C_{36:2}$, $C_{36:5\text{-}36:6}$
PC[b]	$C_{30:0\text{-}30:1}$, $C_{31:1}$, $C_{32:0\text{-}32:4}$, $C_{33:1}$, $C_{34:1\text{-}34:2}$, $C_{34:4\text{-}34:5}$, $C_{35:1\text{-}35:2}$, $C_{35:5}$, $C_{36:2}$, $C_{36:5\text{-}36:6}$, $C_{37:2}$, $C_{37:4\text{-}37:6}$, $C_{38:2}$, $C_{38:4\text{-}38:6}$, $C_{39:5\text{-}39:6}$, $C_{40:6}$	$C_{31:8}$, $C_{32:4}$, $C_{33:8\text{-}33:10}$, $C_{34:1}$, $C_{34:4\text{-}34:5}$, $C_{35:1}$, $C_{35:4\text{-}35:5}$, $C_{36:6\text{-}36:7}$, $C_{37:5\text{-}37:6}$, $C_{37:9}$, $C_{37:12}$, $C_{38:5\text{-}38:6}$, $C_{38:9}$, $C_{39:0}$, $C_{39:2\text{-}39:3}$, $C_{39:11}$, $C_{40:5\text{-}40:6}$, $C_{40:8}$, $C_{41:0\text{-}41:4}$, $C_{42:6}$
PE[b]	$C_{32:1}$, $C_{34:1\text{-}34:2}$	ND[c]
SQDG	$C_{14:0}$, $C_{16:0\text{-}16:2}$, $C_{18:0\text{-}18:3}$	$C_{16:0\text{-}16:3}$, $C_{20:4\text{-}20:5}$
DGTA	$C_{14:0}$, $C_{16:0\text{-}16:1}$, $C_{18:0\text{-}18:2}$, $C_{19:0\text{-}19:1}$, $C_{20:5}$	$C_{14:0\text{-}14:1}$, $C_{16:0\text{-}16:1}$, $C_{17:0}$, $C_{18:1\text{-}18:2}$, $C_{20:1}$

[a] For structures and abbreviations of IPLs, see Fig. 1 and 2.
[b] Combined total number of carbon atoms and total amount of double bond equivalents for both fatty acid moieties.
[c] ND, not detected.

The distributions of the IPL classes over the different chromatographic fractions for the Mokbaai sediment extract were similar to those observed for the microbial mat (Table 2). The majority (61% ± 5%) of the MGDGs eluted in the acetone fraction (versus 39% ± 5% in the methanol fraction). The DGDGs eluted nearly completely (96% ± 5%) in the methanol fraction, with the remainder (4% ± 5%) eluting in the acetone fraction. The PCs and PGs eluted in the methanol fraction, with a small percentage of the PGs (9% ± 2%) eluting in the acetone fraction. For the SQDGs, 67% ± 3% eluted in the methanol fraction and 33% ± 3% in the acetone fraction. All DGTAs were found in the methanol fraction (Table 2). For recovery, results similar to those for the microbial mat sediment were found. For example, 90% to 230% of the SQDGs, MGDGs, and the DGTAs were recovered after separation, while about 20% of the PGs and PCs could not be recovered. The lowest recovery was for the DGDGs, at 55% (Fig. 3B).

Our results show that, independent of sample type, a large part of the phospholipids elute in the "phospholipid" fraction as expected. However, crucially, a major part of the glycolipids also elute in this fraction and not, as anticipated, in the glycolipid fraction. Furthermore, "nonphospholipids" such as SQDGs and DGTA also elute in the "phospholipid" fraction, while SQDGs are also found in the "glycolipid" fraction. Both lipid classes contribute to the membrane lipid pool, especially under phosphate limitation (28, 29), and therefore have to be considered when looking at membrane lipids as biomarkers for living biomass. Neither the "glycolipid" nor the "phospholipid" fraction thus consists of glyco- or phospholipids only. These results indicate that using this common separation method, a complete separation between glycolipid and phospholipid cannot be obtained and that the "PLFAs" reported are not derived exclusively from phospholipids but also are derived from glycolipids, betaine lipids, and SQDGs. Therefore, we experimented with a slightly modified elution scheme in an attempt to optimize separation.

TABLE 2 Elution of different IPL classes after separation of BDE on a silica column

Lipid class[a]	% (mean ± SD) Microbial mat standard separation: Acetone fraction	MeOH fraction	Marine sediment standard separation: Acetone fraction	MeOH fraction	Microbial mat modified separation: Acetone fraction	MeOH fraction
MGDG	54 ± 5	46 ± 5	61 ± 5	39 ± 5	65 ± 1	35 ± 1
DGDG	0 ± 0	100 ± 0	4 ± 5	96 ± 5	0 ± 0	100 ± 0
PG	9 ± 1	91 ± 1	9 ± 2	91 ± 2	6 ± 1	94 ± 1
PC	0 ± 0	100 ± 0	0 ± 0	100 ± 0	0 ± 0	100 ± 0
PE	11 ± 1	89 ± 1	ND[b]	ND	18 ± 4	82 ± 4
SQDG	32 ± 1	68 ± 1	33 ± 3	67 ± 3	60 ± 8	40 ± 8
DGTA	0 ± 0	100 ± 0	0 ± 0	100 ± 0	1 ± 2	99 ± 2

[a] For structures and abbreviations of IPLs, see Fig. 1 and 2.
[b] ND, not detected.

Adaptation of the separation method. To assess the possibility of obtaining better separation between glyco- and phospholipids, we used a mixture of acetone and methanol (99:1, vol/vol) as a second eluent. This slightly more polar solvent should result in a slightly different separation, with possibly more of the glycolipids, especially MGDGs, ending up in the second fraction. This modified elution scheme was tested on the microbial mat extract. Indeed, slight differences in the separation of the different lipid classes were observed. For example, the major part of the MGDGs now ended up in the acetone-methanol fraction and 35% ± 1% in the methanol fraction (Table 2). The separation of DGDGs was the same as that in the standard method, with all lipids eluting in the methanol fraction. As in the standard method, all PCs eluted in the methanol fraction, with slightly less PG (6% ± 1% compared to 9% ± 1%) but slightly more PE (18% ± 4% compared to 11% ± 1%) in the acetone-methanol fraction. The largest difference was observed for the SQDGs, with twice as much being found in the acetone-methanol fraction as in the original acetone fraction (60% ± 8% versus 32% ± 1%). Concerning the DGTAs, 99% ± 2% was still found in the methanol fraction (Table 2). Compared to those in the standard separation, recoveries for DGDGs, PCs, PEs, and PGs were similar, but for MGDGs and SQDGs, the recoveries were increased to 153% to 184% and to 75% to 120%, respectively. The apparently high recovery of SQDG could again be due to ion suppression and therefore a suboptimal ionization of the SQDGs in the complex total BDE, similar to what is observed for the MGDGs. In contrast, the recovery of the DGTAs had decreased by 40% (Fig. 3C).

These results show that an increase in the polarity of the second eluent changes the elution patterns of some of the IPL classes but does not result in an improved separation of glyco- and phospholipids. Further adaptations of the solvent mixture ratios are unlikely to succeed in providing a better separation, as either more of

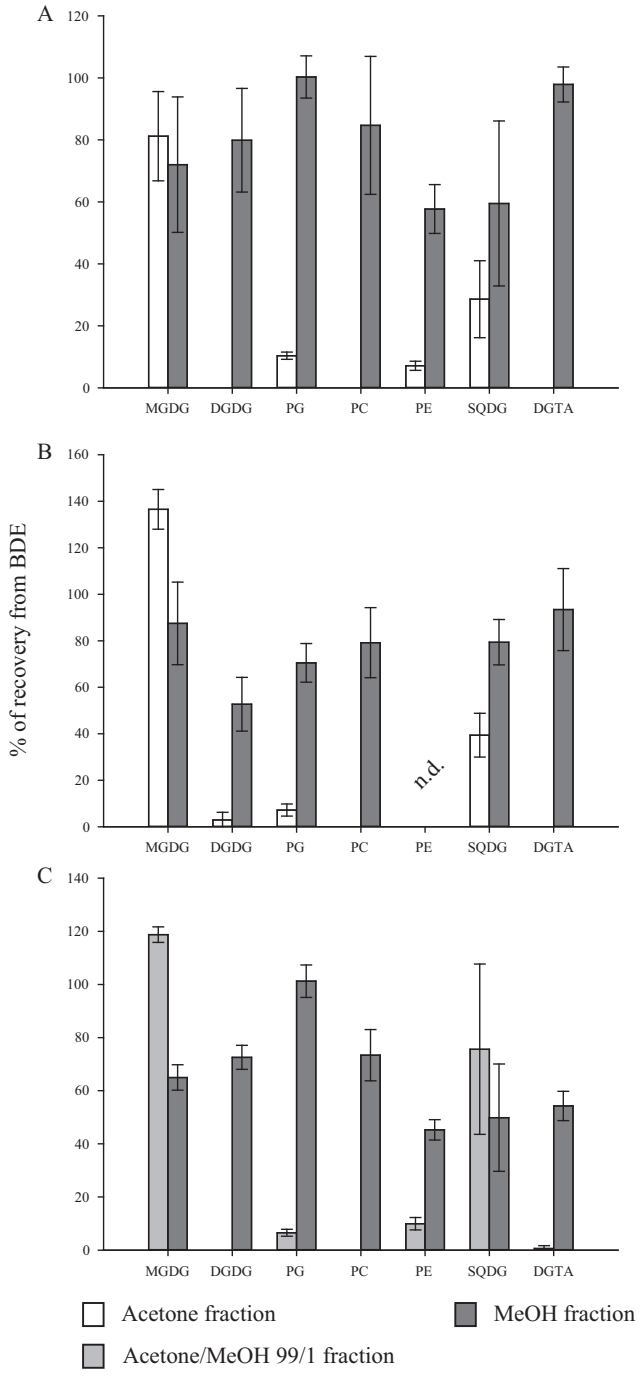

FIG 3 Recovery of different IPL classes after separation on a silica column for the Schiermonnikoog microbial mat (A and C) and the Mokbaai sediment (B), normalized to the abundance in the original extract. For structures of IPLs and definitions of abbreviations, see Fig. 2 and the legend to Fig. 1. n.d., not detected.

the glycolipids will end up in the phospholipid fraction or phospholipids will elute in the glycolipid fraction. This is due to the fact that the separation on a silica column is based on the polarity of the head groups. Therefore, MGDGs will always elute together with PCs and SQDGs.

Implications. Our results show that it is not possible to obtain a complete separation between phospho- and glycolipids using the commonly used silica acid chromatography separation method. Furthermore, the recovery varies between different IPL classes, with some of the phospholipids (e.g., PE) not fully recovered in the "phospholipid" fraction. Thus, previous studies examining PLFAs might have analyzed fractions that also contained fatty acids derived from glycolipids, betaine lipids, and to some extent SQDGs, while missing a certain amount of phospholipids and SQDGs. In the marine environment, different IPLs can contain different fatty acids (25–27, 35), and specific fatty acids do not necessarily always derive from the same lipid class. This will give a biased view of fatty acids present in microbial communities *in situ*. Furthermore, PLFA analysis of microbial isolates will also give an incomplete picture of the full diversity of fatty acids derived from IPLs. Therefore, microbial fingerprints based on PLFA fractions are unlikely to reflect the true fatty acid pattern coming from phospholipids only. Future studies using PLFA fingerprints of environmental samples and microbial biomass have to at least be aware of this less-than-perfect separation and, preferably, combine this with direct analysis of IPLs. Alternatively, one can just separate a neutral and a polar fraction (using DCM and methanol), with the latter containing the full suite of intact polar lipids.

Conclusion. Silica column chromatography results in incomplete separation of glyco- and phospholipids, leading to the presence of "nonphospholipids" in the PLFA fraction. Therefore, studies of microbial activity using stable-isotope analysis of PLFAs may be biased by fatty acids derived from glycolipids, SQDGs, and betaine lipids. Because of the imperfect separation and the fact that other IPL classes such as SQDGs and betaines (and even MGDGs and DGDGs) are also part of the membrane lipids of living and active microorganisms, a separation in only two fractions, a neutral fraction containing free fatty acids of dead biomass and a polar fraction containing all intact polar lipids, is preferable. The "polar lipid"-derived fatty acids can then be used to study microbial communities and their activity.

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