1 Improved sensitivity of sedimentary phospholipid analysis
2 resulting from a novel extract cleanup strategy

3 Rong Zhu^{a,*}, Thomas Williams Evans^a, Lars Wörmer^a, Yu-Shih Lin^{a,b}, Chun Zhu^a,
4 Kai-Uwe Hinrichs^a

5 ^a *Organic Geochemistry Group, MARUM Center for Marine Environmental Sciences*
6 *& Department of Geosciences, University of Bremen, Leobener Str., 28359 Bremen,*
7 *Germany*

8 ^b *Department of Oceanography, National Sun Yat-Sen University, No. 70 Lienhai Rd,*
9 *Kaohsiung 804, Taiwan*

10 * Corresponding author. *Email address:* rongzhu@uni-bremen.de (Rong Zhu).

11

12 ABSTRACT

13 The detection of intact phospholipids (PLs) in sedimentary extracts using liquid
14 chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) is
15 commonly limited by ion suppression caused by complex matrices; the effect
16 typically cannot be satisfactorily reduced using traditional column chromatographic
17 cleanup procedures. We therefore implemented a cleanup strategy recently introduced
18 for serum and plasma samples and adapted it for sedimentary lipid extracts. It
19 involves selective retention of PLs on a zirconia-coated silica phase, available as a
20 HybridSPE[®]-Phospholipid cartridge. Compared with conventional silica gel liquid
21 chromatography, the HybridSPE-based protocol strongly enhances the detection of
22 PLs using LC-ESI-MS as a result of more effective removal of complex
23 environmental matrices, with negligible PL loss. The strength of the protocol was

demonstrated by analysis of marine sediment samples from various depths and of different total organic carbon (TOC) content, for which we observed 1.2-2.7 times more intense signals from sedimentary PLs compared with the untreated reference. This new cleanup protocol significantly improves PL fingerprinting of microbial communities in sediments.

1. Introduction

Phospholipids (PLs) consist of phosphate-based head group(s), glycerol backbone(s) and hydrophobic chain(s) that are linked to the glyceryl moiety via ester or ether bonds. They are principal constituents of microbial cytoplasmic membranes. As they bear chemotaxonomic information (Fang et al., 2000; Zink et al., 2003; Sturt et al., 2004; Koga and Morii, 2005) and decompose rapidly upon cell lysis (White et al., 1979; Harvey et al., 1986; Logemann et al., 2011), PLs have been widely employed as a culture- and gene-independent approach for decoding microbial community composition and total biomass (e.g. Bobbie and White, 1980; Vestal and White, 1989; Zelles et al., 1992; Sundh et al., 1997; Lipp et al., 2008; Peterse et al., 2011).

PLs are conventionally analyzed using gas chromatography-mass spectrometry (GC-MS) through characterizing their ester-linked fatty acid (FA) derivatives after hydrolytic cleavage of the polar head groups and ester linkages between acyl groups and the glycerol backbones (e.g. White et al., 1979; Delong and Yayanos, 1986; Guckert et al., 1986; Frostegård et al., 1993; Elvert et al., 2003; Mills et al., 2006). However, this GC-based approach requires a PL fraction purified from free and matrix-bound FAs, a precondition that needs critical evaluation case by case. With

48 liquid chromatography-electrospray ionization-MS (LC-ESI-MS), intact PLs are
49 directly determined without breakdown of the compounds (e.g. Fang and Barcelona,
50 1998; Rütters et al., 2002; Sturt et al., 2004), thereby providing more comprehensive
51 information for deciphering signals from microbial communities in environmental
52 samples. Unfortunately, LC-ESI-MS-based PL analysis suffers significant
53 interference from the co-eluting sample matrix, which reduces PL ionization
54 efficiency and consequently detection (Wörmer et al., 2013). Indeed, few studies have
55 successfully identified intact PLs in deeply buried marine subseafloor sediments (e.g.
56 Zink et al., 2003; Fredricks and Hinrichs, 2007), where the reduced biomass density
57 creates low signal/high matrix conditions unfavorable for PL detection.

58 Silica gel columns have been widely used for fractionating samples and
59 reducing sample matrix for the analysis of intact polar lipids (IPLs) in environmental
60 samples (e.g. Rütters et al., 2002; Zink and Mangelsdorf, 2004; Pitcher et al., 2009;
61 Schubotz et al., 2009; Liu et al., 2011; Rossel et al., 2011). However, a substantial
62 loss of PLs (up to 80%) has been observed after elution from a silica gel column,
63 presumably because phosphatidic head groups are selectively adsorbed to the silica
64 (Lengger et al., 2012). Thus, it is critical to develop a reliable PL cleanup protocol for
65 effective removal of the sedimentary matrix with minimum PL loss.

66 Studies using HybridSPE[®]-Phospholipid cartridges have recently demonstrated
67 successful isolation of PLs from biological samples, such as serum and plasma (Pucci
68 et al., 2009; Jiang et al., 2011; Moriarty et al., 2012). The solid phase's silica gel in
69 the cartridges is coated with zirconia and exhibits high affinity for PLs due to the
70 Lewis acid-base interaction between zirconia and phosphate moieties, while the
71 retention of a wide range of basic, neutral and acidic compounds is minimal.
72 Subsequently, the retained PLs can be recovered with a strong basic solution. We

have adapted this approach for applications with environmental samples and compared results with those obtained with conventional silica gel chromatography. The results demonstrate better recovery and greatly improved detectability of PLs in various sediments using the approach.

2. Material and methods

2.1. Material

In order to examine any possible cleanup discrimination among different PLs, we investigated the performance of the HybridSPE[®]-Phospholipid cartridge for diverse PLs with (i) different polar head groups (e.g. phosphocholine, PC; phosphoethanolamine, PE; phosphatidylglycerol, PG; phosphoserine, PS), (ii) varying side chain length (e.g. C₁₆, C₁₈, C₂₁) and (iii) varying core structure type (e.g. diacylglycerol, DAG; dietherglycerol, DEG; archaeol, AR). PL standards were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL, USA), Matreya LLC (Pleasant Gap, PA, USA) and Sigma-Aldrich (Munich, Germany). Isopropanol (IPA), dichloromethane (DCM), HCO₂H (98%), NH₄OH (> 25%), and NH₄CO₂H were from Sigma-Aldrich. The HybridSPE[®]-Phospholipid cartridges (500 mg; 6 ml) and the CHROMABOND[®] SiOH glass columns (500 mg; 6 ml) were from Supelco Chemical Co. (Munich, Germany) and Macherey-Nagel GmbH (Düren, Germany), respectively. The vacuum manifold was from Carl Roth GmbH (Karlsruhe, Germany). Sediment samples from different burial depth, ranging from surface (0-1 cm) to subsurface (113.60 m below seafloor, mbsf), and of different total organic carbon (TOC) content (0.3 to 5.5%) were used for method development (listed in Table 1).

97 2.2 *Standard mixture*

98 A PL standard mixture with equal amounts of di-C₁₆-PE, PE-AR, di-C₁₆-PC,
99 di-C₁₈-PC, di-C₂₁-PC, di-O-C₁₆-PC, PC-AR, di-C₁₆-PS and di-C₁₆-PG was prepared
100 for method development. To evaluate recovery after the cleanup protocols, 300 ng of
101 each PL was split into three aliquots. One was directly analyzed as the reference using
102 LC-ESI-MS, whereas the other two were processed with the HybridSPE cartridge or
103 silica gel column before analysis. The amount injected for LC-ESI-MS was 10 ng of
104 each PL for the reference or a volume fraction equivalent to 10 ng of originally added
105 PL in the treated standard mixture. To obtain reliable results, each treatment was
106 carried out in triplicate. The recovery of PLs was determined by normalizing the peak
107 area of the treated samples against the reference (as % peak area of untreated sample).

108

109 2.3. *Sediment samples*

110 Sediment samples (27.4-44.9 g wet wt.; Table 1) were extracted using a
111 modified Bligh and Dyer method described by Sturt et al. (2004). Briefly, each
112 sample was dispersed in a mixture of DCM/MeOH/phosphate buffer (pH 7.4; 1:2:0.8,
113 v/v/v) and ultrasonically extracted twice for 10 min, followed by duplicate extraction
114 with DCM/MeOH/CCl₃CO₂H (pH 2; 1:2:0.8, v/v/v). The mixture was centrifuged
115 after each extraction step and the supernatants were combined in a separation funnel
116 for phase separation. Combined with the DCM used for extraction of the aqueous
117 phase, the organic phase was further washed with MilliQ water 3x and evaporated to
118 dryness as the total lipid extract (TLE). The TLE was treated as follows with each
119 treatment being carried out in triplicate.

(i) To compare PL recovery over the two cleanup protocols in the presence of sedimentary matrix, the TLE of Sample #1 (Table 1) was spiked with the PL standard mixture containing 300 ng of each PL and subjected to the same procedure outlined in Fig. 1a. The recovery of PLs was determined by normalizing the peak area of the treated samples against the reference.

(ii) TLEs of Samples #2 and #3 were selected as sedimentary background to examine the efficiency of the HybridSPE protocol for recovering different concentrations of PLs and/or in different types of matrices. The TLEs were split into three aliquots. Each was spiked with a different amount of PL standard mixture (0.4, 4 and 40 µg), respectively, and split into two sub-aliquots for direct LC-ESI-MS measurements or analysis after the HybridSPE cartridge treatment (Fig. 1b). The mixture of PL standards was prepared with equal amounts of di-C₁₆-PE, PE-AR, di-C₁₈-PC, di-C₂₁-PC, di-O-C₁₆-PC and PC-AR. Since the experiment also aimed to compare quantification of PLs via both GC (as PLFAs) and LC analysis, di-C₁₆-PC, di-C₁₆-PS and di-C₁₆-PS, which contain the same FAs in the core structure as di-C₁₆-PE, were excluded from this standard mixture. Due to analytical difficulty related to low hydrolysis yield (i.e. independent of the procedure introduced here), PLFA data are not reported here. A proportion (1%) of each sub-aliquot (equivalent to 0.2, 2 and 20 ng of each PL, respectively) was used for LC-ESI-MS.

(iii) TLEs of Samples #4 and #5 were selected as study cases to demonstrate the capability of the HybridSPE protocol for detection of indigenous sedimentary PLs. Each TLE was split into two aliquots for direct LC-ESI-MS measurements as reference and cleanup with the HybridSPE cartridge prior to analysis.

For analysis of TOC, ca. 0.5 g of each sediment sample was decalcified with 10%

HCl, washed with MilliQ water and freeze-dried. Aliquots were weighed and measured with a Delta V elemental analyzer (Thermo Finnigan MAT GmbH, Bremen, Germany).

2.4. Sample fractionation

The HybridSPE cartridge was rinsed 2x with 5 ml MeOH prior to use. Samples were dissolved in ca. 500 μ l MeOH:HCO₂H (98%; 99:1, v/v), loaded onto the cartridge and eluted under vacuum. The cartridge was thereafter washed with 3 ml MeOH containing 1% NH₄CO₂H (wt.%) followed by 3 ml MeOH, both of which were collected and combined as the first fraction (F1). PLs were then eluted from the cartridge using three different eluents including 6 ml IPA containing 10% of NH₄OH solution (wt.%), followed by 6 ml IPA containing 15% of NH₄OH solution (wt.%) and finally 6 ml DCM:IPA:NH₄OH (5:9:6, v/v/v) under vacuum. All three eluents were collected and combined as the second fraction (F2, the PL fraction).

For comparison, the silica gel columns were also employed to fractionate TLEs using the method described by Mills et al. (2006) after slight modification. In brief, the columns were pre-conditioned with 10 ml DCM. Non-PL fractions were eluted with 10 ml DCM followed by 10 ml acetone, while PLs were subsequently collected with 10 ml MeOH. All fractions were evaporated to dryness under a stream of N₂ in a water bath at 37 °C and stored at -20 °C until analysis.

2.5. Instrumentation

The LC-ESI-MS analysis was performed with a Dionex Ultimate 3000 ultra-high performance LC instrument coupled to a Bruker maXis ultra high

resolution orthogonal acceleration quadrupole time-of-flight mass spectrometer, equipped with an ESI source and operated in positive mode (Bruker Daltonik, Bremen, Germany). Separation of PLs was achieved with a Waters Acquity UPLC BEH amide column (2.1×150 mm, $1.7 \mu\text{m}$; Waters Corporation, Eschborn, Germany) based on Wörmer et al. (2013). To investigate the compatibility of the cleanup protocol for different LC methods, a Waters Acquity BEH C_{18} column (2.1×150 mm, $3 \mu\text{m}$; Waters Corporation, Eschborn, Germany) was additionally employed (Wörmer et al., 2013). Samples were either dissolved in DCM/MeOH 9:1 for the former column or DCM/MeOH 1:9 for the latter. Compounds were assigned and integrated through Data Analysis 4.0 software (Bruker Daltonik). Compound assignment was based on retention time, accurate mass (< 1 ppm) and diagnostic fragments (Sturt et al., 2004; Schubotz et al., 2009; Rossel et al., 2011). The extraction window of individual ion chromatograms was ± 0.01 m/z units. All samples were analyzed in duplicate with LC-ESI-MS.

3. Results and discussion

3.1. Recovery of PLs after cleanup

PLs were exclusively detected in F2, indicating the high affinity between zirconia-coated sorbents and phosphate-based head groups. The strength of the Lewis acid-base interaction was influenced by head group and side chain length; therefore, three eluents were applied to achieve maximum PL recovery. Our results showed that PL standards with different head groups and side chains were all well retrieved (78-110%), with an average recovery of 98% compared with the signals from untreated standards (Fig. 2). Insignificant discrimination among PLs of different types

was found through the paired *t*-test (P 0.002; statistical analysis using SPSS 20). The ester-bound lipid di-C₁₆-PG showed the lowest recovery (78%), suggesting that increased number of OH groups in the PG head group strengthened the retention of the PL on the HybridSPE cartridge. For comparison, the recovery of PL standards after the silica gel column ranged from 17% to 83% (avg. $63 \pm 20\%$), i.e. substantially and systematically lower than that from the HybridSPE cartridge (*t*-test, P 0.950; Fig. 2). Such low recovery of PLs from the silica gel columns is consistent with the findings of Lengger et al. (2012).

We further examined the performance of the HybridSPE using the PL-spiked TLE from a surface sediment sample from the Wadden Sea (Sample #1). The results showed that signals from the spiked standards (Fig. 3a) and sedimentary PLs (Fig. 3b) were significantly improved in all cases after the HybridSPE cartridge, with a yield of 126-463% (avg. 240%) and 142-268% (avg. 205%), respectively, compared with the untreated aliquot. Noticeably, the greatest signal enhancement was for di-O-C₁₆-PC, PC-AR and PE-AR, followed by di-C₁₆-PC, di-C₁₈-PC and di-C₁₆-PE, whereas di-C₁₆-PS and di-C₁₆-PG showed only minor enhancement (Fig. 3a). The difference in signal enhancement for individual PL standards between pure standards (Fig. 2) and standards in a TLE (Fig. 3a) is tentatively attributed to the varied efficiency of the HybridSPE cartridge in removing sedimentary matrices that co-elute with specific PLs during LC-ESI-MS analysis. The yield of indigenous sedimentary PLs approximated that of spiked PL standards with the same head groups except for C_{32:1}-PE (Fig. 3b), indicating no discrimination between spiked and sedimentary PLs.

Except for di-C₂₁-PC, the yield of spiked standards and sedimentary PLs ranged from 78% to 277% (avg. 162%) and 39% to 172% (avg. 101%), respectively, after fractionation with the silica gel column. The yield of PG-DAGs, e.g. di-C₁₆-PG,

C_{31:1}-PG and C_{34:4}-PG, was similar to the yield with the HybridSPE cartridge, while others showed significantly lower recovery than the HybridSPE-PL fraction (Fig. 3). Unlike other PC-bearing lipids, di-C₂₁-PC displayed an unexpectedly high yield after the silica gel column, with the underlying mechanism remaining unclear. Taking di-C₂₁-PC as an exception, we found that the difference in PL recovery between two cleanup protocols was related to head group, with PE showing the greatest discrepancy. Moreover, when evaluated with average recovery, the HybridSPE protocol was a more effective method than silica gel chromatography, particularly in the presence of sedimentary matrices (cf. Figs. 2 and 3).

3.2. Application of the HybridSPE cartridge to marine sediments

In order to assess the impact of different PL abundances and matrices on cleanup efficiency with the HybridSPE protocol, sediment samples from two sites (Sample #2 and #3) were extracted and spiked with different amounts of PL standards as described above. Sample #2 from the Peru Margin represents a marine organic-rich sediment (5.5% TOC), whereas the sapropelic layers from the Eastern Mediterranean Sea (Sample #3) offer a moderately elevated TOC content (1.5%; Table 1). In accordance with the results described above, the signals of all PL standards were consistently increased after cleanup with the HybridSPE cartridge at all concentration levels (Fig. 4). Greater enhancement was observed for Sample #3 than Sample #2, with yields of 151-266% relative to the untreated TLEs for the former and 110-189% for the latter. Moreover, with 0.2 ng of each spiked PL standard, some PLs were detectable only after cleanup (e.g. di-C₁₆-PE, PE-AR; Fig. 4), demonstrating the improved detectability of PLs after the HybridSPE treatment.

In view of significantly enhanced signals of spiked PLs, we additionally applied the HybridSPE protocol to two organic-rich samples (Sample #4, 4.0% TOC and Sample #5, 4.4% TOC; Table 1) for detection of indigenous PLs. The majority of organic compounds were not retained on the cartridge in both cases, resulting in a relatively matrix-free PL fraction that could be concentrated to a desired level for trace PL analysis. We noted that glycolipids (GLs) were barely detectable in the HybridSPE-PL fraction, suggesting that the HybridSPE protocol can successfully separate PLs from GLs. However, we observed that relatively polar GLs (e.g., diglycosidic compounds) were partly retained on the HybridSPE sorbent (data not shown) and therefore recommend that a dedicated glycolipid analysis should be performed with another aliquot of the TLE. In Sample #4, signals of DEGs with PC, PE and PME head groups in the purified PL fraction were 124-135% compared to the untreated TLE (Fig. 5a). Likewise, signal enhancement of up to 250% relative to the TLE for PE-DEG and PME-DEG was found for Sample #5 (Fig. 5b). Different degrees of improvement after the proposed cleanup protocol may result from various sample matrices that co-eluted with specific PLs. Nevertheless, significantly enhanced PL signals in all tested samples suggest that the HybridSPE cartridge is highly advantageous for cleanup of a wide range of sediment samples.

Notably, the high sensitivity resulting from the HybridSPE treatment was also associated with the observation of a greater diversity of PLs. Trace PLs, which were not detectable in the reference aliquot, could be identified after the HybridSPE protocol (e.g. Fig. 5; PL compounds labeled with *). In Sample #4, PC-DAGs and PC-AEGs were exclusively found in the HybridSPE-PL fraction (Fig. 5a). Together with trace PC-DEGs, PE-DEGs and PME-DEGs (e.g. PE-DEG C_{34:1}, PME-DEG C_{33:1}), they accounted for 14% of the total PLs identified in the untreated sample.

More remarkably, PE-DEGs and PME-DEGs, which were only identified in the HybridSPE-PL fraction, constituted 106% of the total PLs in Sample #5, i.e. an absolute amount exceeding the total PLs quantified in the untreated TLE. Such significant improvement in PL detection resulted in highly different lipid patterns after cleanup and should provide more accurate and comprehensive information on sedimentary PLs. It also suggested that previously published work might underestimate the population and diversity of sedimentary PLs.

3.3. HybridSPE protocol in combination with different LC-MS methods

The results presented above are based on measurements using a HILIC amide column, which separates PLs according to head group polarity (Wörmer et al., 2013). Reversed phase (RP) LC provides an alternative approach for lipid fingerprinting by ESI-MS via separation based on chain length and hydrophobicity (Wörmer et al., 2013; Zhu et al., 2013) and would potentially benefit from an effective cleanup protocol. We therefore also analyzed the PL-spiked Samples #2 and #3 using a Waters Acquity BEH C₁₈ column. The results showed that peak area of each PL in the post-cartridge fractions was improved by 3.3-7.6 and 7.6-16.4 times, respectively, compared to the untreated TLEs of Samples #2 and #3. Enhanced detection was also reflected in significantly improved signal/noise ratios for the spiked PL standards (Fig. 6). Noticeably, none of PL standards was detected in the non-treated samples when 0.2 ng of each PL was injected on column, which is 2-40 times higher than the reported detection limit of the LC method in matrix-free solvents (Wörmer et al., 2013). This indicates a greater sensitivity of the BEH C₁₈ column to matrix-related ion suppression than of the HILIC amide column. It also implies that the cleanup procedure is beneficial to RP-MS-based analysis of PLs at low concentration. In

addition, the PLs quantified in the F2 had up to ca. 90% of the intensity of authentic PL standards in pure solvent at the same concentration, suggesting that matrix effects were significantly reduced by the cartridge. In this regard, it is promising to combine the HybridSPE protocol with RP separation on LC-ESI-MS to more comprehensively analyze distributional patterns within a lipid class.

4. Conclusions

A cleanup protocol based on HybridSPE[®]-Phospholipid cartridge for diverse PLs was adapted to environmental samples prior to LC-ESI-MS analysis. The resulting new protocol is characterized by (i) strongly improved detectability of PLs, (ii) insignificant discrimination among various PL types, and (iii) negligible PL loss. Removal of extract matrix by the HybridSPE cartridge clearly enhances PL ionization, resulting in significantly lowered detection limits of PLs compared to the untreated aliquots. With sediment samples, some indigenous trace PLs were only detectable after cleanup with the HybridSPE cartridge, providing additional clues for tracking the microbial community. With these advantages, the method offers more accurate and comprehensive PL fingerprints for the study of microbial PLs in marine sediments.

Acknowledgements

Samples used in this research were taken during two field trips to the estuary of White Oak River Basin (North Carolina, USA) and a tidal flat of Wadden Sea (North Sea, Germany), respectively, and during RV Meteor cruise M84/1 (DARCSEAS), and Leg 201 of the Ocean Drilling Program (ODP), which is sponsored by the US

316 National Science Foundation and participating countries under management of Joint
317 Oceanographic Institutions (JOI), Inc. We thank the participating scientists and ship
318 crews for sample recovery; J. Lipp for technical and scientific guidance, and two
319 anonymous reviewers for constructive comments. The study was supported by the
320 Deutsche Forschungsgemeinschaft through Grant Inst 144/300-1 (LC-qTOF system)
321 and the Gottfried Wilhelm Leibniz Price awarded to K.-U.H., and by the European
322 Research Council under the European Union's Seventh Framework Programme -
323 “Ideas” Specific Programme, ERC Grant Agreement No. 247153 (to K.-U.H.). R.Z. is
324 sponsored by the Chinese scholarship Council (CSC) and University of Bremen.

325

326 **References**

- 327 Bobbie, R., White, D.C., 1980. Characterization of benthic microbial community
328 structure by High-Resolution Gas Chromatography of fatty acid methyl esters.
329 Applied and Environmental Microbiology 39, 1212-1222.
- 330 DeLong, E.F., Yayanos, A.A., 1986. Biochemical function and ecological
331 significance of novel bacterial lipids in deep-sea procaryotes. Applied and
332 Environmental Microbiology 51, 730-737.
- 333 D'Hondt, S.L., Jørgensen, B.B., Miller, D.J. and Shipboard Scientific Party, 2003.
334 Proceedings of the Ocean Drilling Program, Initial Reports, vol. 201. Ocean
335 Drilling Program, Texas A & M University, College Station, TX.
336 doi:10.2973/odp.proc.ir.201.2003.
- 337 Elvert, M., Boetius, A., Knittel, K., Jørgensen, B.B., 2003. Characterization of
338 specific membrane fatty acids as chemotaxonomic markers for sulfate-reducing
339 bacteria involved in anaerobic oxidation of methane. Geomicrobiology Journal
340 20, 403-419.
- 341 Fang, J., Barcelona, M.J., 1998. Structural determination and quantitative analysis of
342 bacterial phospholipids using liquid chromatography/electrospray
343 ionization/mass spectrometry. Journal of Microbiological Methods 33, 23-35.
- 344 Fang, J., Barcelona, M.J., Semrau, J.D., 2000. Characterization of methanotrophic
345 bacteria on the basis of intact phospholipid profiles. FEMS Microbiology Letters
346 189, 67-72.
- 347 Fredricks, H.F., Hinrichs, K.-U., 2007. Data report: intact membrane lipids as
348 indicators of subsurface life in cretaceous and paleogene sediments from sites
349 1257 and 1258. Proceedings of the Ocean Drilling Program, Science Research
350 207. doi:10.2973/odp.proc.sr.207.112.2007
- 351 Frostegård, Å., Tunlid, A., Bååth, E., 1993. Phospholipid fatty acid composition,
352 biomass, and activity of microbial communities from two soil types
353 experimentally exposed to different heavy metals. Applied and Environmental
354 Microbiology 59, 3605-3617.
- 355 Guckert, J.B., Hood, M.A., White, D.C., 1986. Phospholipid ester-linked fatty acid
356 profile changes during nutrient deprivation of *Vibrio cholerae*: Increases in the
357 *trans/cis* ratio and proportions of cyclopropyl fatty acids. Applied and
358 Environmental Microbiology 52, 794-801.
- 359 Harvey, H.R., Fallon, R.D., Patton, J.S., 1986. The effect of organic matter and
360 oxygen on the degradation of bacterial membrane lipids in marine sediments.
361 Geochimica et Cosmochimica Acta 50, 795-804.
- 362 Jiang, H., Zhang, Y., Ida, M., LaFayette, A., Fast, D.M., 2011. Determination of
363 carboplatin in human plasma using HybridSPE-precipitation along with liquid
364 chromatography-tandem mass spectrometry. Journal of Chromatography B 879,
365 2162-2170.
- 366 Koga, Y., Morii, H., 2005. Recent advances in structural research on ether lipids from
367 archaea including comparative and physiological aspects. Bioscience,
368 Biotechnology, and Biochemistry 69, 2019-2034.

369 Lengger, S.K., Hopmans, E.C., Sinninghe Damsté, J.S., Schouten, S., 2012.
370 Comparison of extraction and work up techniques for analysis of core and intact
371 polar tetraether lipids from sedimentary environments. *Organic Geochemistry* 47,
372 34-40.

373 Lipp, J.S., Morono, Y., Inagaki, F., Hinrichs, K.-U., 2008. Significant contribution of
374 Archaea to extant biomass in marine subsurface sediments. *Nature* 454, 991-
375 994.

376 Liu, X., Lipp, J.S., Hinrichs, K.-U., 2011. Distribution of intact and core GDGTs in
377 marine sediments. *Organic Geochemistry* 42, 368-375.

378 Logemann, J., Graue, J., Köster, J., Engelen, B., Rullkötter, J., Cypionka, H., 2011. A
379 laboratory experiment of intact polar lipid degradation in sandy sediments.
380 *Biogeosciences* 8, 2547-2560.

381 Mills, C.T., Dias, R.F., Graham, D., Mandernack, K.W., 2006. Determination of
382 phospholipid fatty acid structures and stable carbon isotope compositions of
383 deep-sea sediments of the Northwest Pacific, ODP site 1179. *Marine Chemistry*
384 98, 197-209.

385 Moriarty, M., Lee, A., O'Connell, B., Lehane, M., Keeley, H., Furey, A., 2012. The
386 application and validation of HybridSPE-Precipitation cartridge technology for
387 the rapid clean-up of serum matrices (from phospholipids) for the clinical
388 analysis of serotonin, dopamine and melatonin. *Chromatographia* 75, 1257-1269.

389 Peterse, F., Hopmans, E.C., Schouten, S., Mets, A., Rijpstra, I.C., Sinninghe Damsté,
390 J.S., 2011. Identification and distribution of intact polar branched tetraether
391 lipids in peat and soil. *Organic Geochemistry* 42, 1007-1015.

392 Pitcher A., Hopmans, E.C., Schouten S., Sinninghe Damsté, J.S., 2009. Separation of
393 core and intact polar archaeal tetraether lipids using silica columns: Insights into
394 living and fossil biomass contributions. *Organic Geochemistry* 40, 12-19.

395 Pucci, V., Di Palma, S., Alfieri, A., Bonelli, F., Monteagudo, E., 2009. A novel
396 strategy for reducing phospholipids-based matrix effect in LC-ESI-MS
397 bioanalysis by means of HybridSPE. *Journal of Pharmaceutical and Biomedical*
398 *Analysis* 50, 867-871.

399 Rossel, P.E., Elvert, M., Ramette, A., Boetius, A., Hinrichs, K.-U., 2011. Factors
400 controlling the distribution of anaerobic methanotrophic communities in marine
401 environments: evidence from intact polar membrane lipids. *Geochimica et*
402 *Cosmochimica Acta* 75, 164-184.

403 Rütters, H., Sass, H., Cypionka, H., Rullkötter, J., 2002. Phospholipid analysis as a
404 tool to study complex microbial communities in marine sediments. *Journal of*
405 *Microbiological Methods* 48, 149-160.

406 Schubotz, F., Wakeham, S.G., Lipp, J.S., Fredricks, H.F., Hinrichs, K.-U., 2009.
407 Detection of microbial biomass by intact polar membrane lipid analysis in the
408 water column and surface sediments of the Black Sea. *Environmental*
409 *Microbiology* 11, 2720-2734.

410 Sturt, H.F., Summons, R.E., Smith, K., Elvert, M., Hinrichs, K.-U., 2004. Intact polar
411 membrane lipids in prokaryotes and sediments deciphered by high-performance
412 liquid chromatography/electrospray ionization multistage mass

413 spectrometry--new biomarkers for biogeochemistry and microbial ecology.
 414 Rapid Communications in Mass Spectrometry 18, 617-628.

415 Sundh, I., Nilsson, M., Borga, P., 1997. Variation in microbial community structure in
 416 two boreal peatlands as determined by analysis of phospholipid fatty acid
 417 profiles. Applied and Environmental Microbiology 63, 1476-1482.

418 Vestal, J.R., White, D.C., 1989. Lipid analysis in microbial ecology. Bioscience 39,
 419 535-541.

420 White, D., Davis, W., Nickels, J., King, J., Bobbie, R., 1979. Determination of the
 421 sedimentary microbial biomass by extractable lipid phosphate. Oecologia 40,
 422 51-62.

423 Wörmer, L., Lipp, J.S., Schröder, J.M., Hinrichs, K.-U., 2013. Application of two new
 424 LC-ESI-MS methods for improved detection of intact polar lipids (IPLs) in
 425 environmental samples. Organic Geochemistry 59, 10-21.

426 Xie, S., Lipp, J.S., Wegener, G., Ferdelman, T.G., Hinrichs, K.-U., 2013. Turnover of
 427 microbial lipids in the deep biosphere and growth of benthic archaeal
 428 populations. Proceedings of the National Academy of Sciences, U.S.A. 110,
 429 6010-6014.

430 Zelles, L., Bai, Q.Y., Beck, T., Beese, F., 1992. Signature fatty acids in phospholipids
 431 and lipopolysaccharides as indicators of microbial biomass and community
 432 structure in agricultural soils. Soil Biology and Biochemistry 24, 317-323.

433 Zhu, C., Lipp, J.S., Wörmer, L., Becjer, K.W., Schröder, J., Hinrichs, K.-U., 2003.
 434 Comprehensive glycerol ether lipid fingerprints through a novel reverse-phase
 435 liquid chromatography-mass spectrometry protocol. Organic Geochemistry, in
 436 press.

437 Zink, K.G., Wilkes, H., Disko, U., Elvert, M., Horsfield, B., 2003. Intact
 438 phospholipids-microbial "life markers" in marine deep subsurface sediments.
 439 Organic Geochemistry 34, 755-769.

440 Zink, K.G., Mangelsdorf, K., 2004. Efficient and rapid method for extraction of intact
 441 phospholipids from sediments combined with molecular structure elucidation
 442 using LC-ESI-MS-MS analysis. Analytical and Bioanalytical Chemistry 380,
 443 798-812.

444

445

446 Figure captions

447 **Fig. 1.** Schematic overview of procedures for (a) comparison of HybridSPE®-
448 Phospholipid cartridge and silica gel column for recovery of PLs with/without
449 sedimentary matrix and (b) evaluation of HybridSPE protocol for recovering
450 PLs at different concentrations and/or in different types of matrices. Each
451 treatment was carried out in triplicate with each of the resulting solutions
452 analyzed in duplicate using LC-ESI-MS.

453

454 **Fig. 2.** Recovery of authentic PL standards with the HybridSPE®- Phospholipid
455 cartridges (blue) and silica gel columns (yellow), analyzed using LC-ESI-MS
456 and separated with a HILIC amide column. Recovery was calculated by
457 normalizing the signals from the treated standards relative to the untreated
458 standards, indicated by the dashed green line as 100%. Error bars represent
459 standard error from triplicate cleanup experiments. Amount injected into
460 LC-ESI-MS was 10 ng of each PL. (PE, phosphoethanolamine; PC,
461 phosphocholine; PG, phosphatidylglycerol; PS, phosphoserine; AR, archaeol)

462

463 **Fig. 3.** Yield of (a) spiked PL standards (amount injected: 10 ng of each) and (b)
464 indigenous sedimentary PLs from a TLE of a surface sediment from the
465 Wadden Sea, Germany (Sample #1), after treatment with HybridSPE®-
466 Phospholipid cartridges (blue) and silica gel columns (yellow). PLs were
467 analyzed using LC-ESI-MS and separated with a HILIC amide column. Yield
468 was calculated by normalizing the signals from the treated samples relative to
469 the untreated samples, indicated by the dashed green line as 100%. Error bars
470 represent standard error from triplicate cleanup experiments. Sedimentary
471 PLs were indicated as total carbon number of both side chains followed by
472 unsaturation.

473

474 **Fig. 4.** Peak area of PL standards spiked in TLEs of (a) Sample #2 and (b) Sample #3
475 in different amount (equivalent to 0.2, 2, and 20 ng of each for LC-ESI-MS
476 analysis) before (labeled as TLE) and after (labeled as SPE-PL) cleanup with
477 the HybridSPE®-Phospholipid cartridges. Enhanced signals were normalized

to peak area of untreated samples and labeled as % above each HybridSPE-PL fraction. Concentration of spiked standards covered a typical range encountered during analysis of sediment samples. Error bars represent standard error from triplicate cleanup experiments and analyzed with a HILIC amide column on LC-ESI-MS. (bd, below detection limit).

Fig. 5. Distributions of PLs in marine sediments from (a) White Oak River Basin (Sample #4) and (b) Black Sea (Sample #5) before (labeled as TLE) and after (labeled as SPE-PL) cleanup with the HybridSPE protocol. Samples were separated with a HILIC amide column on LC-ESI-MS. Total PL yield determined from the TLE was defined as 100%, whilst PL yield from the HybridSPE-PL fraction are reported relative to TLE yield for comparison. Filled bars represent PLs identified in both aliquots, while diagonal bars represent PLs exclusively identified after cleanup with the HybridSPE cartridge. Error bars represent standard deviation from triplicate cleanup processes. (* PL detected only after cleanup)

Fig. 6. Composite mass chromatograms of PL standards spiked into marine sediment samples from (a) Peru Margin (Sample #2) and (b) Eastern Mediterranean Sea (Sample #3) before (orange) and after (blue) purification with the proposed method and separated with a Waters Acquity BEH C₁₈ column. Injection amount was equivalent to 20 ng of each PL standard. Selected ions were m/z 692.5225, 714.5044 (di-C₁₆-PE), 706.6109, 728.5928 (di-O-C₁₆-PC), 790.6320, 812.6140 (di-C₁₈-PC), 776.6892, 798.6711 (PE-AR) and 874.7259, 896.7079 (di-C₂₁-PC). Extraction window of the ion chromatograms was $\pm$ 0.01 m/z units.

Table 1

Sample details.

No.	Region	Station	Depth (mbsf ^a)	TOC (%)	Sample weight ^b (g)
1	Wadden Sea	53° 38' 0N, 8° 29' 30E ^c	Surface	0.3	44.9
2	Peru Margin	ODP Leg 201, Site 1227 ^d	8.05; 12.43; 17.15; 92.72; 113.60 ^e	5.5 ^f	27.4
3	Eastern Mediterranean Sea	GeoB 15103-2	3.73; 4.43; 5.59; 5.97 ^e	1.5 ^f	27.6
4	White Oak River Basin	WORB 2010-10	0.24	4.0	35.9
5	Black Sea	GeoB 15105-2	3.98	4.4	38.9

^a m below seafloor; ^b wet wt. ; ^c Xie et al., 2013; ^d D'Hondt et al., 2003; ^e mixed with equal amount of sediment from each depth; ^f value for mixture of samples from different depths.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6