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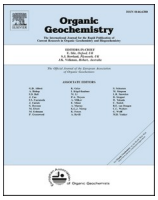

A new and improved protocol for extraction of intact polar membrane lipids from archaea

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ABSTRACT

Knowledge of intact polar membrane lipids (IPLs) is central to investigations of the physiology of archaea in cultures and their prevalence in environmental samples. IPLs are typically extracted using modifications to the 'Bligh and Dyer' protocol developed decades before the chemical complexity of IPLs was fully appreciated. Recent studies have exposed both bias and a low extraction efficiency for archaeal lipids obtained by this technique. Here, we tested different solvent mixtures, mechanical rupture techniques and chemical detergents/enzymatic lysis solutions for their potential to increase the extraction efficiency of archaeal IPLs. Experiments were based on an iterative approach using *Sulfolobus acidocaldarius* biomass as a model organism and incrementally evaluating single-step modifications. Direct acid hydrolysis of biomass was used as a benchmark to assess the recovery of the archaeal lipids. After this initial phase, the resultant protocol was tested on various cultured archaea and environmental samples. Tests with different solvent mixtures revealed the highest *S. acidocaldarius* lipid yields when samples were extracted with a monophasic solvent system that included a trichloroacetic acid buffer. Freeze-thaw cycles before extraction enhanced the extraction efficiency by 1.8 times, while other mechanical rupture techniques showed no increase. Cetrimonium bromide, a quaternary ammonium surfactant, used in combination with freeze–thaw cycles resulted in up to 8 times higher lipid yields in *S. acidocaldarius* and other archaeal test cultures compared to experiments without this pretreatment. Extractions of environmental samples revealed contrasting results: while sediments showed little to no increase in archaeal lipid yields, suspended particulate organic matter yielded elevated abundances compared to the Bligh and Dyer extraction protocol.

1. Introduction

Archaea occur ubiquitously in the marine water column (Karner et al., 2001; Schattenhofer et al., 2009), sediments (Parkes et al., 2014), and terrestrial environments (Timonen and Bomberg, 2009), where they play essential roles in biogeochemical cycling (Offre et al., 2013). A powerful tool to investigate the biomass, composition, and physiology of archaea is the investigation of their intact polar membrane lipids (IPLs). IPLs consist of a polar head group, a glycerol backbone, and a hydrophobic core lipid. Early studies have shown that the labile head group moiety is quickly hydrolyzed after cell death (White et al., 1979; Harvey et al., 1986). Therefore, it has been suggested that IPLs are biomarkers for viable microbial cells in marine sediments (e.g., Lipp et al., 2008; Liu et al., 2011; Evans et al., 2017; Lipsewers et al., 2018), suspended particulate material (SPM; e.g., Schubotz et al., 2018; Sollai et al., 2019; Besseling et al., 2020; Ma et al., 2020) and pure cultures (Sturt et al., 2004; Schouten et al., 2008; Yoshinaga et al., 2015; Elling et al., 2017; Baumann et al., 2018).

The cell envelope of archaea is a complex, multilayered structure that varies among taxa (Albers and Meyer, 2011). However, it commonly consists of a (glyco)protein layer and the lipid membrane bilayer. The protein layer, as well as the lipid membrane, help to protect the cell against lysis due to unfavorable environmental influences such as osmotic pressure or high temperature (Albers and Meyer, 2011). Different types of protein layers have been observed in archaea. Most archaea produce S-layer proteins (e.g., Sulfolobales, Thaumarchaeota; Albers and Meyer, 2011; Stieglmeier et al., 2014). However, other types have been described in archaea, such as two membranes without the S-layer (e.g., *Ignicoccus*), pseudomurein (e.g., Methanobacteriales), polysaccharides (e.g., Thermoplasmatales), glutaminylglycan (e.g.,

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Natronococcus), halomucin (*Haloquadratum*), methanochondroitin (*Methanosarcinales*) and combinations of these components (Albers and Meyer, 2011). The lipid membrane of archaea is formed by IPLs comprising two types of core lipid classes: diphytanyl glycerol diether lipids (archaeol, AR) and/or glycerol dialkyl glycerol tetraether lipids (GDGTs) with 0–8 rings in the structure (Schouten et al., 2013). The polar headgroup of IPLs can be either a sugar (glycolipids) or a phosphate (phospholipids), or a combination of both. The abundance of glyco- and phospholipids varies among different archaea and has been suggested to play an essential role in mediating proton containment and circulation along the surface of energy-transducting membranes (Yoshinaga et al., 2016). Glycolipids with GDGTs as a hydrophobic tail, for instance, are predominant in Thaumarchaeota (e.g., Elling et al., 2017) and *S. acidocaldarius* (Langworthy et al., 1974), while phospholipids with an AR are common in Euryarchaeota, such as haloarchaea (e.g., Kellermann et al., 2016) and *Thermococcus kodakarensis* (Meador et al., 2014).

The crucial step during the extraction of IPLs is the disruption of the cell membrane. The lysis of the cells and the surrounding protein layer can be achieved mechanically (e.g., ultra-sonication or freeze-thawing), chemically (e.g., by detergents), or enzymatically. Different extraction techniques, such as sonication, Soxhlet extraction, saponification, and enzymatic treatments with varying solvent mixtures have previously been tested to increase the extraction efficiency of cultures and environmental samples (Huguet et al., 2010). The most commonly applied extraction technique to investigate IPLs in culture or sediment samples is the modified Bligh and Dyer-protocol (B+D-protocol; Bligh and Dyer, 1959), which uses a solvent mixture of methanol, dichloromethane, and an aqueous buffer (2:1:0.8; v/v/v). Moreover, ultra-sonication is regularly applied during the B+D-protocol to break the cells by friction (sometimes with added sand grains or beads) and increase the extraction efficiency of this protocol. For bacteria and eukarya, several studies have been performed to refine the B+D-protocol and improve the extraction efficiency (see the review by Willers et al., 2015). For instance, the extraction with methyl *tert*-butyl ether resulted in slightly higher recoveries of polar lipids than the B+D-protocol (Matyash et al., 2008; Gorgich et al., 2020). In addition, Frostegård et al. (1991) showed that extraction with a citrate buffer increased the yield of phosphate-bound fatty acids in organic-rich soils. To increase the recovery of archaeal lipids, trichloroacetic acid (TCA) added to the aqueous phase has been employed (Nishihara and Koga, 1987; Sturt et al., 2004). However, over the past 10 years, various studies have revealed a low extraction efficiency of archaeal IPLs by the (modified) B+D-protocol in pure cultures and environmental samples (Huguet et al., 2010; Cario et al., 2015; Weber et al., 2017). These studies showed a much higher recovery of archaeal lipids when sediments or cell pellets were extracted by direct acid hydrolysis than the B+D-protocol. Cario and colleagues (2015) further observed that the B+D-protocol lost GDGTs during their extraction tests with *Thermococcus barophilus*. Similar observations have been made in other archaeal strains (Nishihara and Koga, 1987; Becker et al., 2016; Zeng et al., 2019) and bacterial membrane-spanning lipids in bacterial cultures and environmental samples (Sinninghe Damsté et al., 2011; Weber et al., 2017). However, given that direct acid hydrolysis destroys the majority of IPLs during extraction (Huguet et al., 2010; Evans et al., 2018), the (modified) B+D-protocol remains the primary technique to extract archaeal polar lipids (e.g., Boyer et al., 2020; Ma et al., 2020; Tourte et al., 2020).

In this study, we tested a variety of approaches to evaluate their potential to destroy the cell envelope and, thus, increase the extraction efficiency of archaeal IPLs in pure cultures. To this end, we compared the effect of different solvents, mechanical rupture techniques, and chemical/enzymatic lysis methods to develop a protocol that significantly increases the extraction efficiency of archaeal lipids. The development was based on an iterative approach using biomass from *S. acidocaldarius* as a model organism. Starting from the B+D-protocol, we incrementally improved the extraction by a trial of single modifications. The recovery of archaeal lipids was evaluated by comparing the yields from the modified extractions with acid hydrolysis of the biomass as the control, assuming that hydrolysis yields 100% of the extractable archaeal lipids (Fig. 1; Huguet et al., 2010). After this initial development phase, we tested the protocol with the highest yields from *S. acidocaldarius* on six additional archaeal cultures (two Thaumarchaeota, three Euryarchaeota, and one Crenarchaeota) and environmental samples (two surface sediments and suspended particulate matter) to investigate whether this protocol improves IPL extraction efficiency more widely. The cultures were chosen based on the availability of published IPL-patterns. Thus, we applied the modified protocol on archaea that predominantly produce GDGTs (e.g., Thaumarchaeota; Elling et al., 2017) and AR (primarily detected in most Euryarchaeota, such as *T. kodakarensis* (Meador et al., 2014) or Haloarchaea (Kellermann et al., 2016)). A substantial increase in archaeal lipid yields in the archaeal cultures was observed when biomass was pretreated with chemical detergents combined with freeze–thaw cycles. The environmental samples revealed contrasting results: while sediments showed no increase, archaeal lipid yields were enhanced in marine suspended particulate matter.

2. Material and methods

2.1. Culture samples

Sulfolobus acidocaldarius, due to ease of culturing, high biomass yield and diverse array of intact polar GDGTs, was selected as the model organism. However, the wild-type of this archaeon produces calditol, a GDGT with an ether-bound sugar head group that is not hydrolyzed by the standard methanolic HCl methods (Zeng et al., 2018). Since complete hydrolysis of IPLs was required to compare the lipid yields from different extraction techniques (Fig. 1), a mutant (*S. acidocaldarius* (Δcds)) missing the gene for the production of calditol (Zeng et al., 2018) was employed. *S. acidocaldarius* (Δcds) was grown at 80 °C in 4 L of Brock medium with sucrose (0.2%, w/v), N-Z-Amine (0.1%, w/v), and oxygen as an electron donor (Brock et al., 1972). The pH was adjusted to 3.5 using HCl as described by Zeng et al. (2018). Growth was assessed by optical density measurements at 600 nm, and cells were harvested by centrifugation at 4000 rpm upon reaching stationary phase. The cell pellet was lyophilized, homogenized, and stored at −20 °C until further processing.

After successful testing of different extraction protocols, the methodology was tested on cultures of *Nitrososphaera viennensis* EN76 (DSM 26422), *Nitrosopumilus maritimus* SCM1, *Ignicoccus hospitalis* KIN4/I, *Methanococcus thermolithotrophicus*, *Thermococcus kodakarensis* KOD1 (JCM 12380), and *Haloferax mediterranei* R-4 (ATCC33500) collaborators had supplied. *N. maritimus was* grown at 28 °C in replicates of pH 7.5 HEPES-buffered SCM medium supplemented with 1.5 mM NH_4Cl as described previously (Könneke et al., 2005; Martens-Habbena et al., 2009). *N. viennensis* was grown in replicates at 37 °C in batch cultures in pH 7.5 HEPES-buffered freshwater medium modified from Tourna et al. (2011) adding of 3 mM NH_4Cl and 100 μM dimethylthiourea. *N. viennensis* and *N. maritimus* biomass was harvested in stationary phase using filtration through pre-combusted (450 °C, 6 h) glass fiber filters (GF-75, 0.3 μm pore size, Advantec MFS, Dublin, CA, USA). *I. hospitalis* KIN4/I was grown at 75 °C as described by Jahn et al. (2004). *M. thermolithotrophicus* was grown at 85 °C as described by Liu et al. (2012). *T. kodakarensis* was grown anaerobically at 85 °C in 2 L bottles containing 1 L of ASW-YT medium (Nohara et al., 2014) and harvested in stationary phase using centrifugation (4000*g*, 10 min). *H. mediterranei* was grown at 45 °C in 2 L Erlenmeyer flasks containing 1 L of 25% modified growth medium (MGM; Dyall-Smith, 2009) and harvested in stationary phase using centrifugation (4000*g*, 30 min).

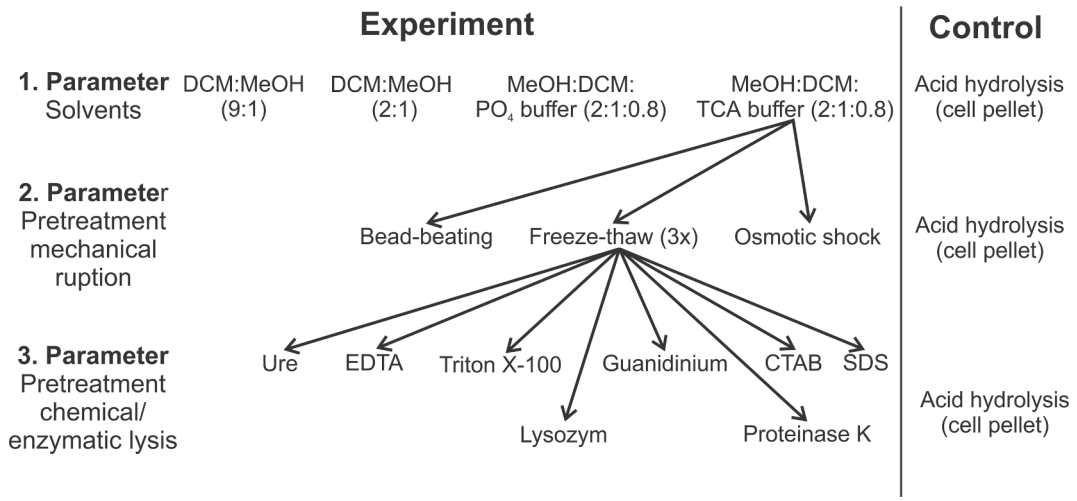

Fig. 1. The iterative approach followed in this study. The arrows represent combinations of treatments that were tested. Initially, monophasic solvent combinations of increasing polarity were tested, including the Bligh and Dyer protocol with a phosphate and trichloroacetic acid buffer. The solvent affording the highest archaeal lipid yields (1:2:0.8; v/v/v; DCM:MeOH:TCA buffer), was then used to test the effect of different methods for mechanical lysis. In the final step, freeze–thaw cycles were performed with different detergents, protein denaturing chemicals, enzymes with Milli-Q-water. The highest lipid yields were achieved using freeze–thaw cycles with a 1% CTAB solution, followed by extraction with the TCA buffer. All experiments were performed in triplicates with *S. acidocaldarius* (Δcds) biomass. Recovery of core lipids after acid hydrolysis of the cell pellet with 1 M HCl, conducted in triplicate, were used as the benchmark for assessments of IPL recovery in each step.

2.1.1. Environmental test samples

2.1.1.1. Sediment test samples. A suite of surface sediments from the Namibian shelf was collected in 2014 (French, 2015). In these experiments, we used material from 'Station 4'. Sediment from the central basin of the Black Sea, Sta. BSK-2 (43°10′N,32°00′E; water depth 2200 m) collected during leg 5 of the 1988 expedition was provided by Dr Stuart Wakeham (6BC; 0–5 and 5–10 cm; Wakeham and Beier, 1991).

2.1.1.2. Suspended particulate matter test samples. Four glass fiber filters (GF/F Whatman) with suspended particulate matter (SPM) were extracted using the improved extraction protocol. The samples were collected during cruise KN210-04 from March to May 2013 on R/V *Knorr*. The SPM filters were sampled at station 15 (2.7°S, 28.5°W; Hurley et al., 2018) at depths of 65 m and 500 m below sea level. For each depth, two filter pore sizes were analyzed: 0.3 μm and 0.7 μm. The filters were halved using solvent-cleaned scissors. One half was extracted using the modified protocol and the other using a solvent mixture of 2 × methanol (MeOH), 1 × dichloromethane (DCM), and 0.8 × trichloroacetic acid (TCA) as described below.

2.2. Set-up of the extraction tests with model organism S. acidocaldarius (Δcds)

Different solvents, mechanical rupture techniques, and chemical/enzymatically lysis protocols were tested to evaluate archaeal lipid extraction efficiency (Fig. 1). All experiments were performed in triplicate unless noted otherwise.

2.2.1. Solvent composition

Four different solvent combinations and their effect on extraction efficiency were investigated (Fig. 1): DCM:MeOH (9:1; v/v), DCM:MeOH (2:1; v/v), MeOH:DCM:PO_4 buffer (K_2HPO_4 in Milli-Q water; 8.7 g L^{-1}; pH 7.4; 2:1:0.8; v/v/v), and MeOH:DCM:trichloroacetic acid buffer (TCA in Milli-Q water; 50 g L^{-1}; pH 2; 2:1:0.8; v/v/v). DCM was purchased from Sigma-Aldrich (Omnisolv, HPLC-grade), MeOH from Sigma-Aldrich (Omnisolv, HPLC-grade), K_2HPO_4 from Merck (99% purity), and TCA from Alfa Aesar (99% purity). For each extraction, 10 mg of freeze-dried biomass, 20 mL of each solvent mixture, and 5 g of combusted sand, to aid mechanical disruption, were added to solvent-cleaned Teflon tubes and sonicated in an ultrasonic bath (US-bath) for 20 min. Subsequently, extracts were centrifuged for 10 min at 175 relative centrifugal force, and the supernatant was transferred to a separatory funnel. The extraction was repeated three times. Afterward, 10 mL of water and 10 mL DCM were added to the separation funnel to allow phase separation, and the organic phase was drawn off. The remaining water phase was washed three times with 20 mL DCM. In the following step, pooled organic layers were washed three times with Milli-Q water (100 mL each). The extracts were dried under a gentle stream of N_2 at 30 °C and stored at −20 °C until analysis

2.2.2. Mechanical disruption

Three protocols that facilitate the mechanical rupture of cells before extraction were tested: osmotic shock, bead-beating, and freeze–thaw cycles (Fig. 1). Osmotic shock experiments were performed by adding 5 mg of the freeze-dried biomass, combusted sand, and 5 mL of a NaCl-saturated solution to the extraction vessel and incubating at room temperature overnight. For bead-beating, 5 mg of the freeze-dried culture was added to PowerBead tubes filled with garnet (Qiagen) and shaken for 20 min at the highest intensity and centrifuged. The supernatant was transferred to an extraction vessel. In the following step, plastic tubes were rinsed 3 times with water and 3 times with MeOH (1 mL for each rinse) to transfer the remaining cell/lipid debris. Before each rinsing step, the tubes were vortexed for 10 sec to dissolve the supernatant. Finally, combusted sand was added. Freeze-thaw cycles were performed in solvent-cleaned Teflon tubes using 5 mL Milli-Q water added to 5 mg of freeze-dried cells and combusted sand. Freeze-thaw cycles consisted of 1.5 h of incubation at 50 °C, 30 min of freezing at −80 °C with three repeats each. Water was removed by lyophilization in all three tested protocols, and samples were extracted as described above using a solvent mixture of MeOH:DCM:TCA buffer (2:1:0.8, v/v/v).

2.2.3. Chemical and enzymatic lysis

Four different detergents including *t*-octylphenoxypolyethoxyethanol (Triton™ X-100; VWR, Proteomics grade), sodium dodecyl sulfate (SDS; VWR, > 99% purity, biotechnology grade), ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich, 99% purity BioUltra), and cetyltrimethylammonium bromide (CTAB; Alfa Aesar, 98% purity), two enzymes (proteinase K (VWR, Biotechnology grade) and

lysozyme (VWR, Ultra-pure grade)) and two protein denaturants (guanidinium chloride (MP Biochemicals, > 99% purity) and urea (Sigma-Aldrich, > 99% purity)) were investigated for their potential to increase the extraction efficiency of archaeal lipids (Fig. 1). A range of concentrations was tested for the chemical detergents and protein denaturants: CTAB (0–4%; w/v), SDS (0–4%; w/v), Triton X-100 (0–6%; w/v), urea (0–10 M), guanidinium chloride (0–4 M) and EDTA (0–0.2 M; Fig. S1 in Supplementary file A). All chemicals were dissolved in Milli-Q water. To dissolve EDTA in Milli-Q water, the pH was adjusted to 11 for dissolution. The enzymes were also dissolved in Milli-Q water. In the case of proteinase K, the final concentration was 0.5 mg mL^{-1} and for lysozyme 0.2 mg mL^{-1}. For each extraction experiment, 5 mL of the detergent-, enzyme- or denaturant-Milli-Q mixture were added to 5 mg freeze-dried biomass, and 5 g combusted sand in solvent cleaned Teflon tubes. In the next step, three freeze–thaw cycles were performed for all tested chemicals, and enzymatic lysis protocols described above, and the liquid was removed by lyophilization. The dried biomass was extracted using the TCA buffer protocol as described above.

2.2.4. Acid hydrolysis

To verify the efficiency of the different extraction protocols, total lipid extracts were split into two aliquots. One contained archaeal IPLs, while the second aliquot was hydrolyzed with 2 mL of 1 M HCl in MeOH solution at 70 °C overnight to remove the polar lipid head groups. On the next day, the 1 M HCl in MeOH solution was dried under a stream of N_2. In addition, for every parameter test, a set of three samples with *S. acidocaldarius* biomass was extracted by direct acid hydrolysis of the cell pellet as a control (Huguet et al., 2010; Fig. 1). Direct acid hydrolysis was performed as described by Evans et al. (2018). In brief, 5 mg of freeze-dried culture, 5 g of combusted sand, and 20 mL of a 1 M HCl in MeOH were added to the solvent-cleaned Teflon tubes, hydrolyzed overnight at 70 °C and combined in a separatory funnel. The sand residue was re-extracted three additional times with 20 mL 5:1 DCM MeOH (v/v) sonicated for 20 min and added to the funnel. 60 mL water was added to the pooled extract to achieve phase separation and the organic phase was collected. The aqueous phase and organic phase were washed as described above. The extract was dried under a stream of N_2 and stored at −20 °C until measurement.

2.3. Analysis of hydrolyzed and intact lipids

Hydrolyzed aliquots and IPLs were measured using a 6520 accurate-mass quadrupole time-of-flight mass spectrometer (MS; Agilent Technologies) equipped with an electrospray ionization source coupled to a 1200 series high-performance liquid chromatography (HPLC; Agilent Technologies). Lipids of both aliquots were separated with an ACE3 C_{18} column (2.1 × 150 mm, 3 μm particle size, Advanced Chromatography Technologies) following a method described by Evans et al. (2017). In brief, the column was maintained at 45 °C. Eluent A comprised MeOH, formic acid and ammonium hydroxide (1000:0.4:1; v/v/v) and eluent B was isopropanol, formic acid and ammonium hydroxide (1000:0.4:1; v/v/v). The gradient was programmed as follows: first 10 min 100% A, then 76% A (15 min), 44% A (60 min), and reached 10% A after 61 min. 10% A was held from 61 min to 76 min. The column was equilibrated for 15 min before the next run. Hydrolyzed lipids and IPLs were detected in positive ionization mode scanning from 150 Da to 2000 Da. Lipids were identified by retention time, characteristic MS^2 fragmentation patterns, and accurate mass (error below 1 ppm) of the possible parent ions (H^+, NH_4^+, and Na^+ adducts). The areas of the three different adducts for the hydrolyzed core lipids and the IPLs were summed to quantify the detected archaeal lipids and normalized to the response of an injection standard (C_{46}-GTGT; Huguet et al., 2006). Quantification of the absolute concentration of archaeal core lipids and IPLs was not possible due to a lack of authentic archaeal lipid standards. The instrument responses of core lipids of the hydrolyzed aliquots from the different experiments were normalized to the control experiments (direct acid hydrolysis of the biomass) to investigate the different yields of total extractable archaeal lipids as shown previously by Huguet et al. (2010). IPL results are displayed as response units per gram biomass or liters of culture (e.g., Besseling et al., 2019; Sollai et al., 2019).

3. Results

3.1. Roles of solvent and mechanical disruption

The role of solvent composition on extraction efficiency was tested using the *S. acidocaldarius* (Δcds) mutant as a model archaeon. Fig. 2A shows lipid extraction yields (core AR + core-GDGT) using different solvent mixtures normalized to acid hydrolysis of the cell pellet. Yields without normalization (in response units) are shown in Supplementary Fig. S2A. Extractions with a MeOH:DCM:TCA buffer (2:1:0.8, v/v/v) showed the highest yield (9.5 ± 0.3%) of the different investigated solvent systems, followed by extractions using MeOH:DCM:PO_4 buffer (2:1:0.8; v/v/v; 5.9 ± 0.4%). Extractions that employed lower polarity solvent mixtures resulted in lower recoveries (0.7– 0.2%). In the next step, the effect of three different mechanical pretreatments on lipid extraction efficiency was investigated. Experiments using bead-beating (6.9 ± 2.2%) or osmotic shock (8 ± 1.3%) before extraction showed similar or lower yields than the samples extracted without pretreatment (7.9 ± 1.6%; Fig. 2B; Supplementary Fig. S2B). Freeze-thaw cycles, in contrast, increased the lipid recovery (14.1 ± 1.4%) compared to the extraction without pretreatment. In the next step, freeze–thaw cycles were performed with six different chemical reagents in different concentrations (Supplementary Fig. S1) and two enzymes to investigate their effect on the extraction efficiency in the model organism (Fig. 2C; Supplementary Fig. S2C). Enzymes afforded lower yields (lysozyme 10.7 ± 2%; proteinase K 10.3 ± 0.6%) than freeze–thaw cycles without any additives (Milli-Q; 11.5 ± 1.4%). Highest yields were achieved when 1% CTAB was dissolved in Milli-Q water (65.8 ± 8.6%), followed by EDTA 0.1 mM (54.8 ± 9.7%), Triton X-100 (6%; 28 ± 2.1%), and SDS (4%; 27.2 ± 7.8%).

The comparison of IPLs extracted with 1% CTAB (freeze–thaw), with Milli-Q (freeze–thaw) and without any pretreatment is shown in Fig. 3. The experiments without pretreatment showed the lowest abundances for IPLs and core lipids of the three extractions. Freeze-thawing with Milli-Q increased the individual IPL classes and enabled the detection of phosphoinositol-AR (PI-AR) and PI-GDGT. Experiments with 1% CTAB incubation and freeze–thaw cycles before extraction showed the highest response units across all detected lipids (Fig. 3). Moreover, 2G-(diglycosidic)-PI-GDGT was only detected in the extractions using 1% CTAB.

3.2. Testing the modified protocol on additional archaeal cultures

The modified extraction protocol was tested on six different taxa covering a range of phyla. The lipid recoveries normalized to acid hydrolysis are shown in Fig. 4 and yields without normalization (in response units) are shown in Supplementary Fig. S3. The hydrolyzed aliquots showed a 2.5–8 fold increase in detection of archaeal lipid yields in the cultures treated with 1% CTAB and freeze–thaw cycles compared to the samples that were only extracted with the TCA buffer (no pretreatment; Fig. 4). The most significant increase of archaeal lipid yields was observed in *N. maritimus* (6.7 ± 0.8% to 52.6 ± 11%), followed by *I. hospitalis* (2.1 ± 0.3% to 15.8 ± 2.3%). Extractions of *M. thermolithotrophicus* revealed the smallest increase of the yields (20.9 ± 3.1% to 56 ± 10.7%). The IPL results (Fig. 5; Supplementary file B) showed higher response units for all investigated IPL classes in the tested cultures for extractions with 1% CTAB and freeze–thaw cycles than extractions without the pretreatment.

3.3. Testing the modified protocol on sediments and SPM

The hydrolyzed aliquots of the collected sediments from the Black

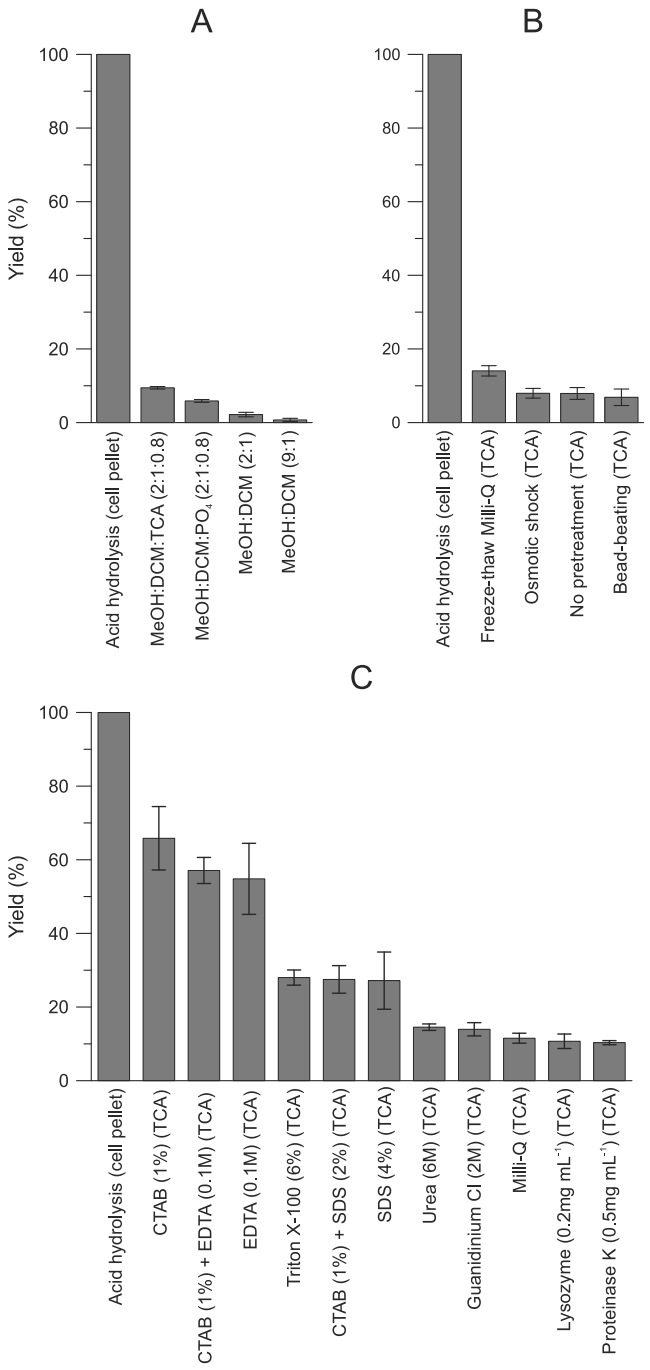

Fig. 2. Total archaeal lipid yields (core-AR and core-GDGT) of a hydrolyzed aliquot of the extracted lipids from *S. acidocaldarius* (Δcds) compared to the direct acid hydrolysis of the cell pellet. (A) Tests with four monophasic solvent mixtures. (B) Trials with three different mechanical pretreatments compared to an experiment without additional pretreatment (TCA). (C) Bar plot summarizing the *S. acidocaldarius* (Δcds) lipid yields using freeze–thaw cycles with different detergents and enzymatic lysis chemicals dissolved in Milli-Q water. Milli-Q represents freeze–thaw cycles without additional detergents or enzymes. The experiments in panels B and C were extracted using the B+D-protocol with a TCA buffer solvent system. All experiments, including the acid hydrolysis control, were performed in triplicate.

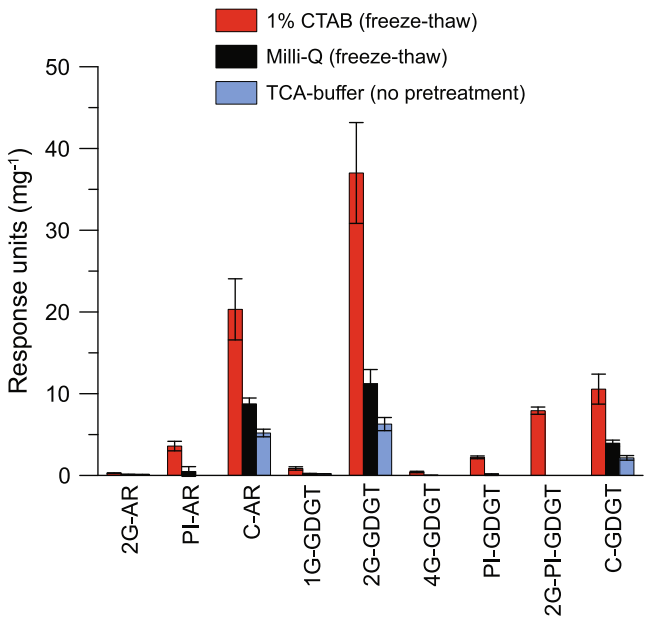

Fig. 3. Bar plot comparing the *S. acidocaldarius* (Δcds) IPL compositions after three freeze–thaw cycles with 1% CTAB in Milli-Q, freeze–thaw cycles only with Milli-Q, and without an additional pretreatment (B+D with TCA buffer system). All extractions were performed in triplicate. Due to lack of response factors, results are given as response units per mg biomass.

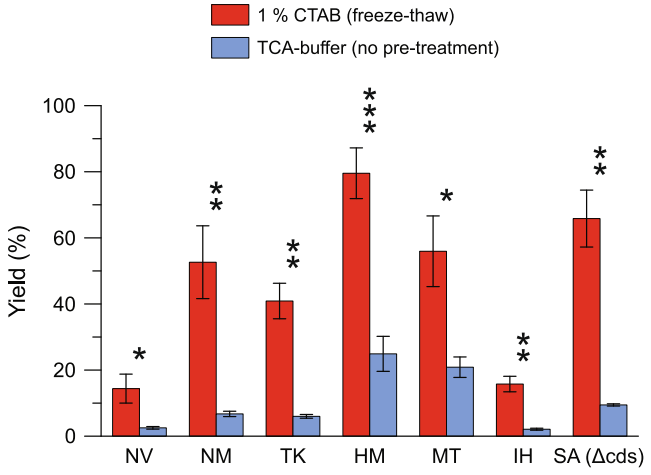

Fig. 4. The yields of total hydrolyzed archaeal lipids (core-AR and core-GDGT) in the test cultures compared to the direct acid hydrolysis of the cell pellet. Test cultures include: *N. viennensis* (NV), *N. maritimus* (NM), *T. kodakarensis* (TK), *H. mediterranei* (HM), *M. thermolithotrophicus* (MT), *I. hospitalis* (IH) and *S. acidocaldarius* (SA (Δcds)). Stars above the bars represent the p-values of two-tailed t-tests (*p < 0.05, ** p < 0.01 and *** p < 0.001).

Sea showed no difference between the freeze–thaw cycles with 1% CTAB (55 ± 1.6%) and without pretreatment (52.3 ± 7.4%), while the lipids from the Namibian Shelf experienced a slight increase due to the incubation with 1% CTAB (59.2 ± 8.6% to 73.4 ± 3.4%; Fig. 6). In the SPM samples, strong contamination by CTAB in chromatograms between 0 and 40 min was observed (Supplementary Fig. S4). This resulted in a strong ion suppression of the C_{46}-GTGT standard signal and core-AR which could not be detected in the samples incubated with CTAB. Therefore, only the peak area per g extracted filter is presented (Fig. 7). Nevertheless, the results showed a higher abundance of GDGTs in the extractions with 1% CTAB and freeze–thaw cycles than the samples without pretreatment.

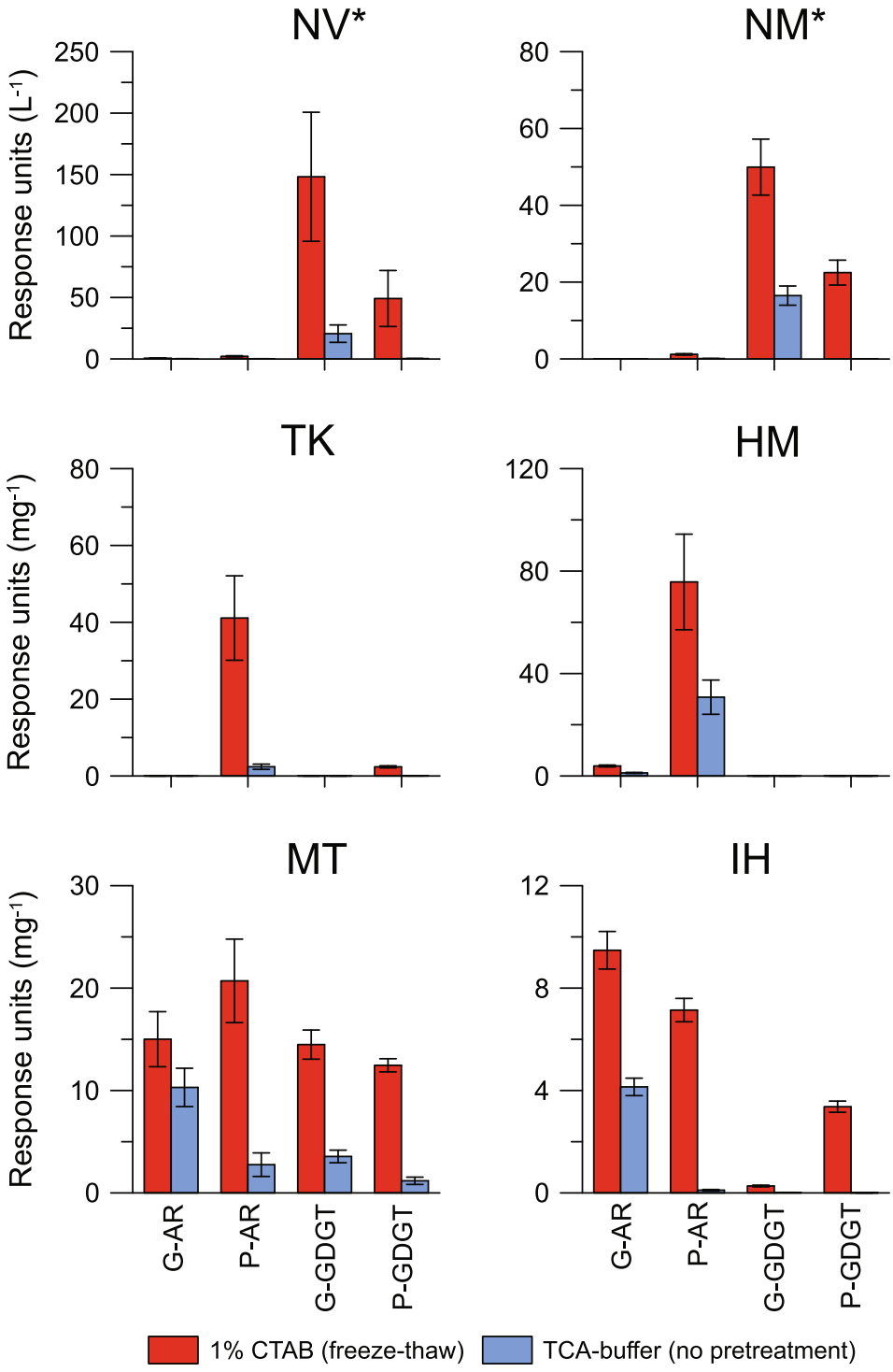

Fig. 5. Response of glycosidic (G) and phosphatic (P) archaeols (AR) and glycerol dialkyl glycerol tetraethers (GDGT) in the test cultures. *N. viennensis** and *N. maritimus** were harvested by filtration, and, therefore, the lipid abundances are presented as response units per liter for these two cultures. Taxa are indicated as in Fig. 4. Due to lack of response factors, results are given as response units per mg biomass or liter*.

4. Discussion

4.1. Method development

Improved extraction efficiency for archaeal lipids (Fig. 1) was investigated through an iterative approach in an *S. acidocaldarius* calditol deletion mutant (Δcds; Zeng et al., 2018) as a model organism. The selection of optimal parameters for the final method was based on the highest yields from the hydrolyzed aliquots compared to the direct acid hydrolysis of the cell pellet in our model organism. We decided to base our decision on the hydrolyzed aliquots since archaeal lipid standards for the diverse range of IPLs found in cultures were unavailable. Accordingly, evaluating the yields from two compounds (core-AR and core-GDGTs) gives more reliable results than comparing a set of different IPLs. Moreover, it must be considered that all experiments were conducted using the calditol deletion mutant and not the wild type, which might affect the observed extraction efficiencies. However, given that both possess the protein layer, we expect similar results among both

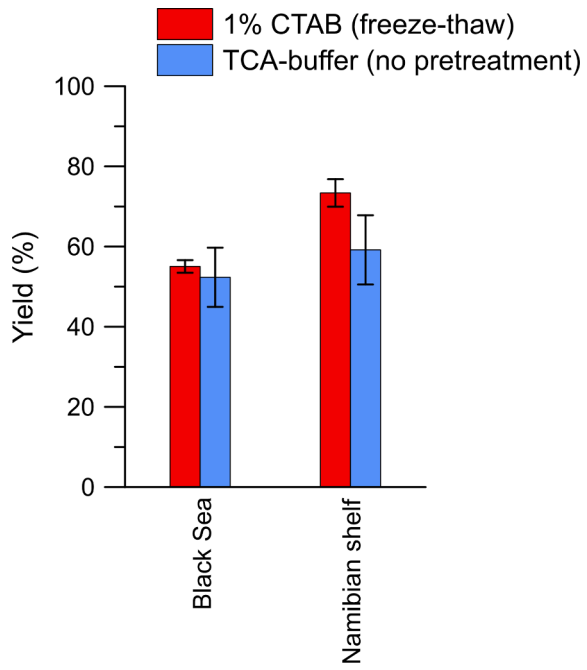

Fig. 6. The yields of total hydrolyzed archaeal lipids (core-AR and core-GDGT) from two sediment samples compared to the direct acid hydrolysis of the cell pellet. Test sediments were collected from the Black Sea and the Namibian Shelf.

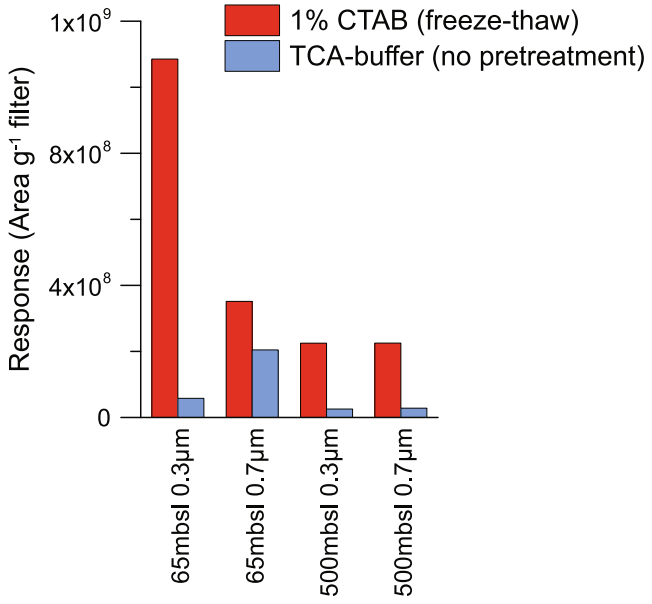

Fig. 7. Recoveries of archaeal lipids from suspended particulate material extracted with 1% CTAB and freeze–thaw cycles compared to extractions without additional pretreatment (B+D with TCA buffer system). For each depth, two filter pore sizes were analyzed (0.3 and 0.7 µm). Due to strong contamination evident in the HPLC-MS data for the CTAB samples, only areas per gram filter are shown. No replicates were performed due to sample limitations. Extracts were hydrolyzed before analysis.

species.

Direct acid hydrolysis of the cell pellet yielded an approximately 10 fold or higher recovery of archaeal core lipids than any other extraction method (Fig. 2A), demonstrating that most archaeal lipids in *S. acidocaldarius* (Δcds) are not recovered with the B+D-protocol alone. This agrees with previous studies in other pure cultures and environmental samples (Huguet et al., 2010; Cario et al., 2015; Becker et al., 2016; Weber et al., 2017). Biomass extracted using the trichloroacetic acid modification afforded higher archaeal lipids yields than extractions with a phosphate buffer (Fig. 2A). The use of the TCA buffer has been controversial. Nishihara and Koga (1987), who introduced this variation, observed an increased extraction efficiency in extractions with *Methanobacterium thermoautotrophicum*. Moreover, a study on SPM from Lake Lugano revealed higher archaeal and bacterial GDGT yields using the TCA buffer compared to the PO_4 buffer (Weber et al., 2017). Other studies, however, showed no positive effect of the TCA buffer in experiments with *N. maritimus* and *T. barophilus* (Huguet et al., 2010; Cario et al., 2015). This suggests that the use of different buffer solutions might lead to varying extraction efficiencies among distinct archaeal species, as shown previously for phosphate-bound fatty acids in organic-rich soils (Frostegård et al., 1991). Given that our goal was to increase lipid yields in the model organism, we decided to use a MeOH:DCM:TCA buffer (2:1:0.8, v/v/v; Fig. 1) solvent system. Finally, less polar solvents were tested since Cario et al. (2015) observed enhanced extracted dry lipid weights with DCM:MeOH (2:1, v/v) than the modified B+D-protocol. However, contrary to their observations, extraction with less polar solvents decreased lipid yields in the experiments described herein (Fig. 2A). This agrees with a previous study that compared the extraction efficiencies in different sediments and observed lower IPL yields when using apolar solvents for extraction (Lengger et al., 2012b).

Three mechanical pretreatments, including osmotic shock, bead-beating, and three freeze–thaw cycles with Milli-Q water, which should facilitate cell lysis (e.g., Prabakaran and Ravindran, 2011) were evaluated next (Fig. 2B). Applying osmotic shock and bead-beating before extraction did not improve the extraction efficiency in *S. acidocaldarius* (Δcds) compared to the extraction without a pretreatment. It must be noted that extraction efficiency in other archaeal strains might benefit from these treatments. For instance, incubating halophilic archaea with Milli-Q water could trigger a reverse flow of water into the cell and facilitate the mechanical destruction of the cell. However, previous studies have shown that washing halophilic archaea with Milli-Q water will release the DNA and RNA during cell lysis and lead to the formation of an intractable gel from which lipids cannot be extracted (Corcelli and Lobasso, 2006). Further studies are required to investigate whether lipid extraction efficiency in halophilic archaea would benefit from this treatment.

The lower yield of archaeal lipids when cells were disrupted by bead-beating was initially surprising since this technique is widely employed to increase the yields of DNA in pure cultures (e.g., de Boer et al., 2010) and in environmental samples (e.g., Miller et al., 1999). An explanation may lie in the polarity contrast between DNA and lipids. DNA and other polar cell components occur in the water phase, whereas IPLs, in contrast, are distributed preferentially in the organic solvent phase. Nevertheless, yields did not improve even if bead-beating tubes were rinsed several times with MeOH after the initial extraction. Washing the tubes with DCM may have increased the recovery of lipids in the extractions where bead-beating was applied as a pretreatment. However, since bead-beating tubes are made from polypropylene, DCM could dissolve the plastic resulting in contamination. Therefore, tubes were not washed with this solvent.

Freeze-thaw cycling was the only mechanical pretreatment that increased archaeal lipid yields. A higher GDGT yield attributable to freeze-thawing, compared to the bead-beating, has also been observed in SPM material collected in Puget Sound (Northwest Pacific Ocean; Huguet et al., 2010). Although Huguet et al. (2010) applied more cycles (seven) than this study (three), the results indicate that freeze-thawing might be generally beneficial for the extraction of archaeal lipids from both cultures and environmental samples. Since freeze–thaw cycles were the only mechanical treatment that increased archaeal lipid yields, it was included in the extraction protocol for the next phase of tests (Fig. 1).

In the next phase, freeze–thaw cycles were performed with six chemical reagents and two enzymes dissolved in Milli-Q to determine whether protein denaturation and membrane disruption would further increase lipid extraction yields in the model organism (Fig. 2C). Moreover, one set of freeze–thaw cycles was performed that contained only Milli-Q. Freeze-thaw cycles with proteinase K and lysozyme resulted in slightly lower lipid yields than freeze–thaw cycles conducted with Milli-Q (Fig. 2C). Previous results had shown a substantial increase of the archaeal lipids in *N. maritimus* when biomass was incubated with proteinase K at 70 °C for 3 h (Huguet et al., 2010), indicating that the effect might be highly specific among different archaeal species.

Guanidinium chloride and urea have both been described as protein denaturants (e.g., Neurath et al., 1944; Monera et al., 1994) and used to either extract proteins (Etienne et al., 1999; Tan and Yiap, 2009; Karan et al., 2019) or for their removal to improve the extraction of DNA (see review by Tan and Yiap, 2009). However, although a range of different concentrations was investigated (Supplementary Fig. S1), urea and guanidinium chloride showed minimal impact on the extraction efficiency (Fig. 2). Triton-X 100, a detergent that disrupts the cell envelope (Harrison, 1991), showed no increase when freeze–thaw cycles were performed with 2% or 4% of this detergent (Supplementary Fig. S1). The addition of 6% of Triton X-100, on the contrary, increased lipid yields (Fig. 2C). However, the LC–MS analyses of these extracts were compromised by a strong contaminant in the detergent, even when very diluted samples (< 0.1% TLE) were injected. Therefore, Triton X-100 was not considered for additional tests. SDS and EDTA have both been shown to facilitate cell lysis (e.g., Watt and Clarke, 1994; Ciccolini et al., 1998; Chourey et al., 2010) and to denature proteins in environmental and culture biomass samples (Janecki and Reilly, 2005; Nowakowski et al., 2014). Here, freeze–thaw cycles with both detergents showed the second-highest lipid yields (Fig. 2C). The increased extraction efficiency using SDS agrees with a previous study that observed an improved lipid yield from multilamellar phospholipid vesicles (Burton et al., 1985).

Using a 1% CTAB in Milli-Q solution afforded the highest recovery of archaeal lipids among all tested detergents and enzymes (Fig. 2C). One explanation for the high yields of archaeal lipids due to the pretreatment with CTAB may lie in its ability to precipitate glycoproteins (Abel and Good, 1966; Wold and Midtvedt, 1977). The surface S-layer protein in *S. acidocaldarius* is predominantly built up of two glycoproteins: the SlaA and SlaB proteins, which have been shown to be very resistant to denaturation (Simonin et al., 2020). Particularly, the SlaB protein, which anchors the S-layer into the lipid membrane, has resisted isolation when extracted with SDS at room temperature (Weiss, 1974; Simonin et al., 2020). Accordingly, CTAB may denature the glycoproteins before extraction by precipitation and, thus, increase archaeal lipid yields. A previous study that investigated the potential of CTAB to improve the harvesting of microalgae cultures by foam floatation for biodiesel applications also observed a higher recovery of saturated and mono-unsaturated fatty acids compared to cells harvested by centrifugation without CTAB (Coward et al., 2014). This implies that CTAB addition may not only result in increased extraction efficiency of lipids in archaeal cultures but may also be applied to lipid extractions in a broader range of cultured microbes, such as bacteria, algae, and plants.

Our experiments further showed that the extraction was strongly dependent on the concentration of CTAB. While 0.5% and, particularly 1%, of added CTAB, resulted in enhanced lipid yields, higher concentrations of this detergent (2– 4%; Supplementary Fig. S1) decreased yields compared to the control (Milli-Q). The lower yields may result from the degradation of lipids at higher CTAB concentrations, an aspect that was not investigated further. Moreover, no extraction efficiency enhancement was observed when combining 1% CTAB with SDS or EDTA (Fig. 2C).

Based on the observations described, a final extraction protocol was devised. This comprised: pretreatment of the biomass with a 1% CTAB in Milli-Q solution with three freeze–thaw cycles followed by extraction of the IPLs with a solvent mixture of MeOH:DCM:TCA buffer (2:1:0.8, v/v/v).

4.1.1. Comparison of IPLs composition for the different extraction protocols in S. acidocaldarius (Δcds)

The composition of *S. acidocaldarius* (Δcds) IPLs showed differences across the protocols tested (Fig. 3). In the extractions without pretreatment, phospholipids were below the detection limit. Previously, phospholipids, such as PI-AR, have been reported in extractions using chloroform and MeOH (2:1; v/v) for 3 h at room temperature (Langworthy et al., 1974). However, Langworthy et al. (1974) used much higher quantities of biomass (15 g) than this study (5 mg or 10 mg). Phospholipids were detected in *S. acidocaldarius* when either freeze–thaw cycles with Milli-Q (PI-AR and PI-GDGT) or freeze–thaw cycles with 1% CTAB (PI-AR, PI-GDGT, 2G-PI-GDGT) were applied as a pretreatment (Fig. 3). An increased phospholipid yield has also been observed in microalgae (*Chlorella* sp.) extracted in the presence of CTAB compared to the same culture extracted without this detergent (Coward et al., 2014). These authors hypothesized that the enhanced detection of phospholipids in *Chlorella* sp. might result from the ability of CTAB, as a positively charged ion, to break the association between the predominantly negatively charged phospholipids and the anchor proteins.

The glycolipid abundance was also enhanced due to 1% CTAB pretreatment compared to extractions without chemical lysis buffers in *S. acidocaldarius* (Fig. 3). However, the increase was less pronounced than for the phospholipids. This suggests that glycolipids could have weaker or different types of associations with the anchor protein compared to phosphate-bound IPLs (Lee, 2003; Eichler and Adams, 2005; Laganowsky et al., 2014; Yeagle, 2014). Accordingly, mechanical cell lysis by sonication could represent a suitable method to extract a significant fraction of glycolipids. An additional factor may be that some glycolipids exist as larger molecules. Large GDGTs with up to 24 sugar head groups, for instance, have been observed in *S. acidocaldarius* (Nicolaus et al., 1993) and *Thermoplasma acidophilum* (Mayberry-Carson et al., 1974, 1978). These lipids are outside the analytical window of the Q-ToF in the IPL aliquot but may be included in the hydrolyzed aliquot detected as core-GDGTs.

We also observed an enhanced abundance of core-AR and core-GDGTs in the experiments with CTAB, which were more than two times higher than the extractions without the detergent (Fig. 3). This could either suggest that the treatment with 1% CTAB and freeze–thaw cycles may destroy IPLs during the pretreatment or that a fraction of core lipids are bound to the protein layer, which are released by the treatment.

4.2. Test of the modified protocol on other archaeal cultures

The modified protocol revealed an increase of the total lipids in all investigated strains, which was between 2.5 and 8 times higher than in the extractions without additional pretreatment (Fig. 4). This demonstrates that CTAB and freeze–thaw cycles not only increase the extraction efficiency in the model organism *S. acidocaldarius* (Δcds), but that this modified protocol is also applicable to other archaea. Nonetheless, extractions with direct acid hydrolysis of the biomass yielded higher core lipid concentrations in all test cultures (yields: 15–78%; Fig. 4), suggesting that a large pool of archaeal lipids remain unrecoverable, as suggested earlier (Huguet et al., 2010). This could result from strong covalent bonds between IPLs and the protein layer that the detergents cannot break (Huguet et al., 2010) or large IPLs outside the analytical window (Mayberry-Carson et al., 1974, 1978; Nicolaus et al., 1993). Additionally, some IPLs might not be dissolved in the applied solvent system or ionized by the ESI source. The highest increase in lipid yields, by the modified protocol compared to the extractions without pretreatment, was observed in the two cultured Thaumarchaeota (*N. viennensis* and *N. maritimus*) and *I. hospitalis* (Fig. 4). *M. thermolithotrophicus* and *H. mediterranei,* on the other hand, showed lower enhancement. One factor influencing lipid yields might be the

types of lipids an archaeon produces. *H. mediterranei* constructs its lipid membranes exclusively with (unsaturated) archaeols, while Thaumarchaeota and Sulfolobales produce GDGTs predominantly (Supplementary Figs. S2 and S3). Accordingly, the relatively high efficiency of the B+D-protocol in *H. mediterranei* possibly indicates that archaeols might be more effectively recovered by this route than GDGTs (e.g., Cario et al., 2015; Becker et al., 2016; Weber et al., 2017). Additionally, differences in the protein layer surrounding the cell may explain some of the observed variations in the archaeal lipid yields. However, the cell envelope of six of the seven investigated archaeal cultures, except *I. hospitalis*, is composed of S-layer proteins (Nußer and König, 1987; Albers and Meyer, 2011; Esclapez et al., 2013). This indicates that factors other than cell envelope type may influence variations in lipid recovery among different archaea. Future experiments on other archaeal strains are needed to understand the observed differences in extraction efficiency.

4.2.1. Comparison of IPL abundance in the different extraction protocols

We observed enhanced IPL abundances for all investigated cultures when the modified protocol was applied compared to the control without the pretreatment (Fig. 5). Although no additional IPLs were detected due to the pretreatment, we observed that IPL patterns changed. Similar to the experiments with the model organism, we noticed high response units and higher relative abundances for IPLs that contained a glyco-phospho head group (Supplementary file B). In *I. hospitalis* and *T. kodakarensis*, for instance, we measured high abundances of PI-AR, while in *M. thermolithotrophicus,* N-acetylhexoseamine phosphatidyl (NAcHP)-AR became the predominant IPL when the modified protocol was applied. This could indicate that glycophospholipids might be particularly susceptible to the applied pretreatment. However, it must be noted that the lack of available response factors for the various identified IPLs in the cultures impairs a quantitative comparison of the two extraction techniques. Therefore, care should be taken when comparing IPLs abundances of the two methods without acid hydrolysis.

For the two investigated Thaumarchaeota, we detected high abundances of hexose, phosphose-hexose (HPH) GDGTs when samples were extracted by the modified protocol. The extractions without a pretreatment, on the contrary, showed only low abundances of HPH-GDGTs in *N. viennensis*, while this compound was below the detection limit in *N. maritimus*. A low abundance of HPH-GDGT in *N. viennensis* has been described earlier (Elling et al., 2017). However, the non-detection of HPH-GDGTs in the extraction without the CTAB pretreatment of *N. maritimus* is surprising, given that earlier studies have reported this lipid in *N. maritimus* (Schouten et al., 2008; Elling et al., 2014). Particularly, the study by Schouten et al. (2008) reported high abundances of these compounds. However, there are some significant differences between the two studies. Here, *N. maritimus* was extracted four times with the TCA buffer, while Schouten et al. (2008) used only the PO_4 buffer. This could indicate that the TCA buffer, which was selected based on the highest lipid yield in the model organism, could degrade phospholipids during the extraction process resulting in an underestimation of phospholipids in the experiments without additional pretreatment. A further explanation for the low abundance of HPH-GDGTs could be that we used less biomass (1 L) than Schouten et al. (2008; 4 L) and that for our study, *N. maritimus* was harvested in the late stationary phase. It has been shown that HPH-GDGTs abundances decrease from early growth to stationary phase (Elling et al., 2014). Finally, it must be noted that Schouten et al. (2008) measured the IPL-patterns with a normal-phase HPLC–MS method, while here, IPLs were measured by reversed-phase HPLC–MS. Variations of IPL responses among different separation techniques and mass spectrometers have been described before (Schröder, 2015), which could also lead to the low abundance of HPH-GDGTs in *N. maritimus* in our study.

H. mediterranei was the only tested archaeon that showed a higher increase in G-AR (~3.5×) than in P-AR (~2.5×; Fig. 5). Nevertheless, PG-AR with up to 5 double bonds remained the predominant IPL type in both extraction methods (Supplementary file B). Moreover, we detected an unknown archaeol with a mass of 1091.78 Da (NH_4^+-adduct; Supplementary Fig. S5), particularly enriched in the extractions performed after the modified protocol (5× higher). The molecular mass matches with a sulfated diglycosyl archaeol (Becker, 2015), reported in some halophilic archaea and hypersaline environments (Kushwaha et al., 1982; Kates, 1993; Becker, 2015). The tentative identification as a sulfated diglycosyl archaeol is further supported by two prominent fragments at 373 Da and 653 Da. However, the characteristic ion fragment 994 Da (loss of the SO_3-group), as described by Becker (2015), was not observed in the fragmentation pattern. Instead, we detected a 999 Da fragment that could not be readily rationalized, thereby precluding a definitive identification.

4.3. Test of the modified protocol on environmental samples

4.3.1. Sediments

Two samples comprising surface sediments from the Black Sea and the Benguela upwelling system were tested. Little to no increase in archaeal lipid yields were observed in these samples (Fig. 6). Given that significantly higher archaeal yields were observed in the cultures, this suggests that most archaeal lipids in marine surface sediments are not associated with intact cells. Previous studies have shown that proteins, the main target of degradation using freeze–thaw cycles with CTAB, are quickly degraded during export from the water column (Keil and Kirchman, 1999). Accordingly, the protein layer is likely degraded before the archaeal biomass reaches the ocean floor or consumed quickly within the sediments, making protein-denaturing detergents, such as CTAB unnecessary. Our results support the notion that a significant fraction of archaeal IPLs in marine sediments may represent detrital archaeal lipids transported from the water column (Schouten et al., 2010; Lengger et al., 2012a; Xie et al., 2013).

Alternatively, the complex sediment matrix could play an important role and affect the efficiency of the modified protocol. For instance, CTAB, as a strong cationic compound, might be deactivated by sedimentary minerals. Moreover, the complex organic matrix in environmental samples could lower the efficiency of CTAB in these samples. Finally, the envelope composition of benthic archaea may differ from those of archaea grown in pure culture. Future experiments are needed to verify if detergents, such as CTAB, can help to denaturize the cell envelope of archaea in sediment samples and whether the modified protocol requires further adjustments for these types of samples. For instance, a combination of different additives during the freeze–thaw, e.g., combining CTAB with EDTA, which gave the second-highest yields in the model organism, could result in higher archaeal lipid yields in environmental samples (Fig. 2C). Furthermore, the application of additional solvents on mechanical pretreatments that were not tested in this study, such as methyl *tert*-butyl ether (Matyash et al., 2008; Gorgich et al., 2020) and/or a French press, could facilitate the extraction efficiency in marine sediments.

4.3.2. Suspended particulate matter

The test extractions with SPM collected on filters from two depths in the South Atlantic showed an increase in the total archaeal lipid abundance due to the pretreatment with CTAB and freeze–thaw cycles compared to experiments extracted solely with the TCA buffer (Fig. 7). Nevertheless, only traces of glycosidic IPLs were detected in the filters. A previous study by Hurley et al. (2018) using samples from the same location reported glycosidic and phosphatidic GDGTs after microwave extraction with DCM:MeOH (9:1; v/v). However, Hurley et al. (2018) measured archaeal IPLs by multiple reaction monitoring with a triple quadrupole-MS system allowing more sensitive detection of targeted compounds.

In the experiments with the modified method, we further observed strong contamination of CTAB in the HPLC–MS analysis of the SPM

filters (Supplementary Fig. S4). In the filter samples, contamination of CTAB was detected for the first 40 min of the run, thus hindering the detection of archaeols and the injection standards in these samples (Supplementary Fig. S4). An evaluation of the culture extracts showed that CTAB was also detectable in the LC–MS runs, particularly when high TLE quantities (> 20% TLE) were injected (Supplementary Fig. S4). However, in the culture extracts, CTAB commonly eluted within the first 20 min of the analysis. The reason for the particularly strong contamination in the SPM filters samples is unclear. One explanation might be the nature of the filter material. CTAB could be adsorbed onto the glass fiber surface or particles on the filters during the pretreatment and then released during ultrasonic extraction, which could be solved in future analysis by scraping off the biomass from the filter. Moreover, the contamination could originate from the low quantities of biomass on the SPM filters collected in the oligotrophic Central Atlantic Ocean (e.g., Gasol et al., 2009). Due to the low amounts of biomass on the SPM filters, less CTAB might be bound on the protein layer, resulting in a high concentration of 'free CTAB'. The 'free CTAB' can be readily extracted by the solvent mixture and, thus, injected into the HPLC–MS system. Although the contamination needs to be considered for future analysis, the substantial increase in tetraether lipids demonstrates the advantage of the modified method.

5. Conclusions

The extraction of archaeal IPLs using conventional modifications to the Bligh and Dyer protocol presents an incomplete picture for cultured archaea. A significant fraction, particularly GDGTs, is not recovered using the Bligh and Dyer extraction technique. Our improvements of the protocol enhanced archaeal lipid yields by up to 8 times in cultures. Further, we observed that the abundances of some IPL classes increased by more than an order of magnitude, and certain IPLs were only detected when the modified method was applied. This study demonstrates the benefit of detergents, such as CTAB to more comprehensively assess the lipidomes of archaea. However, the results further show that there is potential for future studies to increase archaeal lipid recoveries. For instance, exchanging the TCA buffer, which might degrade phospholipids during extraction, with novel, less aggressive buffers, could increase the extraction efficiency of phospholipids. Additionally, removing the CTAB after the extraction, which hinders the analysis of IPLs in the SPM-filters, further improves the proposed modified protocol. We anticipate that the methodology presented here will reduce sample size requirements for archaeal cultures, enable the discovery of novel IPLs, and thus contribute to enhanced understanding of archaeal lipids.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.orggeochem.2021.104353.

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