Intakte polare Lipide
–
Möglichkeit und Grenzen ihrer Verwendung zur Analyse mikrobieller Gemeinschaften.

Intact polar lipids
–
chances and limitations of their application to the analysis of microbial communities.

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Kurzfassung

Im Rahmen dieser Doktorarbeit wurden grundlegende Fragen zur Analytik und Verwendung von intakten polaren Lipiden als Biomarker für lebende Mikroorganismen bearbeitet. Zu Beginn wurden die zur Analyse von intakten polaren Lipiden bekannten Methoden überprüft und Untersuchungen zu ihrer Verbesserung vorgenommen. Es wurde gezeigt, dass eine Verbesserung der Wiederfindung von intakten polaren Lipiden (IPL) bei säulenchromatographischer Auftrennung von Sedimentextrakten an Kieselgel durch die Zugabe eines geringen Anteils von Wasser zum Eluenten möglich ist. Aufgrund der chemischen Diversität innerhalb der Gruppe der intakten polaren Lipide war es trotz aller Anstrengungen jedoch nicht möglich, die Reproduzierbarkeit der Analysen so zu verbessern, dass die Ergebnisse nicht durch die Aufarbeitung beeinflusst werden. Daher sollte, sofern es die zu untersuchenden Proben und die analytischen Möglichkeiten zulassen, auf eine Fraktionierung von Lipidextrakten verzichtet werden.

Ein weiteres zentrales Thema dieser Arbeit war die grundsätzliche Frage der Stabilität von intakten polaren Lipiden nach dem Tod von Mikroorganismen. Für die Anwendbarkeit von intakten polaren Lipiden zur Charakterisierung von Mikroorganismengemeinschaften ist es essenziell, dass diese Lipide rasch nach dem Tod der Zelle abgebaut werden, da sonst die Untersuchungsergebnisse durch die IPL vor längerer Zeit abgestorbener Organismen verfälscht werden. Ein besonderer Fokus bei der Bearbeitung dieses Themas lag dabei auf der Frage, ob sich die Stabilität der intakten polaren Lipide von Bakterien von denen der Archaeen unterscheidet und welchen Einfluss die neuen Ergebnisse auf Untersuchungen von intakten polaren Lipiden in Sedimenten der tiefen Biosphäre haben. Die Ergebnisse dieser Studie zeigten deutliche Unterschiede in der Stabilität der beiden untersuchten Membranlipidgruppen, wobei Archaeen-Lipide eine höhere Stabilität aufweisen. Allerdings wurde nicht abschließend geklärt, inwiefern dies die tatsächliche Anwendbarkeit von intakten polaren Lipiden als Biomarker zur Charakterisierung von Mikroorganismengemeinschaften einschränkt. Zwar wiesen die archaeellen Lipide im Zeitraum des Versuchs eine deutlich höhere Stabilität auf, es ist jedoch unklar, wie sich diese Ergebnisse auf Sedimente mit vergleichsweise kleinen mikrobiellen Umsatzraten wie in der tiefen Biosphäre übertragen lassen.

Ein weiteres Projekt dieser Arbeit beschäftigt sich mit den Membrananpassungsstrategien des Tiefseebakteriums *Desulfovibrio indonensiensis* an verschiedene Inkubationsbedingungen. Dabei wurde eine abgestufte Anpassung der Membranlipide festgestellt. Bei niedriger Inkubationstemperatur (25°C) wurde von *Desulfovibrio indonensiensis* ein hoher Anteil an

ungesättigten verzweigten und unverzweigten Fettsäuren synthetisiert. Bei einer höheren Inkubationstemperatur (35°C) wurde an Stelle der ungesättigten Fettsäuren ein größerer Anteil an verzweigten gesättigten Fettsäuren und bei der höchsten Temperatur (45°C) ein höherer Anteil an unverzweigten gesättigten Fettsäuren produziert. Neben der Fettsäurezusammensetzung wurden zusätzlich auch die IPL-Gehalte bestimmt. Die stärksten Unterschiede bei den unterschiedlichen Inkubationsbedingungen zeigten IPL mit Phosphatidylglycerol und Phosphatidylethanolamin. Dabei war der Einfluss der Inkubationstemperatur größer als der Inkubationsdruck. Der Einbau von Ornithinlipiden war vorwiegend temperaturgesteuert und nicht, wie zuvor von einigen Autoren vermutet, durch Phosphatlimitierung bedingt. Neben den für Bakterien typischen IPL wurden auch vier neue und bisher unbeschriebene Lipide identifiziert und massenspektrometrisch charakterisiert.

Im letzten Projekt dieser Arbeit wurde die mikrobiologische Gemeinschaft im hypersalinen See Lake Tyrrell (Victoria, Australien) auf ihre Fettsäure- und IPL-Zusammensetzung untersucht. Die im Lake Tyrrell vorkommenden IPL-Kopfgruppen (PGP-Me, PGS, PGP-Me, S-DGD, S_2-DGD und PG) unterscheiden sich zu großen Teilen von denen aus weniger extremen Habitaten. Neben vielen bekannten IPL-Kopfgruppen wurden zwei weitere (PI und PIP) identifiziert, die bisher in hypersalinen Habitaten unbekannt waren. Die im Lake Tyrrell vorkommenden IPL wurden mit denen anderer hypersaliner Habitate verglichen. Dabei wurde eine weitgehende Überlappung des IPL-Pools festgestellt. Zusätzlich wurde anhand von Literaturdaten eine Datenbank mit IPL-Daten von Reinkulturen hypersaliner Archaeen erstellt und mit den in Lake Tyrrell vorhandenen IPL verglichen.

Abstract

This work covers a broad spectrum of investigations regarding methodical problems, developments and applicability in the analysis of intact polar lipids as biomarkers for microbial life. Moreover, suggestions for the use and application of intact polar lipids (IPLs) in the field of organic geochemistry are being made.

Traditionally, crude extracts of sediments or microbial biomass are separated in to different fractions before being further analyzed with a combined LC-MS method. To improve the separation various experiments were performed and evaluated. It turned out that the recovery of IPLs can be improved by the addition of a small proportion of water to the eluent of the IPL fraction. By comparison of many separations using pure standard material it appeared that the achieved reproducibility nevertheless was low and no clear reason for this behaviour was found. The results suggest that, whenever possible, extracts of IPLs should be investigated directly by LC-MS analysis without any previous sample fractionation.

To use IPLs as biomarkers for living cells the fundamental precondition is that these lipids degrade quickly after cell death. Furthermore, to answer the interesting question if Bacteria or Archaea dominate certain habitats it is important that the IPLs of both groups of organisms degrade at approximately the same rate. Since Bacteria and Archaea possess structurally different IPLs empiric results are crucial for the use of IPLs for this purpose. The results of this study indeed show differential degradation rates for IPLs with bacterial or archaeal origin. However, it needs to be demonstrated how these results influence the interpretation of IPL investigations from the deep biosphere, because the results obtained during this work cover a very small timescale of only three months. Since the turn-over of microbial biomass especially in the deep biosphere is very low, it is likely that the chemical signal of these communities is not biased by fossil IPLs. In any case, this study warrants caution in order not to overrate the results of IPL investigations.

In a further project the changes in IPL and fatty acid composition of the deep sea bacterium *Desulfovibrio indonensiensis*-affiliated strain P23 were investigated according to changes in incubation pressure and temperature. At both investigated incubation pressures a stepwise change in the fatty acid pattern of *Desulfovibrio indonensiensis* was observed with increasing temperature. At low incubation temperatures (25°C) many unsaturated banched and unbranched fatty acids were produced by the organism. At elevated temperatures (35°C) higher amounts of saturated branched fatty acids and subsequently at the highest incubation temperatures (45°C) more saturated unbranched moieties were incorporated into the IPLs. Additionally, changes in IPL head groups were monitored as function of the cultivation

conditions. The head groups phosphatidylglycerol and phosphatidylethanolamine appeared to be major regulating lipids in sustaining appropriate fluidity. The incorporation of ornithine lipids was mainly temperature dependent and not, as suggested before, related to phosphate limitation. Additionally, four previously undescribed IPLs were found.

The last project of this thesis investigates the microbial community in hypersaline Lake Tyrrell (Victoria, Australia). This community is dominated by Archaea but also some Bacteria are present. The most abundant known archaeal IPLs are PGP-Me-DPG, PGS-DPG, PGP-Me-PSG, S-DGD, S_2-DGD and PG-DPG. Also abundant, but reported for the first time from hypersaline systems, are the inositol-based IPLs PI-DPG and PIP-DPG as well as an unknown archaeal IPLs carrying two sugar moieties. A broad overlap was found when the IPL content of Lake Tyrrell was compared to that of other hypersaline habitats. Additionally, a database containing detailed information on IPLs of all cultured hypersaline Archaea was created and compared to the IPL content of Lake Tyrrell.

Inhaltsverzeichnis

Verzeichnis eigener Publikationen

Das Kapitel 3 der vorliegenden Arbeit wurde in einer Fachzeitschrift publiziert. Die Kapitel 5 und 7 wurden in Manuskriptform geschrieben und stehen kurz vor der Übermittlung an zwei unterschiedliche Fachzeitschriften.

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

* gleichberechtigte Erstautoren

Logemann, J.: Versuchsdesign, Planung, Projektleitung, Lipidanalytik und Erstellung des Manuskripts.

Graue J.: Versuchsplanung, Kultivierung, mikrobiologische Untersuchungen (RNA, Fermentationsprodukte), Headspace-Analysen und Erstellung des Manuskripts.

Köster, J. und Engelen, B.: Versuchsplanung und Überarbeitung des Manuskripts.

Cypionka, H. und Rullkötter, J.: Überarbeitung des Manuskripts.

K. Fichtel*, J. Logemann*, J. Fichtel, H. Cypionka and B. Engelen: High-pressure adaptation of sulfate-reducing bacteria from fluid-influenced sediments of the deep sub-seafloor biosphere. In Vorbereitung zum Einreichen bei FEMS Microbiology Ecology, Wiley.

* gleichberechtigte Erstautoren

Logemann, J.: Lipidanalytik und Interpretation aller Lipiddaten, Grafikerstellung, Manuskripterstellung und -überarbeitung.

Fichtel, K. und Engelen B.: Projektplanung, Kultivierung und Manuskripterstellung.

Fichtel, J.: Fettsäureanalytik.

Rullkötter, J.: Manuskriptüberarbeitung.

Cypionka, H.: Manuskript-überarbeitung.

J. Logemann, K. Andrade, E. Allen, S. George, K. Heidelberg, J. Banfield, J. Rullkötter and J. Brocks: Microbial membrane lipid dynamics on a day-night cycle in Lake Tyrrell - a hypersaline lake in Victoria, Australia. In Vorbereitung zum Einreichen bei Extremophiles, Springer.

Logemann, J.: Lipidanalytik und Erstellung des Manuskripts.

Brocks, J.: Projektplanung, Probennahme, Manuskriptkonzept und Manuskriptüberarbeitung.

Banfield, J.: Projektplanung, Probennahme und Manuskriptüberarbeitung.

Rullkötter, J., Allen, E. und George, S.: Manuskriptüberarbeitung.

Tagungsbeiträge

K. Andrade, J. Logemann, J. Brocks, C.S. Miller, B. C. Thomas , K. Heidelberg, J.F. Banfield, 2012. Diel periodicity in aquatic hypersaline microbial communities in Lake Tyrrell, Victoria, Australia. 112th General Meeting of the American Society for Microbiology (ASM), San Francisco, USA, Poster.

J. Logemann, J. Graue, J. Köster, B. Engelen, H. Cypionka und J. Rullkötter, 2011. Degradation of intact polar lipids in sandy sediments: insights from a laboratory experiment. 25th International Meeting on Organic Geochemistry (IMOG), Interlaken, Schweiz, Vortrag.

A. Böll, B. Scholz-Böttcher, J. Logemann und J. Rullkötter, 2011. Terrestrial organic matter in the sediments of the German Bight – estimate of relative proportions using the BIT index in comparison to other proxies. 25th International Meeting on Organic Geochemistry (IMOG), Interlaken, Schweiz, Poster.

K.-U. Hinrichs, S. Sievert, N. Buchs, J. S. Lipp, J. F. Biddle, A. Boetius, B. A. Cragg, H. Cypionka, B. Engelen, B. Horsfield, F. Inagaki, J. Kallmeyer, K. Knittel, J. Logemann, K. Mangelsdorf, Y. Morono, R. J. Parkes, J. Rullkötter, A. Schippers, A.P. Teske, G. Webster, A. Weightman, 2011. Culture-independent analysis of prokaryotic biomass in the sub-seafloor biosphere: an interlaboratory and inter-method comparison. 8th International Symposium of Subsurface Microbiology (ISSM), Garmisch-Partenkirchen, Deutschland, Poster.

J. Logemann, K. Andrade, E. Allen, S. George, K. Heidelberg, J. Banfield, J. Rullkötter and J. Brocks, 2011. Microbial membrane lipid dynamics on a day-night cycle in Lake Tyrrell a hypersaline lake in Victoria, Australia. North German Meeting on Organic Geochemistry, MARUM, Bremen, Deutschland, Vortrag.

J. Logemann, J. Graue, J. Köster, B. Engelen, H. Cypionka und J. Rullkötter, 2010. Degradation of intact polar lipids in sandy surface sediments. Arbeitsgruppen-Seminar der Arbeitsgruppe "Organische Geochemie" von Prof. Kai-Uwe Hinrichs, MARUM, Universität Bremen, Deutschland, Vortrag.

J. Logemann, J. Graue, J. Köster, B. Engelen, H. Cypionka und J. Rullkötter, 2010. Degradation of intact polar lipids in sandy surface sediments. Gordon Research Conference on Organic Geochemistry, Holderness, NH, USA, Vortrag.

J. Logemann, M. Seidel, T. Riedel, J. Köster und J. Rullkötter, 2009. Lipid distribution patterns in a 19 m sediment core from the tidal flat system of Northwest Germany. 24th International Meeting on Organic Geochemistry (IMOG), Bremen, Deutschland, Poster.

J. S. Lipp, N. Buchs, J. F. Biddle, A. Boetius, B. A. Cragg, H. Cypionka, B. Engelen, A. Weightman, B. Horsfield, F. Inagaki, J. Kallmeyer, K. Knittel, J. Logemann, K. Mangelsdorf, L. McKay,Y. Morono, R. J. Parkes, J. Rullkötter, A. Schippers, A. P. Teske, G. Webster, S. Sievert, K.-U. Hinrichs, 2009. Analysis of prokaryotic biomass analysis in the sedimentary biosphere: an interlaboratory comparison of methods and analytical techniques. 24th International Meeting on Organic Geochemistry (IMOG), Bremen, Deutschland, Poster.

K. Fichtel, J. Logemann, J. Fichtel, H. Cypionka and B. Engelen, 2009. High-pressure adaptation of sulfate-reducing bacteria from fluid-influenced sediments of the deep sub-seafloor biosphere. Annual Meeting of the Association for General and Applied Microbiology (VAAM), Bochum, Deutschland, Poster.

Abbildungsverzeichnis

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Abkürzungsverzeichnis

AEG	*Acyl-/Etherglycerol*
CARD-FISH	*Catalyzed reporter deposition-fluorescence* in-situ *hybridization*
CL	*Cardiolipin oder Diphosphatidylglycerol*
DAG	*Diacylglycerol*
DG	*Diglycosyllipid*
DGGE	*Denaturierende Gradientengelelektrophorese*
DNA	*Desoxyribonukleinsäure*
DPG	*Diphytanylglyceroldiether*
ESI	*Elektrosprayionisierung*
FAME	*Fettsäuremethylester*
GC-FID	*Gaschromatographie Flammenionisierungsdetektor*
GC-MS	*Gaschromatographie Massenspektrometrie*
GDGT	*Glyceroldiphytanylglyceroltetraether*
HPLC	*Hochleistungsflüssigkeitschromatographie*
LC-MS	*Flüssigkeitschromatographie Massenspektrometrie*
IPL	*Intaktes polares Lipid / Intakte polare Lipide*
MG	*Monoglycosyllipid*
OL	*Ornithinlipid*
PA	*Phosphatidylsäure*
PC	*Phosphatidylcholin*
PDME	*Phosphatidyl-(N,N-dimethyl)-ethanolamin*
PE	*Phosphatidylethanolamin*
PG	*Phosphatidylglycerol*
PGP-Me	*Phosphatidylglycerolphosphatidylmethylester*
PGS	*Phosphatidylglycerolsulfat*
PI	*Phosphatidylinositol*
PIP	*Phosphatidylinositolphosphat*
PLFA	*Phospholipid fatty acid*
PMME	*Phosphatidyl-(N-monomethyl)-ethanolamin*
PS	*Phosphatidylserin*
PSG	*Phytanylsesterterpanylglyceroldiether*
q-PCR	*Quantitative Polymerasekettenreaktion*
rRNA	*Ribosomale Ribonukleinsäure*
SL	*Sulfonolipid*
SQ	*Sulfochinovosyllipid*
SRB	*Sulfatreduzierendes Bakterium*
TeG	*Tetragylcosyllipid*
TG	*Triglycosyllipid*
TOC	*Total organic carbon*

1. Einleitung

1.1. Die Bedeutung von Mikroorganismen

Mikroorganismen sind ubiquitär. Sie existieren auf der Erde bereits seit mehreren Milliarden Jahren und sind damit evolutionärer Ursprung für alle höheren Lebewesen. Erst durch mikrobielle Aktivität wurden die Voraussetzungen für das heutige Gesamtökosystem Erde wie beispielsweise die Präsenz von Sauerstoff geschaffen. Damit hängen die Existenz und das Fortbestehen aller höheren Lebensformen untrennbar mit dem Vorhandensein von Mikroorganismen zusammen (Madigan et al., 2006).

Aufgrund ihrer Bedeutung ist es erstrebenswert, die Funktionen, Lebensweise und Interaktionen von Mikroorganismen und Mikroorganismengemeinschaften eingehend zu untersuchen, um auf diese Weise ein besseres Verständnis für unsere Umwelt zu erlangen.

1.2. Charakterisierung von mikrobiellen Gemeinschaften

1.2.1. Kultivierungsabhängige und molekularbiologische Charakterisierung von Mikroorganismen

Die Beschreibung von mikrobiologischen Gemeinschaften kann auf unterschiedliche Arten erfolgen. Dabei stellen kultivierungsabhängige und kultivierungsunabhängige Methoden zwei grundsätzlich unterschiedliche Herangehensweisen dar.

Die Grundlage der kultivierungsabhängigen Charakterisierung von mikrobiellen Gemeinschaften ist die Gewinnung von Mikroorganismen in Reinkulturen. Auf diesem Weg soll die Rolle des einzelnen Vertreters einer komplexen Gemeinschaft beurteilt werden (Giovannoni & Stingl, 2007). Die Suche nach optimalen Kultivierungsbedingungen für bestimmte Mikroorganismen ist jedoch zeit- bzw. arbeitsaufwendig und gelingt nicht immer. Schon die Wahl der Kultivierungsbedingungen für die Gewinnung von Reinkulturen beinhaltet eine unvermeidliche Diskriminierung oder Bevorzugung einzelner oder Gruppen von Mikroorganismen. Problematisch ist dabei, dass eine ganze Reihe von Mikroorganismen sich aufgrund des Fehlens syntropher Partner nicht in Reinkultur gewinnen lassen. Dies hat zur Folge, dass nur wenige Prozent aller Mikroorganismen als Reinkultur vorliegen (Amann et al., 1995; D'Hondt et al., 2004). Weiterhin kann sich das Verhalten eines Organismus in Reinkultur von seiner Rolle in der natürlichen Gemeinschaft unterscheiden (Zak et al., 1994; Chowdhury & Dick, 2012).

Die phylogenetische und zum Teil die physiologische Charakterisierung mikrobieller Gemeinschaften erfolgt heute ohne Kultivierung, im Wesentlichen über DNA- oder RNA-basierte Methoden. Eine Möglichkeit ist die Extraktion und Vervielfältigung von bestimmten

RNA- oder DNA-Abschnitten durch PCR (engl. *polymerase chain reaction*) oder qRT-PCR (engl. *real-time quantitative PCR*) und nachfolgender Auftrennung der erhaltenen Abschnitte durch DGGE (engl. *denaturing gradient gel electrophoresis*). Eine Sequenzierung, also die Bestimmung der Nukleotid-Abfolge der aufgetrennten PCR-Produkte und ein Vergleich dieser Informationen mit bekannten und kultivierten Mikroorganismen erlaubt es, die häufigsten Vertreter in der untersuchten Gemeinschaft zu bestimmen. Diese Methode bietet so die Möglichkeit, einen vergleichsweise schnellen Überblick über die wichtigsten Vertreter in einer Mikroorganismengemeinschaft zu erhalten. Ein Nachteil dieser Methode ist jedoch, dass nicht zwischen DNA aus lebenden und abgestorbenen Zellen unterschieden werden kann (Coolen & Overmann, 1998; Dell'Anno et al., 1998). Zusätzlich ist es möglich, dass es bei der Vervielfältigung der extrahierten DNA-Fragmente zu der Bildung von Artefakten kommt oder Teile der extrahierten DNA stärker vervielfältigt werden als andere (v. Wintzingerode et al., 1997).

Dem gegenüber stehen Methoden, bei denen mit einem Fluoreszenzfarbstoff versehene molekulare Sonden in intakte Zellen eingebracht werden und dort mit der ribosomalen RNA reagieren (z.B. Amann et al., 1992; Pernthaler et al., 2002). Mit diesen als FISH- (engl. *fluorescence in situ hybridization*) oder CARD-FISH-Verfahren (engl. *catalyzed reporter deposition fluorescence in situ hybridization*) bezeichneten Untersuchungsmethoden können so anhand ihrer RNA-Signatur ausgewählte und fluoreszenzmarkierte Zellen mit einem Fluoreszenz-Mikroskop sichtbar gemacht werden (Madigan et al., 2006). Diese Methode eignet sich besonders gut, wenn die Verteilung von bestimmten Mikroorganismengruppen untersucht werden soll. Die Grundvoraussetzung für die Anwendung dieser Methode ist allerdings das mit der Aktivität von Zellen verknüpfte Vorhandensein von RNA. Inaktive oder Zellen mit sehr langsamem Stoffwechsel können nur schlecht erfasst werden (Souza et al., 2007). Weiterhin lassen sich die molekularen Sonden aufgrund von Strukturunterschieden der äußeren Zellmembran unterschiedlich gut in Zellen verschiedener phylogenischer Stämme einschleusen (Amann et al., 1992). Da eine mikrobielle Gemeinschaft natürlicherweise eine große Vielzahl verschiedener Mikroorgansimengruppen enthält, besteht die Möglichkeit, dass ihre Zusammensetzung nicht korrekt wiedergegeben wird.

Die Spezifizität der CARD-FISH-Methode lässt sich über die Auswahl und Länge der an den Fluoreszenzfarbstoff gebundenen Genabschnitte steuern. Besonders bei spezifischen Gensonden muss sichergestellt werden, dass der gewählte Genabschnitt für den Zielorganismus indikativ ist (DeLong et al., 1989; Yilmaz & Noguera, 2004; Amann & Fuchs, 2008). Eine entsprechende Vorbereitung und Absicherung dieser Analysenmethode ist damit notwendig und zeitintensiv.

1.2.2. Analyse von intakten polaren Lipiden als alternative, kultivierungs-unabhängige Methode zur Charakterisierung von Mikroorganismen

Der Hauptgegenstand dieser Arbeit ist die Analyse von intakten polaren Lipiden (IPL), die eine alternative und kultivierungsunabhängige Methode zur Untersuchung von Mikroorganismen in Reinkulturen oder komplexen Gemeinschaften darstellt (Black et al., 1997; Fang & Barcelona, 1998). Im Gegensatz zu den zuvor dargestellten Methoden wird hier nicht ein Teil der in jeder Zelle vorkommenden genetischen Information als Basis genutzt, sondern eine Unterscheidung von Mikroorganismengruppen anhand der Lipidzusammensetzung ihrer Cytoplasmamembranen angestrebt. Zwar ist die erreichte (chemo)-taxonomische Auflösung dieser Methode im Vergleich zu DNA- oder RNA-basierten Methoden geringer, ein bedeutender Vorteil ist jedoch die mögliche gleichzeitige Erfassung des „chemischen" Signals von komplexen Mikroorganismengemeinschaften. So können beispielsweise die Verhältnisse von Bakterien und Archaeen (Biddle et al., 2006) und zusätzlich das Auftreten methanogener Archaeen (Rossel et al., 2008) durch eine Analyse bestimmt werden. Dies wäre bei DNA- oder RNA-basierten Methoden aufwendiger.

Eine wichtige grundsätzliche Voraussetzung für die Anwendung intakter polarer Lipide als Werkzeug zur Beschreibung lebender Mikroorganismengemeinschaften ist ein nach dem Zelltod zeitlich schneller und mit vergleichbaren Raten ablaufender Abbau dieser zum Teil strukturell unterschiedlichen Verbindungen (Rütters et al., 2002). Ohne diese Voraussetzung würde es zu einer Verfälschung des Untersuchungsergebnisses durch fossile Lipide kommen (Lipp et al., 2008).

Die erste Veröffentlichung der Analyse von intakten polaren Lipiden aus Mikroorganismen über Massenspektrometrie mit Elektrosprayionisierung wurde von Smith und Mitarbeitern (1995) verfasst. Zuvor wurde die Analyse von intakten Membranlipiden überwiegend durch dünnschichtchromatographische Auftrennung von Zellextrakten und anschließende Anfärbung mit unterschiedlichen Reagenzien erreicht (z.B. White & Frerman, 1967). So können Lipide, die Phosphat in ihren Kopfgruppen enthalten, sehr gut von Lipiden mit zucker- und oder sulfathaltigen Kopfgruppen unterschieden werden. Durch den Vergleich der Auftrennungsergebnisse unbekannter Proben mit denen von Standardsubstanzen ist eine vergleichsweise exakte Bestimmung vieler Lipide möglich. Diese Methode wird auch heute für die schnelle und einfache Charakterisierung unbeschriebener Bakterien und Archaeen verwendet (Feng et al., 2005; Gutiérrez et al., 2011). Im Gegensatz zu modernen LC-ESI-MS Verfahren ist es mit dieser Methode jedoch nicht möglich, die Zusammensetzung der Fettsäureseitenketten oder die Verteilung von intakten polaren Lipiden innerhalb von intakten polaren Lipiden mit derselben Kopfgruppe zu bewerten. Auch die Strukturaufklärung unbekannter Substanzen ist nicht möglich.

Alternativ ist die Hydrolyse von intakten polaren Lipiden zu Fettsäuren (PLFA, engl. *phospholipid fatty acid*) mit anschließender gaschromatographischer Untersuchung möglich (z.B. Marr & Ingraham, 1962; Langworthy et al., 1974; Guckert et al., 1985). Diese Methode wird zum Teil bei der Beschreibung bisher unbekannter Stämme verwendet, um Fettsäureprofile zu erstellen. Die Profile werden als chemischer Fingerabdruck für die Identifizierung von Mikroorganismen genutzt (Zelles, 1997; Buyer & Sasser, 2012). Da sich die Fettsäuremuster in vielen Mikroorganismen überschneiden, können mit dieser Methode nur Aussagen über vergleichsweise einfach zusammengesetzte mikrobielle Gemeinschaften getroffen werden. In komplexen Gemeinschaften ist die Identifizierung von Mikroorganismen möglich, die sehr spezielle und seltene Markerfettsäuren produzieren (Zelles, 1999). Ein weiterer Nachteil der PLFA-Methode ist der Informationsverlust, der durch das Abspalten der apolaren Seitenketten vom Glycerolgrundgerüst mit der jeweiligen Kopfgruppe entsteht (Rütters et al., 2002).

1.2.3. Gegenseitige Kontrolle zur Überprüfung von Analysenergebnissen

Ein Problem bei der Anwendung der Methoden zur Charakterisierung von Mikroorganismengemeinschaften stellen mögliche gegensätzliche Aussagen dar. So zeigten die Untersuchungen von Schippers et al. (2005) und Biddle et al. (2006) bei der Analyse von identischem Probenmaterial mit zwei verschiedenen Untersuchungsmethoden gegensätzliche Ergebnisse. Schippers et al. (2005) kamen aufgrund von molekulargenetischen Untersuchungen auf der Basis von CARD-FISH- und q-PCR-Methoden zu dem Schluss, dass die mikrobielle Gemeinschaft in den untersuchten Sedimentproben überwiegend aus Bakterien bestand. Nach Ergebnissen, die auf der Basis der Analyse von intakten polaren Lipiden und der FISH-Methode an denselben Sedimentkernen erzielt wurden, war die mikrobiologische Gemeinschaft jedoch von Archaeen dominiert (Biddel et al. 2006).

Unabhängig von der Richtigkeit der Ergebnisse beider Studien zeigt diese kontroverse Interpretation der im betreffenden Habitat vorherrschenden mikrobiellen Gemeinschaft die Notwendigkeit der Kontrolle von Ergebnissen über komplementäre Methoden. Eine solches, sich gegenseitig kontrollierendes Paar von Methoden stellt die Analyse von intakten polaren Lipiden und die DNA- oder RNA-basierte Untersuchung von Mikroorganismengemeinschaften dar.

1.3. Die Cytoplasmamembran

Intakte polare Lipide sind integraler Bestandteil der Cytoplasmamembranen aller lebenden Zellen. Diese Membran sorgt für die chemische Abgrenzung einer Zelle von ihrer Umgebung. Je nach Organismus kann der strukturelle Aufbau der Cytoplasmamembran auf zwei unterschiedliche Arten erfolgen – als Lipid-Doppelschicht oder Lipid-Einfachschicht (Cypionka,

2006; Madigan et al., 2006). Es existieren zwei unterschiedliche Typen von intakten polaren Lipiden, wobei in beiden Typen Glycerol das Rückgrat der Moleküle darstellt. Im ersten Fall sind an das Grundgerüst zum einen eine sogenannte polare Kopfgruppe und zum anderen zwei apolare Acyl- oder Alkylreste gebunden. Diese Moleküle formen Cytoplasmamembranen als Lipid-Doppelschicht. Dem gegenüber stehen Lipide, die aus zwei Glycerolmolekülen aufgebaut sind. Die Glycerole sind hier über zwei langkettige Alkylreste kovalent miteinander verknüpft, und die dritte Hydroxyl-Gruppe der Glycerolmoleküle ist mit je einer polaren Kopfgruppe gebunden. Organismen, die diese Moleküle synthetisieren, verfügen über eine einschichtige Cytoplasmamembran (Lipid-Einfachschicht) (Cypionka, 2006; Madigan et al., 2006).

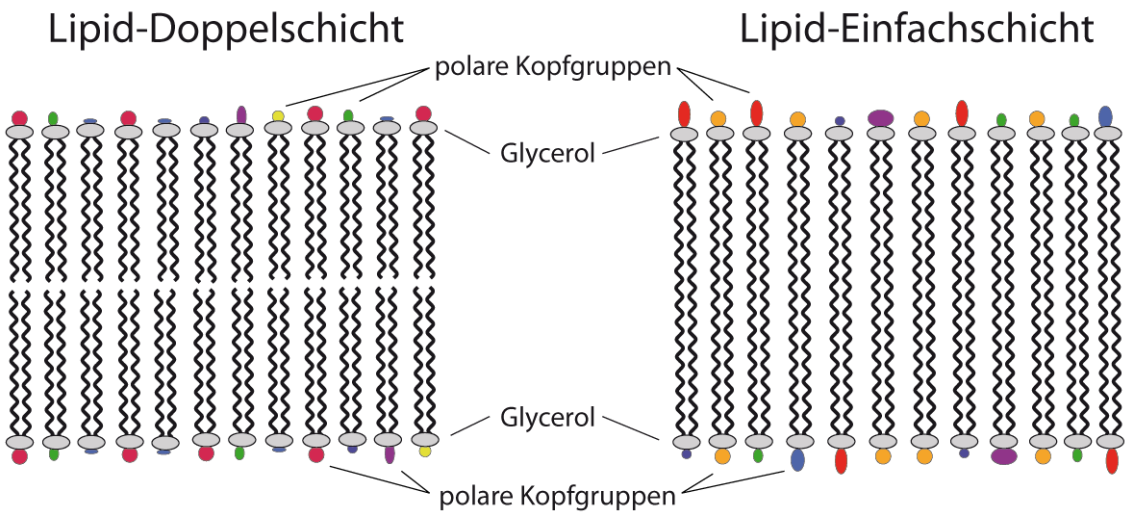


Abbildung 1.1: Schematischer Aufbau von Cytoplasmamembranen (verändert nach Cypionka, 2006, sowie Madigan et al., 2006).

Die Lipid-Doppelschicht ist sehr weit verbreitet und kommt in allen Eukaryonten, der Mehrheit der Bakterien sowie in manchen Archaeen wie z.B. Mitgliedern der Familie der Halobacteriaceae (De Rosa et al., 1986) vor. Das Auftreten der Lipid-Einfachschicht ist beschränkt auf vergleichsweise wenige Bakterien wie z.B. Acidobakterien (Dedysh et al., 2012) sowie einen Großteil der Archaeen (De Rosa et al., 1986).

Cytoplasmamembranüberspannende Proteine sorgen für den interzellulären Transport von Substanzen. Zusätzlich sind Moleküle wie Steroide (bei Eukaryonten) und Hopanoide (bei Bakterien) in die Cytoplasmamembran integriert. Sie werden auch als Membranversteifer bezeichnet und sorgen für eine größere Festigkeit der Cytoplasmamembran (Campbell & Reece, 2003).

1.3.1. Intakte polare Lipide in Cytoplasmamembranen von Bakterien und Eukaryonten

Innerhalb der Domänen der Bakterien und Eukaryonten gibt es in Bezug auf die vorkommenden Molekülstrukturen von intakten polaren Lipiden eine weitreichende Überlappung. Im überwiegenden Teil aller Organismen dieser zwei Domänen werden intakte polare Lipide mit der in Abbildung 1.2 dargestellten chemischen Struktur gefunden. Die Kopfgruppe von bakteriellen und eukaryontischen intakten polaren Lipiden ist stets an der *sn*-3-Position des Glycerolgrundgerüsts gebunden und die apolaren Seitenketten an den *sn*-1- und *sn*-2-Positionen (Kates, 1977; De Rosa et al., 1986).

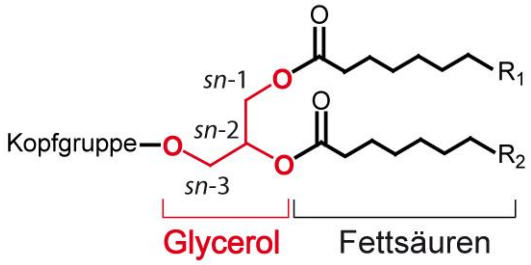


Abbildung 1.2: Chemische Struktur vieler bakterieller und eukaryontischer Cytoplasmamembran-Moleküle. $R_{1,2}$: Alkyl- oder Alkenketten mit einer Länge von 5 bis 17 Kohlenstoffatomen.

Einige wenige Vertreter der Bakterien besitzen Cytoplasmamembranen, die aus speziellen Lipiden aufgebaut sind (Abb. 1.3). So wurden beispielsweise von Rütters und Mitarbeitern (2001) sulfatreduzierende Bakterien gefunden, deren intakte polare Lipide je eine ether- und estergebundene apolare Seitenkette aufweisen (Abb. 1.3, oben). Weiterhin wurden für Archaeen typische membranüberspannende Lipide in Torfproben gefunden, die jedoch einem bakteriellen Ursprung zugeordnet wurden (Abb. 1.3, unten; Weijers et al., 2006). Nach neueren Forschungsergebnissen werden diese sogenannten bakteriellen Tetraether von Bakterien synthetisiert, die der Gruppe der Acidobakterien angehören und in Böden vorkommen (Sinninghe Damsté et al., 2011).

Das Auftreten dieser beiden Membranlipidtypen ist damit grundsätzlich diagnostisch für die entsprechenden Organismengruppen. Allerdings ist zu beachten, dass auch sulfatreduzierende Bakterien gefunden wurden, die ausschließlich estergebundene Seitenketten in ihren intakten polaren Lipiden enthalten (Rütters et al., 2001). Weiterhin können nur intakte bakterielle Tetraether als Anzeiger für das Vorhandensein von Acidobakterien dienen. Aufgrund der Stabilität des Grundgerüsts dieser bakteriellen Tetraether ergeben sich aber auch neue Anwendungsmöglichkeiten als Biomarker für den Eintrag terrestrischen organischen Materials in marine Systeme (Hopmans et al., 2004). So können diese Verbindungen durch Niederschlag zusammen mit anderen organischen Molekülen aus Böden herausgelöst werden, über die

Flüsse in marine Systeme gelangen und so zur Abschätzung des Eintrags von terrestrischem Material genutzt werden.

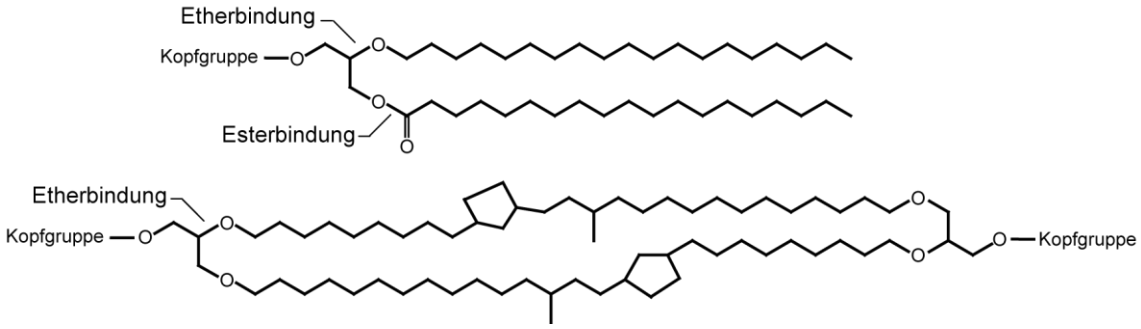


Abbildung 1.3: Spezielle Strukturen von intakten polaren Lipiden aus Bakterien und Eukaryonten.

Kopfgruppen von bakteriellen und eukaryontischen intakten polaren Lipiden

Die Kopfgruppen von intakten polaren Lipiden, die in Bakterien und Eukaryonten auftreten, enthalten häufig einen an das Glycerolgrundgerüst gebundenen Phosphatrest, der wiederum über ein Sauerstoffatom an unterschiedliche Reste gebunden sein kann (Abb. 1.4, oben). Diese sogenannten Phospholipide sind sehr weit verbreitet und kommen zusätzlich zu den beiden Hauptdomänen der Bakterien und Eukaryonten auch in der Domäne der Archaeen vor, oft als dominierender Kopfgruppentyp. Nach gegenwärtigem Stand des Wissens gibt es dabei für den Großteil der Phospholipidkopfgruppen keine eindeutige Zuordnung zu bestimmten Organismengruppen.

Weiterhin existiert eine ganze Reihe von Kopfgruppen, die kein Phosphat enthalten. Lipide mit Betainkopfgruppen treten vorwiegend in photosynthetisch aktiven Bakterien, Eukaryonten oder Pilzen auf (Dembitsky, 1996). Auch Sulfochinovosyllipide kommen in photosynthetisch aktiven Organismen wie beispielsweise höheren Pflanzen, Moosen, Farnen, Algen oder Cyanobakterien vor (Benning, 1998). Dabei hängt das Auftreten dieser Lipide nicht automatisch mit der Fähigkeit zur Photosynthese zusammen (Cedergren & Hollingsworth, 1994).

Glycoglycerolipide wie vor allem Monoglycosyl- (MG) und Diglycosyl-Kopfgruppen (DG) werden häufig in den Membranen von Chloroplasten höherer Pflanzen, eukaryotischer Algen und in Cyanobakterien gefunden (Hölzl & Dörmann, 2007). Bei Diglycolsyl-Kopfgruppen führt die Kombination von verschiedenen Zuckern wie α-, β-Glucose, α-, β-Galaktose oder α-Mannose durch unterschiedliche Verknüpfungen (1 --> 2, 1 --> 3, 1 --> 4 oder 1 --> 6) zu einer Vielzahl von Kopfgruppen. Diese strukturelle Variabilität, die die molekulare Masse der Glycolipide nicht und die Retentionszeit bei gängigen HPLC-Methoden kaum verändert, stellt eine besondere Herausforderung für die LC-ESI-MS-Analyse dar.

Ornithinlipide wurden in vielen Gram-negativen Bakterien nachgewiesen. In Gram-positiven Bakterien tritt dieses Membranlipid deutlich seltener auf (Geiger et al., 2010). Bei dem Bakterium *Sinorhizobium meliloti* wurde ein Zusammenhang zwischen Phosphatlimitierung und vermehrter Produktion von Ornithinlipiden gefunden (Weissenmayer et al., 2002). Die Autoren Dees und Shively (1982) fanden in *Thiobacillus thiooxidans* eine höhere Konzentration an Ornithinlipiden in der äußeren Membran als in der Cytoplasmamembran und vermuteten einen positiven Einfluss auf die Säureresistenz dieses Bakteriums. Die genaue Funktion dieser in ihrer chemischen Struktur besonderen Lipide ist bislang nicht geklärt (Geiger et al., 2010).

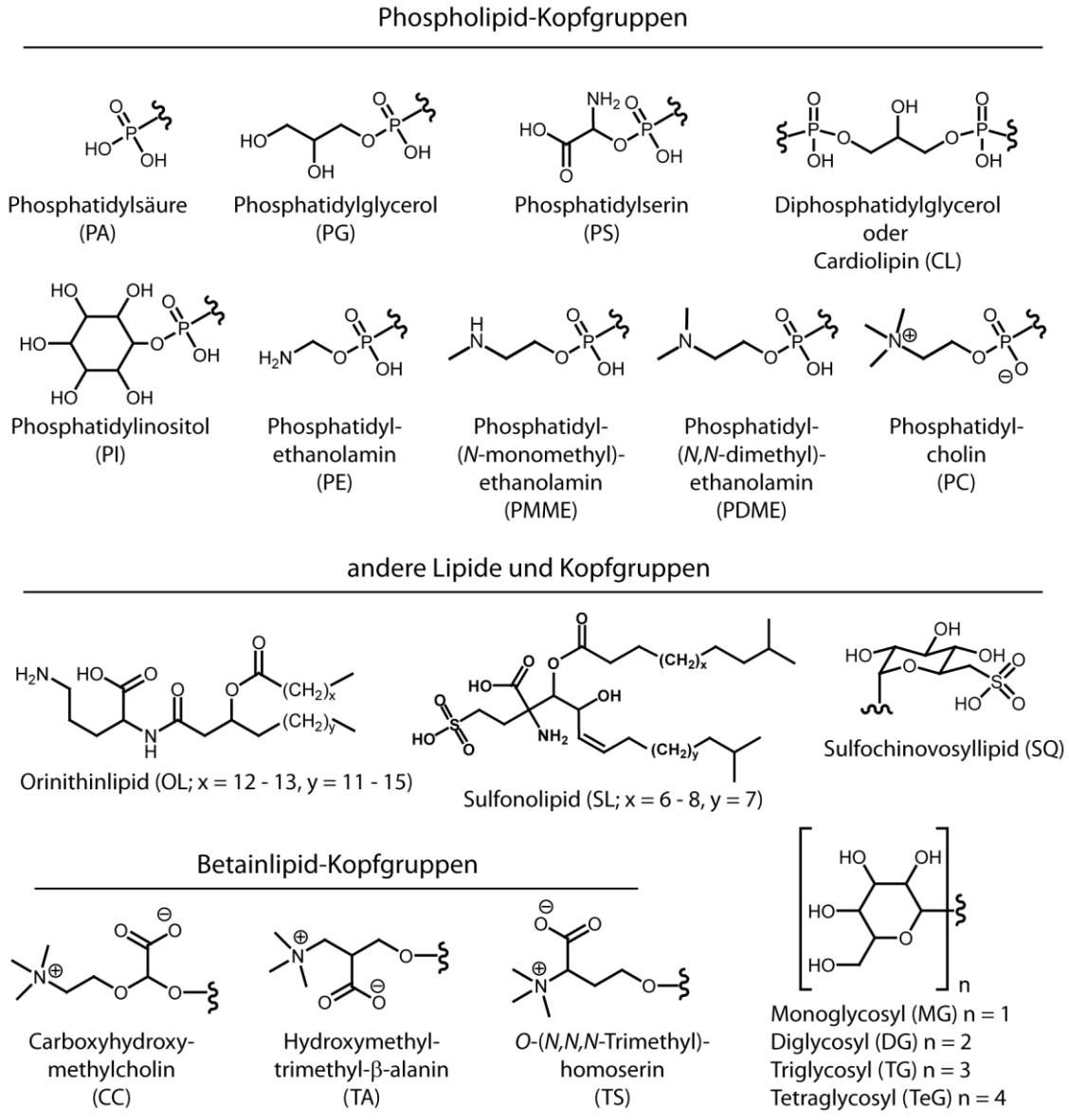


Abbildung 1.4: In Bakterien und Eukaryonten vorkommende Kopfgruppen intakter polarer Lipide mit den in dieser Arbeit verwendeten Abkürzungen.

Die für diese Arbeit wichtigen Sulfonolipide (SL) kommen ausschließlich in hypersalinen Systemen vor und sind den Ornithinlipiden strukturell sehr ähnlich. Sulfonolipide (SL) nehmen aufgrund ihres sehr beschränkten Auftretens eine Sonderrolle ein. Nach gegenwärtigem Stand

des Wissens kommen diese Verbindungen ausschließlich in *Salinibacter ruber* (Corcelli et al., 2004) und *Salisaeta longa* (Baronio et al., 2010) vor. Dabei benötigt *S. ruber* einen Salzgehalt von mindestens 15 % (Anton et al., 2002) und *S. longa* höchstens 20 % (Vaisman & Oren, 2009). Da sich die optimalen Wachstumsbedingungen für diese beiden Organismen kaum überschneiden, können mit dem Auftreten von Sulfonolipiden in hypersalinen Habitaten in Abhängigkeit vom Salzgehalt Aussagen über das Vorhandensein einer der beiden Bakterienarten getroffen werden. Hierbei gilt allerdings die Einschränkung, dass die Zahl der sulfonolipidsynthetisierenden Bakterien möglicherweise größer ist als bisher bekannt.

1.3.2. Intakte polare Lipide in Cytoplasmamembranen von Archaeen

Die Cytoplasmamembran von Archaeen besteht aus Molekülen, deren apolarer Teil aus Isopreneinheiten zusammengesetzt ist. Die Ursache hierfür ist das im Vergleich zu Bakterien oder Eukaryonten anders aufgebaute Enzymsystem (Daiyasu et al., 2005). Die Cytoplasmamembran besteht entweder aus membranüberspannenden Glycerol-dibiphytanyltetraethern (GDGT, Abb. 1.5 unten) oder aus Diethern mit zwei Phytanylseitenketten (Abb. 1.5 oben). Diese Diether werden auch als Diphytanylglyceroldiether (DPG) oder Archaeole bezeichnet. Eine Mischung von Lipiden mit GDGT- und DPG-Struktur in einem Organismus wurde von Lai und Mitarbeitern (2008) für *Archaeoglobus fulgidus* beschrieben. Ebenfalls unterscheidet sich die Konstitution der von Archaeen produzierten Lipide von denen bakteriellen Ursprungs, da unterschiedliche Biosynthesewege verwendet werden (Kates, 1977; De Rosa et al., 1986). So findet man in Archaeen ausschließlich Lipide, in denen die Kopfgruppe an der *sn*-1-Position des grundgerüstbildenden Glycerols gebunden ist und die ethergebundenen apolaren Seitenketten dementsprechend an den Positionen *sn*-2 und *sn*-3 (Koga & Morii, 2006).

In Archaeen, die in salinen oder hypersalinen Habitaten vorkommen, wurden bisher keine membranüberspannenden Lipide gefunden (Sprott et al., 2003). Neben Diphytanylethern (DPG) sind hier auch Membranlipide zu finden, in denen eine oder zwei Sesterterpanyl-Reste an das Glycerolgrundgerüst gebunden sind und die als Phytanylsesterterpanylglyceroldiether (PSG) bezeichnet werden (De Rosa et al., 1982). Weiterhin wurden in hypersalinen und methanogenen Archaeen Diether gefunden, die eine OH-Funktion aufweisen und auch als Hydroxyarchaeole bezeichnet werden (Abb. 1.5; Hinrichs et al., 1999; Stiehl et al., 2005).

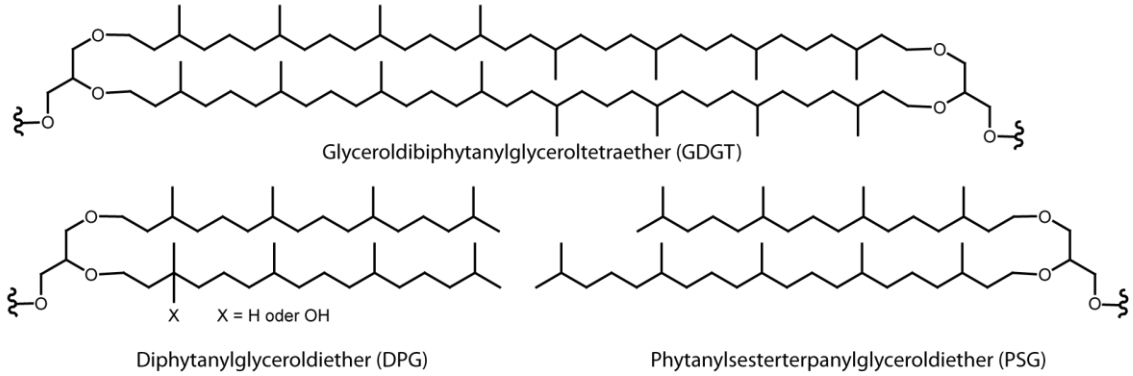


Abbildung 1.5: Unterschiedliche Grundstrukturen von intakten polaren Lipiden in Archaeen und die verwendeten Abkürzungen.

1.3.3. Kopfgruppen intakter polarer Lipide in Archaeen

Die meisten der polaren Kopfgruppen in den Lipiden der Bakterien und Eukaryonten kommen auch in Vertretern der Archaeen vor (Ulrih et al., 2009; Yoshinaga et al., 2011). Ausnahmen bilden Ornithin-, Sulfono- und Sulfochinovosyllipide sowie Lipide mit Phosphatidylcholin als Kopfgruppe. Neben den phosphatenthaltenden Lipiden treten in vielen Archaeen auch Membranlipide auf, die Kopfgruppen mit einer unterschiedlichen Anzahl von Zuckerresten aufweisen. Diese Lipide werden auch als Glycolipide bezeichnet. Die vielfältigen Verknüpfungsmöglichkeiten bei Kopfgruppen, die aus zwei oder mehr Zuckereinheiten bestehen, führt in Kombination mit verschiedenen Zuckerarten wie beispielsweise Glukose, Galaktose oder Mannose zu einer großen Vielzahl an unterschiedlichen Kopfgruppen (Sprott, 1992). Aus diesem Grund übersteigt die Anzahl der verschiedenen Glycolipide die der phosphathaltigen Membranbausteine. Damit einhergehend könnte durch die genaue stereochemische Analyse von Glycolipiden in Umweltproben eine deutlich bessere taxonomische Auflösung erzielt werden. Die hierzu notwendige Analytik ist jedoch sehr aufwendig und lässt sich kaum auf einen größeren Probensatz anwenden.

In hypersalinen Habitaten vorkommende Archaeen stellen spezielle Membranlipide her (Abb. 1.6). Phosphatidylglycerolphosphatmethylester (PGP-Me) als Kopfgruppe kommt in allen archaeellen Organismen vor, die in aquatischen Systemen mit höchster Salinität leben (Kates, 1993). PGP-Me spielt eine zentrale Rolle bei der Membranstabilisierung archaeeller Membranen in diesen Systemen (Tenchov et al., 2006). Lipide mit einer Phosphatidylglycerolsulfatkopfgruppe (PGS) sind ebenfalls in der Gruppe der halophilen Archaeen verbreitet, sie haben jedoch eine geringere Bedeutung als Lipide mit PGP-Me-Kopfgruppe. Das Vorkommen der Kopfgruppen Phosphatidylinositol (PI) und Phosphatidylinositolphosphat (PIP) wurde von Morii und Mitarbeitern (1999) für das Archaeon *Aeropyrumpernix* K1 beschrieben. Im Rahmen dieser Arbeit durchgeführte Untersuchungen

deuten jedoch darauf hin, dass diese Lipide auch in Archaeen hypersaliner Systeme vorkommen können.

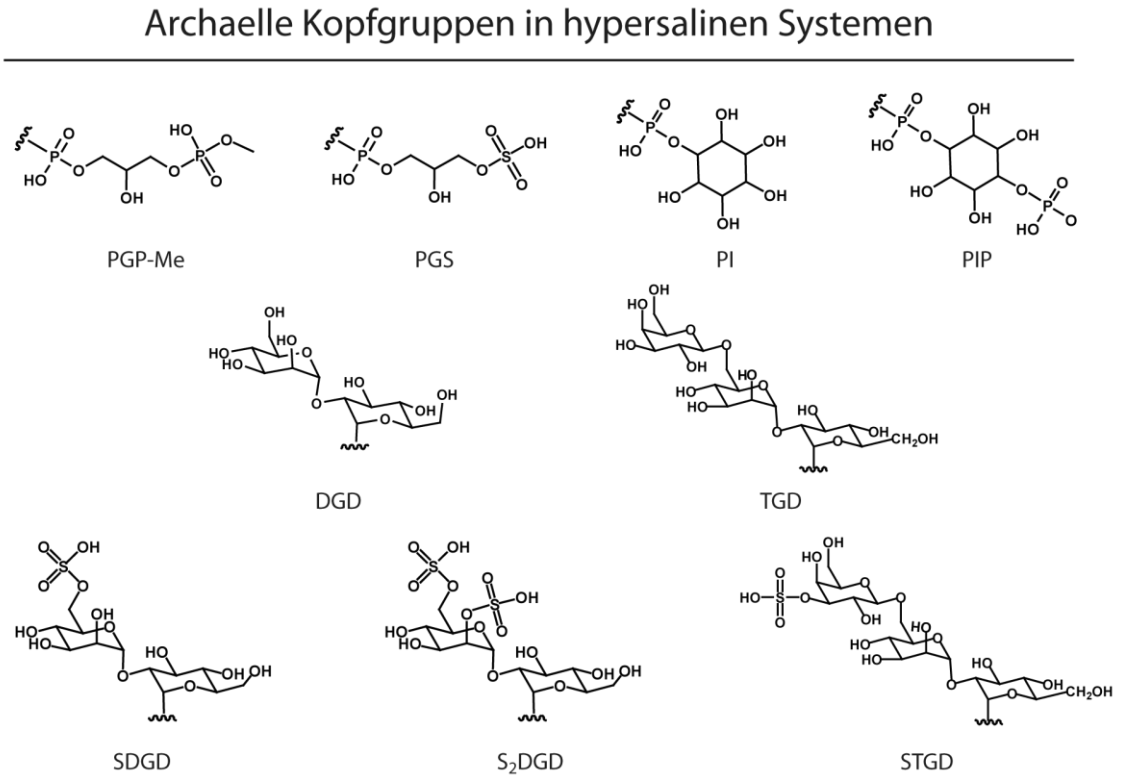


Abbildung 1.6: Molekülstrukturen von Kopfgruppen halophiler Archaeen.

Lipide, die Zuckermoleküle in ihren Kopfgruppen enthalten, sind auch in halophilen Archaeen verbreitet (Kates, 1996). Interessanterweise treten jedoch neben den aus anderen Habitaten bekannten Mono-, Di-, Tri- oder Tetraglyceriden auch Verbindungen auf, die ein oder zwei an die Kopfgruppe gebundene Sulfatreste besitzen (Kates & Deroo, 1973; Trincone et al., 1990; Matsubara et al., 1994).

1.4. Anpassung der Cytoplasmamembran

Die Homöostase und Anpassung der Lipidzusammensetzung der Cytoplasmamembran sind maßgebliche Eigenschaften von Mikroorganismen, die ihren Fortbestand unter wechselnden Umweltbedingungen möglich machen (Zhang & Rock, 2008). So dient die Cytoplasmamembran einerseits der chemischen Abgrenzung einer jeden Zelle von ihrer Umgebung. Andererseits müssen die von einer Zelle benötigten Nährstoffe in die Zelle und nicht benötigte Moleküle wieder aus ihr heraus transportiert werden (Dowhan, 1997). Dieses geschieht in der Regel durch in die Membran eingebaute Proteine, für deren Funktion ein sogenannter flüssigkristalliner Zustand der Membran aufrechterhalten werden muss (Sinensky, 1974). Das Aufrechterhalten dieses flüssigkristallinen Zustands der Cytoplasmamembran bei wechselnden Umweltbedingungen kann durch unterschiedliche Veränderungen auf molekularer Ebene geschehen. Diese Veränderungen sind in Abbildung 1.7 übersichtlich zusammengefasst.

Die Reduktion der Fluidität wirkt einer höheren Umgebungstemperatur entgegen und erhält so den flüssigkristallinen Zustand der Membran (Russell, 1984). So reduziert beispielsweise im Fall von Bakterien eine Kohlenstoffkettenverlängerung der unpolaren Reste von intakten polaren Lipiden die Fluidität der Membran (Marr & Ingraham, 1962). Weiterhin wirkt sich auch die Synthese von Methylgruppen in *iso*- oder *anteiso*-Position am Ende der Kohlenstoffketten im Vergleich zu unverzweigten Ketten negativ auf die Membranfluidität aus. Diese Arten der Membranmodifizierung können nur durch den Einbau von neu synthetisierten Lipiden erfolgen, damit sind sie wachstumsabhängig (Zhang & Rock, 2008).

Neben der Veränderung der Kohlenstoffkettenlängen der apolaren Reste führt der Einbau von Doppelbindungen in *trans*- oder *cis*-Konfiguration zu einer erhöhten Membranfluidität (Zhang & Rock, 2008). Der Einbau von Cyclopropylringen in die Membranlipide von *Escherichia coli* wird nach Chang und Cronan (1999) mit einer erhöhten Resistenz gegen Säuren assoziiert. Im Gegensatz zur Verlängerung oder Verkürzung der Kohlenstoffkettenlängen werden die zuletzt genannten Modifizierungen durch die entsprechenden Enzymsysteme innerhalb einer bestehenden Cytoplasmamembran vorgenommen und sind damit wachstumsunabhängig.

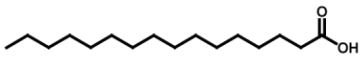
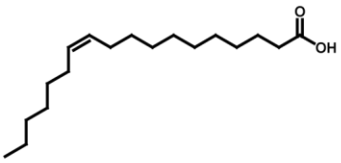
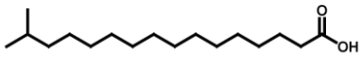
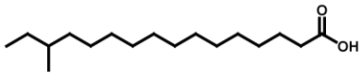
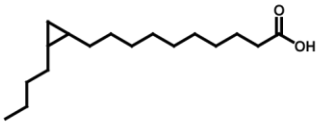
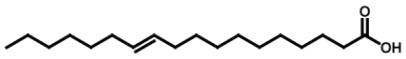
Fettsäure	Molekülstruktur	Einfluss auf die Membranfluidität
n-$C_{16:0}$		Abnahme der Membranfluidität
cis-11-$C_{18:1}$		Zunahme der Membranfluidität
iso-$C_{17:0}$		im Vergleich zu *anteiso*-verzweigten Kohlenstoffketten Abnahme der Membranfluidität
anteiso-$C_{17:0}$		im Vergleich zu *iso*-verzweigten Kohlenstoffketten Zunahme der Membranfluidität
cyclopropan-$C_{17:0}$		verleichbarer Effekt wie Unsättigung in Verbindung mit höherer Stabilität gegen Säurestress
trans-11-$C_{18:1}$		vergleichbarer Effekt wie gesättigte Fettsäuren, in Kultur jedoch erhöhter Schutz gegen Lösungsmittel und steigende Wachstumstemperatur

Abbildung 1.7: Fettsäuren und ihr Einfluss auf die Fluidität von bakteriellen Cytoplasmamembranen (verändert nach Zhang et al. 2008).

Welcher der genannten Mechanismen zum Einsatz kommt, hängt dabei vom jeweiligen Organismus ab und lässt sich nur schlecht generalisieren (Russel 1984). Zusätzlich kann die Fettsäurezusammensetzung von Mikroorganismen in unterschiedlichen Wachstumsphasen

selbst bei gleichbleibender Kultivierungstemperatur variieren (Wang & Cronan, 1994; Grogan & Cronan, 1997).

Neben der Modifizierung der Seitenketten von intakten polaren Lipiden hat auch der Einbau von unterschiedlichen Kopfgruppen einen entscheidenden Einfluss auf die Membraneigenschaften. Zusätzlich zu den molekularen Veränderungen von intakten polaren Lipiden verändert auch der Einbau anderer Moleküle wie beispielsweise von Hopanolen in Bakterien (Kannenberg & Poralla, 1999) oder Sterolen im Fall von Eukaryonten (Cooper, 1978) die Cytoplasmamembranfluidität entscheidend.

Die Membrananpassungsmechanismen von Archaeen unterscheiden sich aufgrund der zu Bakterien und Eukaryonten verschiedenen Biosynthese der Membranlipide (Daiyasu et al., 2005) aus Isoprenbausteinen (2-Methylbuta-1,3-dien). Die Verlängerung der apolaren Kohlenstoffketten wurde bisher nur als Anpassung an hypersaline Habitate mit höchsten Salzgehalten von mehr als 250 g L^{-1} beobachtet (Teixidor et al., 1993). Dabei wird von einigen der hier lebenden Organismen ein Anteil der Membranlipide nicht wie bei halophilen Archaeen üblich aus Diphytanyl-Steitenketten mit je 20 Kohlenstoffatomen aufgebaut (Kates, 1977), sondern unsymmetrisch aus Phytanyl- und Sesterterpanylseitenketten zusammengesetzt (De Rosa et al., 1982). Weiterhin sind einige wenige Organismen bekannt, die Lipide mit Disesterterpanylseitenketten herstellen (Morii et al., 1999).

Vertreter der in der Wassersäule von marinen Systemen lebenden Crenarchaeota nutzen den Einbau einer unterschiedlichen Anzahl an Ringen (1 bis 5) in die Biphytanylseitenketten ihrer Membranlipide, um sich an die Umweltbedingungen anzupassen (Schouten et al., 2002). Eine andere Möglichkeit der Membrananpassung bei archaeellen Organismen, die sowohl Tetraether- als auch Diethermembranlipide besitzen, besteht darin, das Verhältnis dieser beiden Lipidtypen zu verändern (Lai et al., 2008). Der bei niedrigeren Umgebungs-temperaturen zunehmende Einbau von Doppelbindungen wurde als ein möglicher Anpassungsmechanismus beschrieben (Nichols et al., 2004). Dieser Mechanismus scheint nach gegenwärtigem Stand des Wissens auf einige wenige Archaeen beschränkt zu sein, da in der verfügbaren Literatur kaum entsprechende Beispiele beschrieben sind. Für Archaeen sind keine molekularen Analoga zu Hopanoiden oder Steroiden, wie sie in Bakterien und Eukaryonten auftreten, bekannt.

1.5. Zielsetzung der vorliegenden Arbeit

Die Ziele dieser Arbeit gliedern sich in mehrere Teilaspekte. Der erste Aspekt beinhaltet die methodischen Grundlagen der Analyse von intakten polaren Lipiden mit Hochleistungsflüssigkeitschromatographie und gekoppelter Massenspektrometrie mit Elektrosprayionisierung (LC-ESI-MS). Diese Untersuchungen können als Fortführung der in meiner Diplomarbeit begonnenen Untersuchungen gesehen werden. Die in diesem Zusammenhang im Rahmen dieser Doktorarbeit bearbeitete Fragestellung bezieht sich im Wesentlichen auf die genaue Untersuchung des zur Probenvorbereitung genutzten Auftrennungsverfahrens und die möglichen Analytverluste.

Eine grundsätzliche Frage bei der Anwendung von intakten polaren Lipiden als Biomarker für lebende Mikroorganismen ist die Geschwindigkeit und die Vergleichbarkeit des Abbaus von verschiedenen Verbindungen. Dies ist problematisch, da im Falle einer erhöhten Stabilität von einzelnen intakten polaren Lipiden oder Gruppen dieser Verbindungen das Signal der bestehenden mikrobiellen Gemeinschaft von einem fossilen Signal überlagert werden würde. So könnte es zu falschen Aussagen kommen. Die Grundlage zu dieser Frage bildet eine Publikation von White und Mitarbeitern (1979) sowie hauptsächlich eine Arbeit von Harvey und Coautoren (Harvey et al., 1986). Diese zeitlich länger zurückliegenden Veröffentlichungen behandeln den mikrobiellen Abbau von intakten polaren Lipiden vor allem unter den Gesichtspunkten von unterschiedlichen Raten in An- und Abwesenheit von Sauerstoff und des Einflusses von organischem Material (Huminstoffen). Zusätzlich war die mögliche unterschiedliche Stabilität von archaeellen gegenüber bakteriellen intakten polaren Lipiden Gegenstand der Untersuchungen. Es wurde jedoch nicht untersucht, inwiefern innerhalb der Gruppen von archaeellen und bakteriellen Lipiden die Bindungsart der Kopfgruppe an das Glycerolgrundgerüst oder die Verknüpfungsart der apolaren Seitenketten per Ether- oder Esterbindung einen Einfluss auf den Abbau haben. Eine im Vorfeld zu dieser Doktorarbeit durchgeführte umfangreiche Untersuchung von Dr. Michael Seidel und Tatjana Burchart ergab keine befriedigenden Ergebnisse. Daher ist im Rahmen dieser Doktorarbeit ein weiterer Abbauversuch durchgeführt worden, der anhand des vorhergehenden Ansatzes ein überarbeitetes Versuchsdesign aufweist. Die Ergebnisse dieses Projekts wurden im Rahmen eines Fachartikels in Biogeosciences veröffentlicht. Der eigene Anteil an dieser Veröffentlichung lag in der Projektplanung, Projektleitung, Versuchsdurchführung, Lipidanalytik und Manuskripterstellung.

Neben den beschriebenen methodischen Fragen zur Analyse von intakten polaren Lipiden, ist die Fragestellung der Anpassung von Cytoplasmamembranen an unterschiedliche Umweltbedingungen ein weiterer Aspekt dieser Doktorarbeit. Wie bereits in den

vorhergehenden Kapiteln beschrieben, müssen Mikroorganismen eine definierte Fluidität der Cytoplasmamembran aufrecht erhalten, um die Zellfunktionen und damit ihr Überleben zu gewährleisten. Diese Anpassungsstrategien sind anhand einer Reinkultur von *Desulfovibrio indonensiensis* in Zusammenarbeit mit Katja Fichtel untersucht worden. Dieses sulfatreduzierende Bakterium wurde aus Sedimentmaterial isoliert, das aus dem unteren Teil eines ca. 250 m langen Bohrkerns gewonnen wurde, der vom Juan-de-Fuca-Rücken aus einer Wassertiefe von ca. 2600 stammt. *Desulfovibrio indonensiensis* ist somit in der Lage, unter extrem hohen Drücken zu leben. Da dieser Organismus zusätzlich auch unter atmosphärischen Bedingungen leben kann, ist er ein ideales Beispiel für die zentrale Frage von möglichen Anpassungsstrategien auf Cytoplasmamembranlipidebene an hohen Druck und hohe Temperatur. Die Ergebnisse dieses Projekts wurden in Form eines Manuskripts festgehalten und stehen kurz vor der Einreichung zur Veröffentlichung bei FEMS Microbiology Ecology. Der eigene Anteil an diesem Manuskript liegt in der Lipidanalytik und der Manuskripterstellung. Zusätzliche nicht in das Manuskript eingeflossene Daten finden sich in einem weiteren Kapitel dieser Arbeit.

Eine ähnliche, aber wesentlich komplexere Fragestellung soll mit dem abschließenden Projekt dieser Doktorarbeit beleuchtet werden. Im Rahmen eines internationalen Projekts wurde der planktonisch lebende Teil einer Mikroorganismengemeinschaft in einem hypersalinen See mit Salzgehalten nahe der Sättigung (250 g L^{-1} NaCl) beprobt. Die zentralen Fragen dieses Projekts sind, welche Lipide werden von der bestehenden mikrobiellen Gemeinschaft produziert und inwiefern lässt sich das Lipidverteilungsmuster mit anderen hypersalinen Habitaten vergleichen. Weiterhin wird überprüft, ob sich anhand der IPL-Verteilung auf die mikrobielle Zusammensetzung des Habitats zurückgeschlossen werden kann.

Für die beiden zuletzt genannten Projekte zur Untersuchung von Cytoplasmamembranlipiden soll die Kombination aus gaschromatographischer Analyse der durch alkalische Hydrolyse freigesetzten Fettsäuren und LC-ESI-MS-Analyse archaeeller Glyceroldiphytanylether weitere Informationen zu möglichen methodischen Problemen beider Verfahren liefern. Die Ergebnisse dieses Projekts wurden in einem Manuskript verarbeitet. Dieses Manuskript steht vor der Einreichung zur Veröffentlichung in der Fachzeitschrift Extremophiles. Der eigene Beitrag zu diesem Manuskript liegt in der Lipidanalytik, der Erzeugung der IPL-Datenbank und der Erstellung des Manuskripts.

2. Methodenentwicklung zur Analyse von intakten polaren Lipiden

2.1. Einleitung und Ziele

Die Extraktion und Vortrennung von Analyten dient dazu, die Nachweisgrenzen von Analysen zu verbessern und notwendige Reinigungen von Analysengeräten zu reduzieren.

Bei der Analyse von komplexen Substanzgemischen haben die Vollständigkeit der Extraktion und eine verlustfreie sowie reproduzierbare Vortrennung einen entscheidenden Einfluss auf die Verlässlichkeit und Empfindlichkeit der im späteren Verlauf der Analyse eingesetzten analytischen Verfahren. Für die Verwendung eines analytischen Verfahrens und die Interpretation der gewonnenen Ergebnisse ist es wichtig zu wissen, wo mögliche Probleme auftreten können. Dabei stellen Zielanalyten mit heterogenen chemischen Eigenschaften eine besondere Herausforderung dar. So ist es nicht zielführend, die Maximierung der Ausbeute einzelner Vertreter anzustreben, da dies aufgrund der unterschiedlichen chemischen Eigenschaften wie beispielsweise der Polarität unweigerlich zu starken Verlusten anderer Substanzklassenvertreter führen würde.

Ein solches Beispiel bildet die Analyse intakter polarer Lipide. Wie in Kapitel 1 gezeigt, besteht diese Substanzgruppe aus membranbildenden Molekülen, die eine polare und eine apolare Molekülregion aufweisen. Die strukturellen und chemischen Unterschiede zwischen den einzelnen Vertretern der intakten polaren Lipide werden dabei besonders durch ihre polaren Kopfgruppen hervorgerufen. Die apolaren Reste wie Fettsäuren oder Alkylether haben durch ihre Präsenz in vielen verschiedenen intakten polaren Lipiden einen vergleichsweise geringen Einfluss auf intermolekulare Unterschiede.

Im Rahmen meiner Diplomarbeit wurden bereits die Wiederfindungsraten von intakten Glyco- und Phospholipiden bei der Extraktion von Wattsedimenten und der angeschlossenen Extraktauftrennung bestimmt (Logemann, 2007). Dabei wurden die Extraktionsverfahren nach Schouten et al. (2002) , Vancanneyt et al. (1996) und Sturt et al. (2004) miteinander verglichen. Das Extraktionsverfahren nach Sturt et al. (2004) zeigte für einige wenige Lipide leicht verbesserte Extraktionsausbeuten und wurde daher weiterhin verwendet. Eine vergleichende Extraktion eines Crenarchaeenisolats bestätigte die zuvor anhand von Standardmaterial ermittelten Ergebnisse dieser Methode. Die Extraktionseffizienz bzw. der Einfluss der Sedimentmatrix auf die Extraktionseffizienz wurde zusätzlich an Schlick- und Sandwattsedimenten sowie geglühtem Seesand getestet. Hier zeigte sich, dass die Extraktionseffizienz stark von dem untersuchten Sedimenttyp abhängt, da die Wiederfindungen mit kleiner werdender Sedimentkörnung (Seesand>Sandwatt>Schlickwatt)

für alle IPL mit estergebundenen Seitenketten schlechter wurden. IPL mit ether-gebundenen Seitenketten waren von diesem Effekt nicht betroffen.

Im Allgemeinen folgt der Lipidextraktion eine säulenchromatografische Auftrennung des Extrakts (Rütters et al., 2001; Sturt et al., 2004; Zink & Mangelsdorf, 2004; Biddle et al., 2006; Lipp & Hinrichs, 2009). Daher wurden im Rahmen meiner Diplomarbeit drei Fraktionierungsmethoden, bei denen jeweils eine unterschiedliche stationäre Phase zum Einsatz kam, auf Verluste von intakten polaren Lipiden untersucht. Die besten Ergebnisse lieferte hier eine von Zink und Mangelsdorf (2004) entwickelte Methode (Abb. 2.1). Eine weitere Optimierung konnte damals jedoch nicht mehr durchgeführt werden.

Das Ziel im Rahmen dieser Arbeit war es daher zu überprüfen, ob eine Verbesserung der von Zink und Mangelsdorf (2004) entwickelten Auftrennungsmethode möglich ist. Die Methodenoptimierung wurde mit Untersuchungen zum Einfluss der eingesetzten Lösungsmittelmenge auf die Elution der polaren Fraktion, des Deaktivierungsgrades des zur Auftrennung eingesetzten Kieselgels und des Einflusses der Polarität des Elutionsmittels der polaren Fraktion begonnen. Die Ergebnisse dieser Versuche dienten als Grundlage für eine von mir initiierte und betreute Bachelorarbeit (Manecki, 2008).

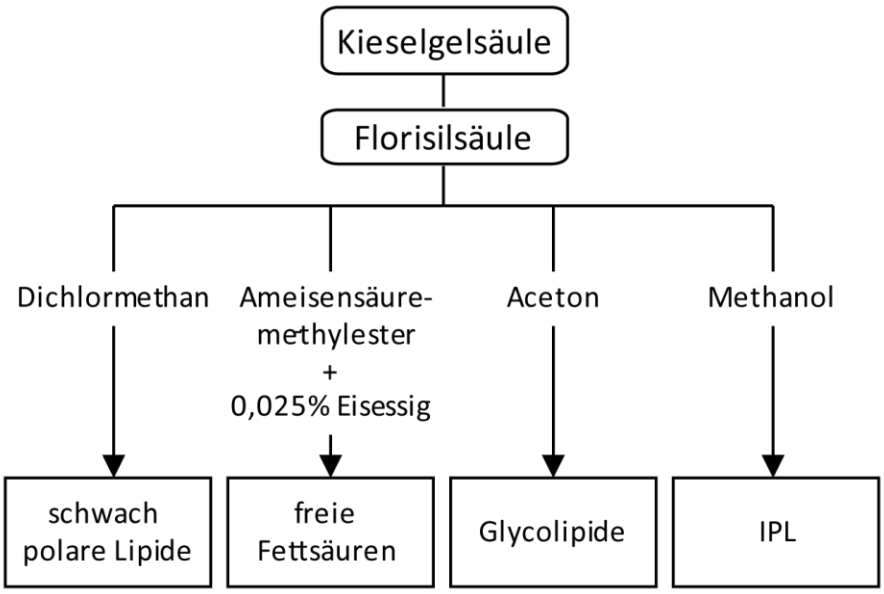


Abb. 2.1: Auftrennung nach Zink und Mangelsdorf (2004). Die Florisilsäule wird vor der Elution der intakten polaren Lipide entfernt.

Es wurden unter anderem die individuellen Mengen an Elutionsmittel der vierten Fraktion bestimmt, die notwendig sind, um die einzelnen Komponenten des IPL-Standards von der Trennsäule zu eluieren. Weiterhin hatten die Vorversuche ergeben, dass die Zugabe von Wasser und die damit einhergehende Steigerung der Polarität des Elutionsmittels der vierten Fraktion (Abb. 2.1) zu verbesserten Wiederfindungen führen. Der vergleichsweise hohe Siedepunkt wirkt sich jedoch negativ auf den Zeitbedarf und die thermische Belastung der

Proben bei der Aufarbeitung aus. Folglich ist es erstrebenswert die eingesetzte Wassermenge auf ein Minimum zu reduzieren.

Wie bereits in Kapitel 1 angesprochen, unterschieden sich die verschiedenen IPL-Kopfgruppen chemisch stark voneinander (vgl. Abb. 1.4 und 1.6). Somit kann davon ausgegangen werden, dass der pH-Wert des Lösungsmittels einen entscheidenden Einfluss auf die Wiederfindungen von IPLs bei der säulenchromatografischen Trennung hat.

Das Ziel dieser Untersuchungen war neben der Verbesserung von IPL-Wiederfindungen auch ökonomische Aspekte wie die Optimierung des Zeitbedarfs und des Lösungsmittelverbrauchs.

2.2. Methoden

2.2.1. Säulenchromatografische Trennung

Bei der Optimierung der säulenchromatographischen Trennung nach Zink und Mangelsdorf (2004) wurden zwei hintereinander geschaltete Glassäulen (8 ml; LiChrolut, Merck, Darmstadt) mit auswechselbarer PTFE-Fritte (LiChrolut, Merck, Darmstadt) verwendet, wobei die obere Säule mit 1 g Kieselgel 60 (63-200 µm, Merck, Darmstadt, 16 Stunden aktiviert bei 110 °C) gefüllt und mit 0,5 cm gereinigtem Natriumsulfat überschichtet wurde. In die untere Glassäule wurde 1 g Florisil (Magnesiumsilikat-Gel, 150-250 µm, Merck, Darmstadt) gegeben und mit ca. 1 cm gereinigtem Natriumsulfat überschichtet. Die Säulen wurden vor der Benutzung mit 20 ml Dichlormethan konditioniert. Nach Aufgabe von 100 µl des IPL-Standards (100 µg ml^{-1} pro Einzelsubstanz) gelöst in Dichlormethan/Methanol 9:1 (v/v) wurden mit in der Polarität aufsteigenden Lösungsmitteln vier Fraktionen eluiert. 1: Fraktion der schwach polaren Lipide mit 20 ml Dichlormethan; 2: Fraktion der freien Fettsäuren mit 50 ml Ameisensäuremethylester mit 0,025 % Eisessig (≥ 99,5 %); 3: Fraktion der Glycolipide mit 25 ml Aceton; 4: Fraktion der intakten polaren Lipiden mit 25 ml Methanol. Im Anschluss wurden die dritte Fraktion der Glycolipide und die vierte Fraktion der intakten polaren Lipide auf die im Standardgemisch enthaltenen intakten polaren Lipide durch ein LC-ESI-MS System (HPLC-Anlage: Waters 2695 Separations Module; Massenspektrometer: Micromass Q-Tof micro, Hersteller beider Geräte: Waters, Manchester, UK) untersucht.

Diese Methode der Auftrennung wurde für alle im Rahmen der Vorversuche durchgeführten Experimente angewendet. Die exakten Veränderungen für die einzelnen Experimente waren: A: Veränderung des Deaktivierungsgrades der eingesetzten Trennphase von 0 %, 5 %, 10 % und 15 % Gew.-% Wasser; B: Reduktion der eingesetzten Elutionsmittelmenge von 100 %, 92,5 %, 75 % und 50 %; C: Variation der Polarität des

Elutionsmittels der vierten Fraktion von 0 % Wasser, 10 % Wasser, 25 % Wasser sowie 50 % Wasser.

Aus Gründen der Zeitersparnis wurde bei den Untersuchungen auf die Eluierung der ersten drei Fraktionen verzichtet. Weiterhin wurde keine Florisilsäule verwendet, da diese lediglich zur besseren Auftrennung zwischen den ersten drei Fraktion benötig wird. Die Kieselgelsäule wurde nun vor dem Aufgeben der Standardmischung mit Aceton vorkonditioniert. Zusätzlich zu den zuvor im IPL-Standardgemisch verwendeten Lipiden wurden in den Experimenten zusätzlich zwei IPL mit ethergebundenen Seitenketten eingesetzt. Für jede Auftrennung wurden 100 µl des IPL-Standardgemischs in einer Konzentration von 25 µg ml^{-1} verwendet. Bei den Untersuchungen zur pH-Wertabhängigkeit des Elutionsverhaltens von IPL wurde der pH-Wert des Elutionsmittels durch die Zugabe von Essigsäure eingestellt.

2.2.2. Eingesetztes IPL-Standardgemisch

Das in den Vorversuchen eingesetzte Standardgemisch bestand aus folgenden Komponenten: Phosphatidylsäure (PA, 16:0/16:0 = 1,2-Dihexadecanoyl-*sn*-glycero-3-phosphat - Natriumsalz, 99 %); Phosphatidylglycerin (PG, 16:0/18:1 = 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-*rac*-(-1-glycerol) - Ammoniumsalz, 99 %); Phosphatidylethanolamin (PE, 16:0/16:0 = 1,2-Dihexadecanoyl-*rac*-glycero-3-phosphoethanolamin - 98 %); Phosphatidylcholin (PC, 16:0/16:0 = 1,2-Dihexadecanoyl-*rac*-glycero-3-phosphocholin - 99 %), alle vertrieben von Sigma-Aldrich Chemie GmbH, München; Digalactosyldiglycerid (DGDG, 18:0/18:0 - 98%); Glyceroldibiphytanylglyceroltetraether (GDGT – 95 %), beide: Matreya, USA - Vertrieb durch Biotrend Chemikalien, Köln; Diphosphatidylglycerin oder Cardiolipin (CL, 4 × 18:2 = 1,3-bis-(1',2'-diacyl-3'-phosphoryl-*sn*-glycerol)-*sn*-glycerol – Natriumsalz, 98 %); Phosphatidylserin (PS, 16:0/16:0 = 1,2-Dihexadecanoyl-*rac*-glycero-3-phospho-L-serin - Natriumsalz, 98 %); Phosphatidylinositol (PI, 18:0/20:4 = 1-Hexadecanoyl-2-octadecadien-*sn*-glycero-3-phospho-inositol - Natriumsalz, 98 %); Lyso-Phosphatidylcholin (Lyso-PC = 1-Heptadecanoyl-2-hydroxy-*sn*-glycero-3-phosphocholin - 99 %), alle Avanti Polar Lipids, USA, Vertrieb durch Otto Nordwald GmbH, Hamburg.

Zusätzlich wurden in den weitergehenden Untersuchungen folgende intakte polare Lipide eingesetzt: Phosphatidylethanolamin (PE-DPG, O-20/O-20 = 1,2-Di-O-Phytanyl-*sn*-glycero-3-phosphoethanolamin und Phosphatidylcholin (PC-DPG, O-20/O-20 = 1,2-Di-O-phytanyl-*sn*-glycero-3-phosphocholin - 99 %), beide Avanti Polar Lipids, USA - Vertrieb durch Otto Nordwald GmbH, Hamburg.

2.2.3. LC-ESI-MS-Messung

Für die Analyse der erhaltenen Fraktionen wurde eine HPLC-Anlage (Waters 2695 Seperations Module, Waters, Manchester, UK) verwendet, an die ein Hybrid-Quadrupol/Flugzeit-Massenspektrometer (Micromass Q-Tof micro) der Firma Waters gekoppelt war. Zur Ionisierung der IPL wurde eine Elektrospray-Ionisierungsquelle (ESI) verwendet. Zur Erzeugung des für den Betrieb der ESI-Quelle benötigten Stickstoffs (98,5% Reinheit bei einer Flussrate von 15 l min^{-1}) wurde ein Stickstoff-Membran-Generator (NGM11-LC/MS, CMC-Instruments GmbH, Eschborn) verwendet. Die Steuerung des gesamten Systems und die Auswertung der Daten erfolgte mit der Software MassLynx 4.1.

Alle massenspektrometrischen Analysen wurden im negativen Ionenmodus durchgeführt. In Tabelle 2.1 sind die gerätespezifischen Parameter aufgeführt.

Tabelle 2.1: Geräteparameter für die LC-MS-Analyse.

MS	Q-Tof *micro*
Capillary voltage:	3016 V
Sample cone voltage:	35 V
Desolvation temperature:	220°C
Source temperature:	110°C
Cone gas:	12 l h^{-1}, N_2
Scanbereich:	*m/z* 500 - *m/z* 2000
Scangeschwindigkeit:	1 scan s^{-1} über 40 min im Fullscanmodus
Datenaufzeichnung:	kontinuierlich (*data format continuum*)
Hexapol:	Argon, 2 bar
Kollisionsenergie:	7 V

Die Quantifizierung der einzelnen intakten polaren Lipide wurde über die Massenspuren vorgenommen. Da die Detektor-Signalstärke für jede Phospholipidklasse spezifisch und mit dem verwendeten Massenspektrometer nur in einem bestimmten Konzentrationsbereich linear ist, wurde eine externe Mehrpunktkalibrierung mit den im Experiment verwendeten Phospholipidstandards in unterschiedlichen Konzentrationen vorgenommen. Zur Massenkalibrierung des Geräts wurden vor jeder Messsequenz eine Kalibrierlösung bestehend aus 10 mM-Natriumformiat in Acetonitril injiziert und die Geräteparameter entsprechend der Ergebnisse angepasst.

2.2.4. LC-ESI-MS Auswertung

Die Identifizierung der intakten polaren Lipide in den jeweiligen Fraktionen erfolgte durch den Vergleich der Retentionszeiten und der Masse-zu-Ladungsverhältnisse der jeweiligen Komponenten mit den Ergebnissen von Einzelsubstanzmessungen. Für die Quantifizierung wurde eine externe Kalibrierung anhand der jeweiligen Massenspuren vorgenommen. Dazu wurde die Lösungen des für die Optimierungsversuche verwendeten Standardgemischs in unterschiedlichen Konzentrationen vermessen. Aus diesen Ergebnissen wurden

Kalibrierkurven erstellt und so die Konzentration jeder Einzelsubstanz in den Proben ermittelt, deren Sollkonzentration 10 µg ml^{-1} betrug. Dieser Wert wurde dann für die weitere Auswertung als eine 100%ige Wiederfindung angesehen.

2.3. Ergebnisse

2.3.1. Ergebnisse der Vorversuche

Unabhängig voneinander wurde drei Veränderungen an der Auftrennungsmethode von Zink und Mangelsdorf vorgenommen. In Abbildung 2.2a sind die Ergebnisse der säulenchromatografischen Trennungen dargestellt, bei denen die eingesetzte Trennphase mit ansteigenden Mengen an Wasser deaktiviert wurde. Es fällt auf, dass die Wiederfindungen der einzelnen intakten polaren Lipide untereinander schwanken. Bei einem Vergleich der vier Einzelversuche sind die Wiederfindungen innerhalb der IPL-Kopfgruppen ähnlich. Die zunehmende Deaktivierung der Trennphase hat nur einen geringen Einfluss auf die Wiederfindungen der unterschiedlichen Lipide. Lediglich bei den intakten polaren Lipiden mit den Kopfgruppen PI, PE und DGDG wurde mit Zunahme der Deaktivierung von 0 bis 10 Gew.-% Wasser eine leicht verbesserte Wiederfindung festgestellt. Zusätzlich wurde das Lipid Lyso-PC erst ab einem Deaktivierungsgrads von 15 Gew.-% Wasser von der Säule eluiert.

Die Variation der für die Fraktion der intakten polaren Lipide eingesetzten Elutionsmittelmenge (Abb 2.2b) sollte in erster Linie dazu dienen, Einsparungspotentiale für die eingesetzten Lösungsmittel zu identifizieren. Die erzielten Wiederfindungen variierten für die Mehrzahl der untersuchten IPL-Kopfgruppen kaum, wobei das IPL Lyso-PC nicht wiedergefunden wurde. Für die IPL-Kopfgruppe Phosphatidylcholin (PC) führte eine abnehmende Lösungsmittelmenge zur stufenweisen Abnahme der Wiederfindungen. Ein ähnliches, aber weniger stark ausgeprägtes Verhalten wurde auch für die Wiederfindungen des IPLs Cardiolipin (CL) ermittelt. Bei einigen anderen Lipiden wie beispielsweise GDGT, PS und PI wurden mit abnehmender Lösungsmittelmenge zunehmende Wiederfindungen festgestellt, was auf andere Probleme schließen lässt, die im Bereich der weiteren Probenaufarbeitung oder der Quantifizierung dieser Substanzen liegen könnten.

Die Steigerung der Polarität des Lösungsmittels zur Elution der Fraktion der intakten polaren Lipide hat zu Ergebnissen geführt, die in Abbildung 2.2c zu sehen sind. Die Zugabe von 10 % Wasser (v/v) zum Elutionsmittel der vierten Fraktion sorgte insgesamt gesehen im Vergleich zur bisher eingesetzten Methode ohne Wasseranteil für eine leichte Verbesserung der Wiederfindungen. Besonders deutliche Verbesserungen wurden jedoch für die IPL-Kopfgruppen PA, PC und Lyso-PC erzielt. Eine Erhöhung des Wasseranteils über 10 % führte für alle Lipide zu schlechteren Wiederfindungen.

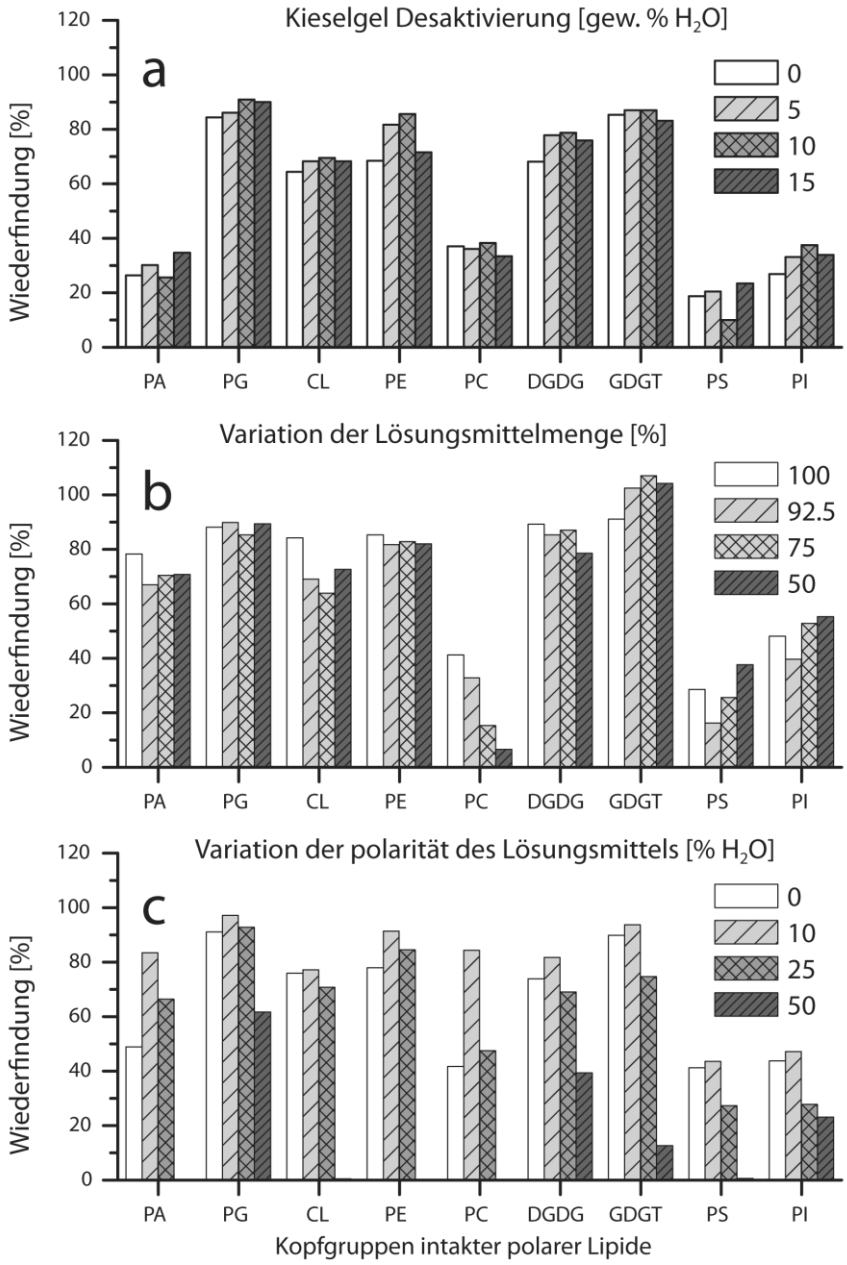


Abbildung 2.2: Ergebnisse der Auftrennung eines IPL-Standardgemischs mit verschiedenen methodischen Veränderungen. a: Variation des Deaktivierungsgerades der eingesetzten Trennphase, Werte in Gew.-% Wasser bezogen auf das Kieselgel, b: Schrittweise Reduktion der zur Elution der IPL-Fraktion eingesetzten Lösungsmittelmenge, Werte in %; c: Erhöhung der Polarität des Elutionsmittels durch Zugabe von Wasser, Werte in %.

Bei dem Vergleich der drei Versuche untereinander (Abb. 2.2a-c) wird deutlich, dass einige IPL-Kopfgruppen wie beispielsweise PA, PS, PI und das IPL Lyso-PC zwar innerhalb der Experimente vergleichbare und konsistente Wiederfindungen liefern, aber in einem neuen Versuchsansatz zum Teil deutlich unterschiedliche Ergebnisse zeigen. Im Gegensatz zeigten einige Lipide wie PG, CL, PE, DGDG und GDGT durchgehend hohe Wiederfindungen mit guter Reproduzierbarkeit.

2.3.2. Weiterführende Ergebnisse

Die Unterteilung der vierten Fraktion der intakten polaren Lipiden in sechs Unterfraktionen (Abb. 2.3a und 2.3b; Doppelbestimmung) zeigt analog zu den vorhergehenden Versuchen eine lösungsmittelvolumenabhängige Elution der im IPL-Standard enthaltenen Lipide. Für die Mehrzahl der intakten polaren Lipide war die maximale Wiederfindung bereits nach Elution mit 5 ml Methanol erreicht. Lipide mit einer Phosphatidylcholinkopfgruppe eluierten erst beginnend mit der dritten Unterfraktion, was auch den vorangegangenen Ergebnissen entspricht (vgl. Abb. 2.2b, 2.3a sowie 2.3b). Besonders bemerkenswert ist, dass ein Anteil von PC-DPG und PC bzw. die gesamte Substanzmenge von Lyso-PC erst in der sechsten Unterfraktion eluiert. Für diesen im Vergleich zur Standardmethode zusätzlichen Schritt wurde ein Lösungsmittelgemisch aus Methanol und Wasser verwendet. Hier wird deutlich, dass die Erhöhung der Elutionsmittelpolarität einen positiven Einfluss auf die Wiederfindungen von Lipiden mit Phosphatidylcholinkopfgruppen hat. Dieser Zusammenhang wird zusätzlich durch die Ergebnisse aus Abbildung 2.2c und 2.3c bestätigt.

In einer detaillierten Untersuchung des Einflusses der Lösungsmittelpolarität auf die Wiederfindungen von intakten polaren Lipiden (Abb. 2.3c) wurden nur geringe Unterschiede zwischen der Verwendung von Elutionsmittelgemischen festgestellt, die aus einem Verhältnis von Methanol zu Wasser von 10:1, 9:1 oder 8:1 (v/v) bestanden. Die Wiederfindungen bei Verwendung von reinem Methanol waren deutlich geringer. Wie aus Abbildung 2.3d ersichtlich ist, hatte das Absenken des pH-Werts für einige wenige intakte polare Lipide wie PA, PE-DPG und PE einen leicht positiven Effekt. Für alle anderen IPLs war jedoch eine deutliche Abnahme der Wiederfindungen zu beobachten.

In einigen Versuchen traten für IPL mit den Kopfgruppen PA und PC sowie das archaeelle IPL GDGT Wiederfindungen von mehr als 100% auf (Abb. 2.3a, b und c). Der Vergleich von Ergebnissen identischer Versuchsdurchführungen aus den verschiedenen Experimenten (Abb. 2.2a: 0% Deaktivierung, Abb. 2.2b: 100% Elutionsmittel, Abb. 2.2c: 0% H_2O, Abb. 2.3a+b: ∑F1-F5 sowie Abb. 2.3c) zeigt, dass sich die Ergebnisse bei wiederholter, identisch durchgeführter Auftrennung zum Teil stark voneinander unterscheiden können.

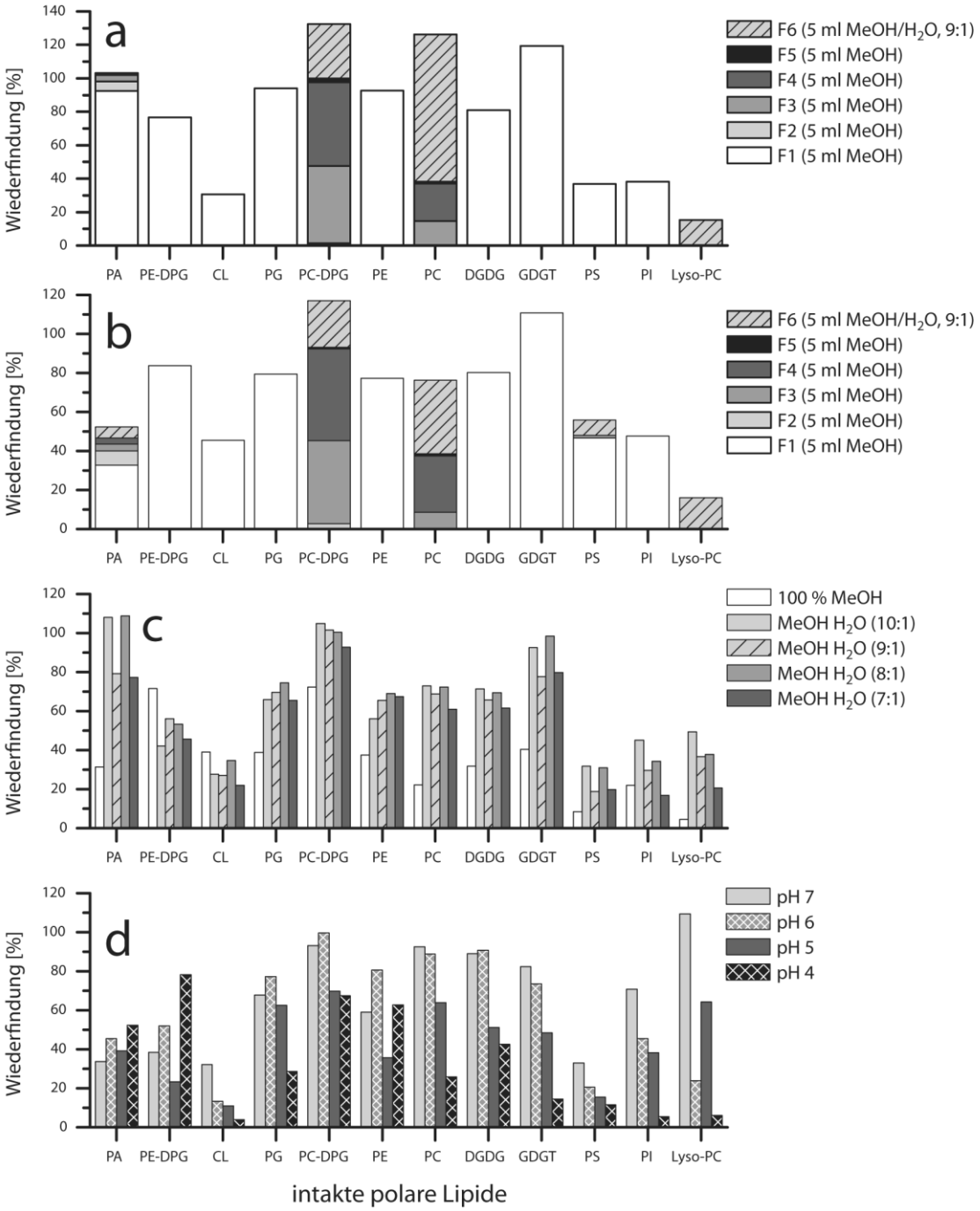


Abbildung 2.3: Ergebnisse der Auftrennung eines IPL-Standards mit unterschiedlichen Veränderungen. a und b: Schrittweise Elution der IPL-Fraktion in sechs 5 ml-Schritten (Doppelbestimmung), abweichend von den vorhergehenden Schritten wurde im letzten Schritt eine Methanol/Wassermischung eingesetzt; c: Variation der Polarität des Elutionsmittels durch Zugabe von Wasser; d: Variation des pH-Werts des Elutionsmittels der vierten Fraktion.

2.4. Diskussion

Die beiden Versuche mit erhöhter Elutionsmittelpolarität (Abb. 2.2c und 2.3c) zeigen, dass durch die Zugabe von 10% Wasser zum Elutionsmittel (MeOH) die Wiederfindungen bestimmter Lipide (PA, PC, PC-DPG und Lyso-PC) signifikant erhöht werden, ohne dass die Wiederfindungen anderer Lipide negativ beeinflusst werden. Dem gegenüber steht aufgrund

des vergleichsweise hohen Siedepunkts von Wasser eine zeitaufwändigere Probenvorbereitung.

Beim Vergleich der Ergebnisse aus Abb. 2.2a, 2.2c und 2.3c fällt auf, dass Wasser als Bestandteil des Elutionsmittels einen positiven Effekt besonders auf die Wiederfindung der IPL PA und PC hat. Die Zugabe von Wasser zum Elutionsmittel zeigt, dass die Affinität der Analyten zum Elutionsmittel von großer Bedeutung ist. Dagegen hat die Deaktivierung des Kieselgels einen geringen Einfluss (Abb. 2.2a). Die unterschiedlich starke Deaktivierung der stationären Phase hat gezeigt, dass die Elution der intakten polaren Lipide im untersuchten Bereich der Deaktivierung kaum vom Wassergehalt des verwendeten Kieselgels abhängt.

Da für die spätere Analyse die Proben im Starteluenten der LC-MS-Analysenmethode gelöst werden und dieser kein Wasser enthält, muss das enthaltene Wasser vor der Analyse abgetrennt werden. Der vergleichsweise hohe Siedepunkt von Wasser erschwert diesen Arbeitsschritt und sorgt durch das notwendige leichte Erwärmen der Probe für eine unerwünschte thermische Belastung. Daher sollte nur so viel Wasser im Elutionsmittel eingesetzt werden wie gerade notwendig ist.

In einigen Fällen traten Wiederfindungen von intakten polaren Lipiden auf, die zum Teil deutlich oberhalb von 100 % lagen. Interessanterweise betrifft dieses Phänomen immer nur einzelne intakte polare Lipide (PA, PC und GDGT), wobei alle anderen IPL im Standardgemisch keine Auffälligkeiten zeigen. Bei der für die Wiederfindungsversuche eingesetzten IPL-Mischung handelte es sich um dieselbe IPL-Stammlösung, die im Anschluss zum Erstellen der Kalibrierreihe zur Quantifizierung verwendet wurde. Da bei der späteren Messung der Kalibrierreihe keine vom Sollwert abweichenden IPL festgestellt wurden, können Pipettierfehler als Ursache ausgeschlossen werden. Die Konzentrationsspanne der zur Quantifizierung verwendeten Kalibriereihen lag jeweils über und unter den zu erwartenden Konzentrationen der Proben, so dass von einer sicheren Quantifizierung ausgegangen werden kann.

In den Versuchen zur fraktionierten Elution in 6 Fraktionen (Abb. 2.3a und 2.3b) ist jedoch von dieser Vorgehensweise abgewichen worden, da zu Versuchsbeginn nicht abzuschätzen war, in welcher der Unterfraktion der Großteil der IPLs zu erwarten ist. Durch die Aufsummierung von möglichen Quantifizierungsfehlern kann es so zu den beobachteten Wiederfindungen von mehr als 100 % gekommen sein. Allerdings sind zu hohe Wiederfindungen auch in anderen Proben zu finden. Dabei handelt es sich augenscheinlich nicht um ein systematisches, sondern eher zufälliges Auftreten, da in keiner Probe die gleichen Substanzen betroffen sind (Abb. 2.2b, 2.3c und 2.3d). In der von mir angefertigten Diplomarbeit (Logemann, 2007) wurde die Signalstabilität von intakten polaren Lipiden durch

die Mehrfachinjektion eines Standards bestimmt. Die Standardabweichung für die untersuchten IPLs in der injizierten Probe lag für alle untersuchten IPLs bei 4% und darunter.

Bei der Ionisierung in der verwendeten Elektrosprayquelle kann ein sogenannter Matrixeffekt auftreten (Tang & Kebarle, 1993; Mei et al., 2003; Taylor, 2005). Dabei können bestimmte Bestandteile einer Probe, die die Probenaufbereitung genauso durchlaufen wie die Analyten, die Effizienz der Ionisierung abschwächen oder verstärken (Souverain et al., 2004). Es ist bekannt, dass Säuren oder Basen die Ionisierungseffizienz für bestimmte Verbindungen verbessern (Kuhlmann et al., 1995). Dieser Effekt wird bei der überwiegenden Mehrheit aller LC-ESI-MS-Methoden durch die Verwendung eines Puffers ausgenutzt (z.B. Stüber & Reemtsma, 2004; Böttcher et al., 2007; Chambers et al., 2007).

In Bezug auf die durchgeführten Versuche kann der Matrixeffekt als Ursache für die beobachteten Schwankungen weitestgehend ausgeschlossen werden, da die Versuche mit reinen Standardgemischen durchgeführt wurden und so die Messbedingungen in Bezug auf Probenmatrix und pH-Wert für alle Proben identisch waren. Es ist jedoch unklar, inwiefern eine geringfügig unterschiedliche Probenhandhabung einen Einfluss auf den Matrixeffekt haben kann. Zusätzlich sollten Quellen für Substanzen, die einen positiven oder negativen Matrixeffekt verursachen bei Verwendung von reinen Standardlösungen nicht vorhanden sein. Somit bleibt unklar, wodurch die erhöhten Wiederfindungen einzelner IPL ausgelöst wurden.

Die Unterteilung der Fraktion der intakten polaren Lipide in mehrere Unterfraktionen (Abb. 2.2b, 2.3a und 2.3b) zeigt deutlich die unterschiedlichen Affinitäten der untersuchten Substanzen zur verwendeten stationären Phase. Der überwiegende Teil der IPL eluiert schon nach Verwendung der Hälfte des eingesetzten Lösungsmittels. Für die Elution von Lipiden mit Phosphatidylcholinkopfgruppen (PC-DPG, PC und Lyso-PC) ist die vorgesehene Lösungsmittelmenge jedoch notwendig. Weiterhin zeigt sich im Vergleich der Ergebnisse aus 2.2a und 2.2b mit 2.2c, dass die nachträgliche Verwendung eines Elutionsmittels mit Wasseranteil die Wiederfindung kaum verbessert. Die Ursache hierfür ist möglicherweise die große Oberfläche, auf der die Lipide in den Versuchen 2.2a und 2.2b bereits verteilt sind, so dass auch das etwas höher polare Elutionsmittel eine Ablösung nicht mehr möglich macht.

Die Absenkung des pH-Werts auf im Vergleich zur Ausgangsmethode (pH 7) niedrigere Werte hat für einige IPL eine bessere Eluierbarkeit hervorgerufen (PA und PE-DPG). Bezogen auf alle untersuchten IPLs stellt dies jedoch keine Verbesserung dar.

Bei allen in diesem Kapitel dargestellten Versuchen wurde jeweils ein Versuchsparameter wie z.B. der Wasseranteil des Elutionsmittel gesteigert. Bei Betrachtung der Ergebnisse fällt unerwarteter Weise auf, dass einige Ergebnisse nicht den Veränderungen innerhalb der einzelnen Versuche folgen. Beispiele hierfür sind der Versuch zur Reduktion der eingesetzten

Elutionsmittelmengen (vgl. Abb. 2.2b). Hier wurden für PS und PI höhere Wiederfindungen bei Durchführungen festgestellt, in denen weniger Elutionsmittel eingesetzt wurde. Ähnliche Ergebnisse wurde für die Untersuchung zum Einfluss der Elutionsmittelpolarität (vgl. Abb. 2.3c) und die Versuche zum Einfluss des pH-Werts auf die Wiederfindungen von intakten polaren Lipiden (vgl. Abb. 2.3d) ermittelt.

Weiterhin fällt beim direkten Vergleich von Untersuchungsergebnissen einzelner IPL in den verschiedenen Versuchsansätzen auf, dass sich die Ergebnisse bei vergleichbaren Versuchsbedingungen signifikant unterscheiden. Dies wird besonders bei einem Vergleich der Wiederfindungen von Lyso-PC, PA und PS unter den Bedingungen der Ausgangsmethode mit 100% Methanol als Elutionsmittel mit einem pH-Wert von 7 deutlich. Durch diese Ergebnisse wird klar, dass nicht nur die gewählten Versuchsbedingungen, sondern auch feine Unterschiede in der Durchführung der Auftrennung einen bedeutenden Einfluss auf die IPL-Wiederfindungen haben. Demgegenüber stehen sehr gut reproduzierbare Wiederfindungswerte (Abb. 2.2a und 2.2b). Durch das partielle Auftreten der Wiederfindungsschwankungen konnte die Ursache für die Variabilität der Werte nicht befriedigend erklärt werden. Weiterhin ist zu bedenken, dass bei diesen Untersuchungen reine Standardlösungen zum Einsatz kamen und dass bei der Verwendung von realen Proben die Schwankungen in den Wiederfindungen möglicherweise noch größer ausfallen könnten.

Die schlecht reproduzierbaren Ergebnisse der säulenchromatografischen Auftrennung von intakten polaren Lipiden an Kieselgelphasen haben zu der Endscheidung geführt, im weiteren Verlauf dieser Arbeit von einer Auftrennung der Lipidextrakte vor der LC-MS-Analyse abzusehen. Der Nachteil dieser Vorgehensweise bestand jedoch in der Notwendigkeit, die Vorsäule der HPLC-Anlage häufiger auszutauschen und das Einlasssystem des Massenspektrometers zu reinigen. Außerdem führte dies zu einer verminderten Nachweisgrenze. Da für die im Verlauf dieser Arbeit durchgeführten Projekte Proben mit vergleichsweise hohen IPL-Konzentrationen zur Verfügung standen, resultierte daraus kein Nachteil.

3. A laboratory experiment of intact polar lipid degradation in sandy sediments

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3.1. Abstract

Intact polar lipids (IPLs) are considered biomarkers for living biomass. Their degradation in marine sediments, however, is poorly understood and complicates interpretation of their occurrence in geological samples. To investigate the turnover of IPLs, a degradation experiment with anoxic sandy sediments from the North Sea was conducted. Intact cells of two organisms that do not naturally occur in North Sea sediments were chosen as IPL sources: (i) *Saccharomyces cerevisiae*, representative for ester-bound acyl lipids that also occur in Bacteria, and (ii) the archaeon *Haloferax volcanii*, representative for ether-bound isoprenoid lipids. Surprisingly, IPLs with phosphoester-bound head groups showed approximately the same degradation rate as IPLs with glycosidic head groups. Furthermore, the results indicate a relatively fast degradation of *S. cerevisiae* IPLs with ester-bound moieties (analogs of bacterial membrane lipids) and no significant degradation of archaeal IPLs with ether bound moieties. Pore water and 16S rRNA-based DGGE analysis showed only a minor influence of the IPL source on microbial metabolism and community profiles. Due to our results, the IPL-based quantification of *Archaea* and *Bacteria* should be interpreted with caution.

3.2. Introduction

Intact polar lipids (IPLs) have widely been used as biomarkers for living organisms in sediments and water columns for several years (e.g. Sturt et al., 2004; Zink & Mangelsdorf, 2004; Biddle et al., 2006; Ertefai et al., 2008; Rossel et al., 2008; Schubotz et al., 2009; Van Mooy et al., 2009). Additionally, IPLs are applied as chemotaxonomic markers as some of these molecules are representative for specific microbial clades. The chemotaxonomic information of IPLs is based on the combination of various head groups with different side chains that are attached to a glycerol backbone by two different bonding types. In general, bacterial and eukaryal cytoplasma membranes contain intact polar lipids with non-isoprenoidal ester-bound fatty acid moieties. In contrast, archaeal IPLs contain ether-bound isoprenoid moieties. This difference between bacterial and archaeal IPLs has been used to quantify *Bacteria* and *Archaea* in sediments and water samples (Lipp et al., 2008; Rossel et al., 2008; Schubotz et al., 2009; Van Mooy et al., 2009). Thus, IPL analysis is valuable as an alternative or complementary

technique to standard microbiological methods. On a higher chemotaxonomic level, ammonia-oxidizing bacteria can be identified by the presence of ladderane lipids (Boumann et al., 2006; Jaeschke et al., 2009). Intact polar lipids with mixed ether/ester-bound moieties attached to the glycerol backbone were found in some strains of sulfate-reducing bacteria (Rütters et al., 2001). Separate $\delta^{13}C$ analysis of polar head groups or non-polar core lipids of IPLs can be used to gain information on the metabolism of their producers (Boschker et al., 1998; Lin et al., 2010; Takano et al., 2010).

One fundamental assumption underlies most of these applications: intact polar lipids are considered to degrade rapidly after cell death (White et al., 1979; Harvey et al., 1986). Harvey et al. (1986) examined the degradation of both, a glycosidic ether lipid and ester-bound phospholipids. Based on their findings, the authors assumed that intact polar lipids with glycosidic head groups show a higher stability against degradation than intact polar lipids with phosphoester head groups. Following the results of Harvey et al. (1986), Lipp and Hinrichs (2009) and Schouten et al. (2010) modeled the potential preservation of fossilized IPLs of planktonic origin after sediment burial. Using different variables significantly influenced the results showing that modeling alone cannot resolve the preservation issue and that experimental data are needed.

The quantification of Bacteria and Archaea in the deep marine biosphere by IPL analysis and fluorescence in situ hybridization (FISH) in comparison to catalyzed reporter deposition fluorescence in situ hybridization (CARD-FISH) and quantitative polymerase chain reaction (q-PCR) analysis has been controversially discussed in several studies (e.g. Schippers et al., 2005; Biddle et al., 2006; Lipp et al., 2008). A reason for the contradictory abundances of *Archaea* and *Bacteria* in these studies may be that ether-bound archaeal IPLs are more stable than their ester-bound bacterial counterparts, which in turn may lead to an overestimation of archaeal cell numbers. On the other hand, q-PCR-based investigations may underestimate the yields of archaeal 16S rRNA genes, as indicated by Lipp et al. (2008) and Teske and Sørensen (2008). However, the lack of a broad study on the degradation of intact polar lipids, which includes lipids with phosphoester and glycosidic head groups as well as ester- and ether-bound moieties occurring in Bacteria and Archaea, respectively, makes it necessary to revisit the degradation of IPLs to ensure the robustness of this proxy.

We designed a degradation experiment to answer three general questions: what are the degradation rates of IPLs? Are there differences between ester- and ether-bound intact polar lipids and what is the influence of the bonding type of the head group upon lipid degradation? The main degradation experiment was accompanied by two controls: the first control was intended to assess any processes that are not mediated by microorganisms but still lead to the

degradation of the added IPLs. This control is subsequently named “abiotic control”. No cell material was added to the second control. It was used to investigate the development of the microbial community without any further substrate addition under laboratory conditions. This control is subsequently named “untreated control”.

3.3. Material and methods

3.3.1. Experimental setup

The incubation vessels for the degradation experiment and the untreated control had a total volume of 2.5 l each and were filled with 3 kg wet sediment (water content 29 % wt, 2.13 kg dry wt). The sediment used in this experiment had been freshly collected in November 2009 on Janssand, a sandplate located approximately 3 km south of Spiekeroog Island, North Sea, Germany (53°44.178´N and 07°41.974´E). For sampling, the top centimeter of oxic surface sediment was removed until only black anoxic sediment was visible. The underlying sediment was transferred into plastic containers, which were sealed by a lid, transported to the laboratory and stored at 4 °C for one week prior to further use. The total organic carbon (TOC) content was 0.23 %. It was calculated as the difference between total carbon (Vario EL Cube, Elementar Analysensysteme GmbH, Germany) and inorganic carbon (UIC CO_2 coulometer). The pore water concentration of sulfate was equal to the sea water concentration (28 mM). A total of 2.5 g *Saccharomyces cerevisiae* biomass (elemental composition, of dry mass: 45.1 % C, 7.9 % H, 7.9 % N and 0.4 % S) as source for ester-bound IPLs and 1.25 g of *Haloferax volcanii* (18.9 % C, 3.6 % H, 4.7 % N and 0.7 % S) as source for ether-bound IPLs were added to the sediment in the incubation vessels used to study IPL degradation.

As calculated from the equation of Adams et al. (1990), the dry weight of a single *Saccharomyces cerevisiae* cell is approximately 60 pg. With a total sediment volume of 1.5 l 2.8×10^7 cells ml^{-1} sediment were added. The cell size of a single *Haloferax volcanii* cell is 1 - 3 × 2 - 3 µm and 0.4 - 0.5 µm thick (Mullakhanbhai and Larsen, 1975). Assuming a water content of 80%, this leads to a dry weight of 0.16 - 0.9 pg per single cell of *Haloferax volcanii*. With 1.25 g of dry Haloferax biomass, approximately 9.3×10^8 - 5.2×10^9 cells ml^{-1} were added. The elemental composition of the dry biomass used in this experiment indicates a contamination with inorganic material. Thus, the calculated cell number may be too high but still in the correct order of magnitude. After addition of cell material to the incubation vessel, the sediment was homogenized for 6 h on a mixing device by slow rotation (12 rpm). The experimental parameters for the untreated control were the same as for the degradation experiment but no inactive cell material was added. To prevent contamination with microorganisms due to sampling, several 100 ml bottles were used instead of a single 2.5 l

incubation vessel for the abiotic control. The bottles contained 50 g of sediment, 50 mg dry inactive biomass of *S. cerevisiae* and 25 mg dry inactive *H. volcanii* biomass and were closed with rubber stoppers. The incubation vessels of the abiotic control were autoclaved after addition of the intact polar lipid-containing cell material.

3.3.2. Source material for intact polar lipids

As sources for intact polar lipids, two different organisms were used which do not occur in North Sea sediment. Ether-derived IPLs (diphytanylglycerols=DPGs) were originated from pure culture of *Haloferax volcanii* (DSM No. 16227) grown at 37 °C in "Haloferax sulfurifontis medium" (DSMZ No. 1018). This archaeon provided two kinds of structurally different molecules – first, two IPLs with ether-bound isoprenoid moieties and a phosphoester head group (PGP-Me-DPG, PG-DPG; Fig. 3.1) and second, an IPL with ether-bound isoprenoid moieties and a glycosidic head group (S-GL-1- DPG; Fig. 3.1). The cells were harvested at the end of the exponential growth phase. None of the *H. volcanii* IPLs occurs in the natural sediment and thus all of them could be used as tracers to monitor the degradation of ether-bound IPLs. Commercially available Saccharomyces cerevisiae (baker's yeast; Fala GmbH, Germany) was used as source for IPLs with ester-bound acylic moieties (diacylglycerols=DAGs) and phosphoester head groups. *S. cerevisiae* cells harbor a broad variety of IPLs which were partly used as tracers because these IPLs are not synthetized by the natural sediment microbial community. *S. cerevisiae* and the harvested archaeal cells were freeze-dried and stored at -20 °C. Before use in the experiment, *H. volcanii* and *S. cerevisiae* cells were pasteurized at 100° C for 30 min. Thereafter, part of the cells was transferred to fresh medium ("Haloferax sulfurifontis medium" and yeast extract medium, respectively) to confirm that the cells were no longer alive and unable to grow.

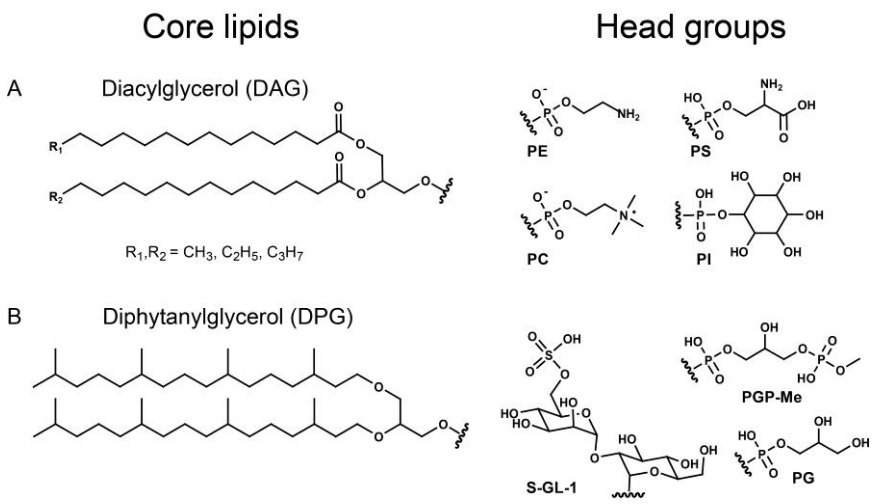

Figure 3.1: Chemical structures of IPLs characteristic for the two organisms used in this study: (A): diacylglycerol ester-bound core lipids (DAGs) of Saccharomyces cerevisiae bound to: phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylinositol (PI) and phosphatidylserine (PS); (B): diphytanylglycerol ether-bound core lipids (DPGs) of Haloferax volcanii bound to: sulfono diglyco dialkylglycerol S-GL-1 (nomenclature according to Sprott *et al*., 2003), phosphatidylglycerol (PG) and phosphatidylglycerol methylphosphate (PGP-Me).

Identification of *S. cerevisiae* lipids was achieved by HPLC-MS/MS experiments and comparison of mass spectral data to commercially available standards. The lipids of *H. volcanii* were identified by MS/MS experiments and comparison with published results of lipid structures (Sprott et al., 2003). In this experiment we monitored the contents of 16 intact polar lipids, 13 of them were ester-bound and 3 ether-bound.

3.3.3. Incubation parameters and sampling

After starting the experiment, the incubation vessels were stored at room temperature in the dark. Before sampling, the sediment was homogenized for a minimum of 20 min on a mixing device. Headspace gas samples were taken with a syringe directly before sampling the sediment. For sediment and pore water sampling, the incubation vessels were transferred into a glove box with an oxygen-free nitrogen (99 %) and hydrogen (1 %) atmosphere. Samples were taken as triplicates, aliquots of 8-12 g sediment for IPL analysis and 3-5 g sediment for RNA extraction. Pore water (1-2 ml) was extracted with rhizones (Rhizon CSS 5 cm, Rhizosphere Research Products, Wageningen, The Netherlands) from the samples that were collected for IPL analysis. Our previous investigations had ensured the absence of IPLs in the pore water samples (data not shown). Until further processing, all samples were stored at -20 °C. The samples for RNA extrac-tion were kept at -80 °C. After sampling, the headspace of the incubation vessels was flushed with nitrogen to remove traces of hydrogen that was used in the glove box to sustain the oxygen-free atmosphere. The constantly increasing pH was adjusted at each sampling point to values between 7 and 7.5 by addition of hydrochloric acid. After day 21, sulfate concentrations were adjusted to approximately 28 mM by the addition of

1 M Na_2SO_4 when concentrations dropped below 10 mM. After day 76, when hydrogen sul.de reached toxic concentrations, it was expelled by flushing the headspace with CO_2. After each CO_2 flushing the headspace was re-placed with N_2.

3.3.4. Lipid extraction

Wet sediment was extracted using the Bligh & Dyer method modified according to Sturt et al. (2004). In the first three extraction steps, a single-phase mixture of methanol, dichloromethane and phosphate buffer (2:1:0.8, v/v/v) was used. In the following three extraction steps, trichloroacetic acid replaced the phosphate buffer. The combined extracts were collected in a separatory funnel. Phase separation was achieved by addition of dichloromethane and water to final mixture of 1:1:0.9 (v/v/v, methanol, dichloromethane, aqueous phase). The organic phase containing the IPLs was removed and the aqueous phase washed three times with dichloromethane. The dry extracts were stored in a freezer at -20 °C until further use.

3.3.5. HPLC-MS

Intact polar lipids were analyzed by HPLC (2695 separation module, Waters, Milfort, USA) coupled to a time-of-flight mass spectrometer equipped with an electrospray source (Micromass, Q-TOF micro, Waters, Milfort, USA). HPLC separation was achieved on a diol phase (Lichro-spher100 Diol 5 µm, CS – Chromatographie Service, Langer-wehe, Germany) using a 2 × 125 mm column. A flow rate of 0.35 ml min^{-1} was employed with the following solvent gradient: 1 min 100 % A, increasing over 20 min to 35 % A, 65 % B using a concave curvature, followed by 40 min of re-conditioning (R¨utters et al., 2001). Eluent A was a mixture of *n*-hexane, *i*-propanol, formic acid and a solution of 25 % ammonia in water (79:20:1.2:0.04 by volume), eluent B was *i*-propanol, water, formic acid and a solution of 25 % ammonia in water (88:10:1.2:0.04 by volume). After addition of an injection standard (O-PE, phosphatidyl ethanolamine diether with two C_{16} alkyl moieties) to every sample, the extracts were dissolved in the starting eluent and directly analyzed. In this study, we exclusively report the change of those compounds that were added with the inactive cell material and did not occur in the natural sediment. Due to the lack of analytical standards for the archaeal glycolipids used, it was not possible to determine the absolute concentrations of these compounds. Instead, ratios of peak areas of the monitored compounds to the peak area of the injection standard for each sample were calculated. To compare the results of ester-bound and ether-bound IPLs the same procedure was applied also to Saccharomyces-derived IPLs. Peak areas were deter-mined by integration of mass traces. Since all samples had the same matrix background, this procedure should give reasonable results without any influence of changing ionization.

The analytical error varied between 0.5 % and 7 % depend-ing on the investigated IPL and was determined by repeated analysis of the same samples taken at three different times. The limit of detection in general depends on the ionization efficiency for every analyzed compound and typically lies between 2 and 10 ng per injection and IPL for the mass spec-trometer used.

3.3.6. Chemical analyses

Sulfate concentrations were measured by an ion chromatograph with an LCA A24 anion separation column (both Sykam, Fürstenfeldbruck, Germany) at 60 °C followed by conductivity detection. The eluent consisted of 0.64 g sodium carbonate, 0.2 g sodium hydroxide, 150 ml ethanol and 2 ml modifier (0.1 g 4-hydroxybenzonitrile/10 ml methanol) filled up to 1 l with distilled water. The flow rate was set to 1.2 ml min^{-1}. Prior to analysis the samples were diluted 1:100 in eluent without modifier.

The concentrations of gaseous compounds were determined by an 8610C gas chromatograph (Schambeck SFD GmbH, Honnef, Germany). Analysis was carried out with argon (1 ml min^{-1}) as carrier gas and at a column oven temperature of 40 °C. For analysis of molecular hydrogen and methane a molecular sieve 13X packed column was used, whereas carbon dioxide was separated by a HayeSep D packed column. A thermal conductivity detector (256 °C) and a flame ionization detector (380 °C) were connected in series for detection of the gases. Sulfide concentrations were determined photometrically as described by Cord-Ruwisch (1985).

3.3.7. Calculation of degradation rates

Degradation rates were calculated with the following equation described by Schouten et al. (2010):

$$C_t = C_i \cdot e^{-k' t} \quad (1)$$

with C_t = concentration at time t, C_i = initial concentration and k' being the kinetic degradation constant. This method allows calculating degradation rates from degradation curves without curve fitting. The degradation constants k' were calculated for every time point separately using data of day zero and the respective time point. Values for the calculation of degradation rates of phosphatidylethanolamine diacylglycerol (PE-DAG) and glycol diphytanylglycerol (GL-DPG) were taken from Figs. 3.2 and 3.3 (beach sediments) in Harvey et al. (1986). For calculating degradation rates of IPLs investigated in this study, we used mean values of esterand ether-bound IPLs. Due to increasing contents of ester and ether-bound IPLs, no degradation rates could be calculated for the first 5 and first 9 days, respectively.

3.3.8. Determination of total cell numbers

Total cell counts were obtained after SYBR Green I staining (Molecular Probes, Eugene, OR, USA) according to a protocol of Lunau *et al.* (2005), which was adapted to sediment samples. For sample fixation, 0.5 cm^3 of sediment was transferred to 4.5 ml of fixing solution (63 ml distilled water, 30 ml methanol, 2 ml of 25 % aqueous glutardialdehyde solution, 5 ml Tween 80) and incubated at room temperature overnight. For detaching cells from particles, the sediment slurries were incubated for 15 min at 35 °C in an ultrasonic bath (35 kHz, 2 × 320 W per period; Sonorex RK 103 H, Bandelin, Mörfelden-Walldorf, Germany). Homogenized aliquots of 20 µl were equally dispensed on a clean microscope slide in a square of 20 × 20 mm. The slide was dried on a heating plate at 40 °C. A drop of 12 µl staining solution (190 µl Moviol, 5 µl SYBR Green I, 5 µl 1 M ascorbic acid in TAE buffer) was placed in the center of a 20 × 20 mm coverslip, which was then placed on the sediment sample. After 10 min of incubation, 20 randomly selected fields or at least 400 cells were counted for each sediment sample by epifluorescence microscopy.

3.3.9. RNA extraction and quantification

Total RNA was extracted from 1 g sediment using the All-Prep DNA/RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For cell disruption, 1 g sediment and 1 ml RLT Buffer were added to 1 g glass beads (0.18 mm diameter, Sartorius, Göttingen, Germany). Samples were homogenized for 90 s using a Mini Beadbeater (Biospec Products, Bartlesville, USA).

For quantification, 100 µl of RiboGreen (Invitrogen, Eugene, USA) solution (diluted 1:200 in TE buffer; pH 7.5) were added to 100 µl of RNA extract (each sample diluted 1:100 in TE buffer; pH 7.5) and transferred to a microtiter plate. Serial dilutions (200 to 1 ng μl^{-1}) of *E. coli* 16S and 23S ribosomal RNA (Roche, Grenzach-Wyhlen, Germany) were treated as described above and served as a calibration standard in each quantification assay. Fluorescence was measured at an excitation of 485 nm and an emission of 520 nm.

3.3.10. Quantitative reverse transcription PCR (qRT-PCR)

Bacterial and archaeal 16S rRNA gene copy numbers were determined by quantitative reverse-transcription PCR using the OneStep RT-PCR Kit (Qiagen, Hilden, Germany). The primer pairs 519f/907r and s D Arch 0025-a-S-17/s-D-Arch0344-a-S-20 were used to quantify bacterial and archaeal RNA, respectively. Primer sequences of these two domains are given in Wilms et al. (2007). Each 25 µl PCR reaction contained 15.9 µl nuclease-free water, 5 × RT-PCR Buffer (Qiagen, Hilden, Germany), 0.4 mM dNTP Mix (Qiagen, Hilden, Germany), 0.2 µM of each primer, 0.1 µl of a 1:500 diluted SYBR Green I solution (Molecular Probes, Eugene, OR, USA),

1 µl One Step Enzyme Mix (Qiagen, Hilden, Germany) and 1 µl standard (10^9 to 10^2 gene copies per µl) or environmental target RNA. Thermal cycling comprised a reverse transcription step for 30 min at 50 °C, followed by an initial denaturation step for 15 min at 95 °C, 40 cycles of amplification (10 s at 94 °C, 20 s at 54 °C for bacterial RNA quantification or 48 °C for archaeal RNA quantification, 30 s at 72 °C and 20 s at 82 °C) and a terminal step (2 min at 50 °C). After each run, a melting curve was recorded between 50 °C and 99 °C to ensure that only specific amplification had occurred.

As standards for bacterial gene targets, 16S and 23S ribosomal RNA of *E. coli* (Roche Diagnostics GmbH, Grenzach-Wyhlen, Germany) were used. A PCR product was used as standard for quantification of Archaea. Archaeal primer sequences and PCR conditions are given in Wilms *et al.* (2007). For each amplification the OneStep RT-PCR Kit was used according to the manufacturer's instructions. All PCRs contained a reverse transcription step (30 min, 50 °C) prior to amplification.

3.4. Results

The monitored microbial processes demonstrated similar trends in the degradation experiment and the untreated control. In contrast, the abiotic control showed no sign of sulfate reduction, methanogenesis, IPL degradation, fermentation or any other microbial activity as demonstrated by the stability of all measured parameters (data not shown).

3.4.1. Sulfate and methane data

Sulfate reduction and methanogenesis are terminal anaerobic mineralization processes. The concentrations of sulfate and methane were monitored to assess the current metabolic status of the experiment.

Sulfate was completely consumed within the first 9 days (Fig. 3.2). Between day 9 and day 20, the sulfate concentration remained below the detection limit, until sulfate was refilled. Sulfate was replenished to mimic the natural environment. At the end of the experiment, sulfate was consumed more slowly than at the beginning, indicating the depletion of electron donors. The sulfate concentration decreased to 9 mM on day 97. Large amounts of methane were only detected in the absence of sulfate. The slightly decreasing values for sulfate consumption were in the range of the analytical error.

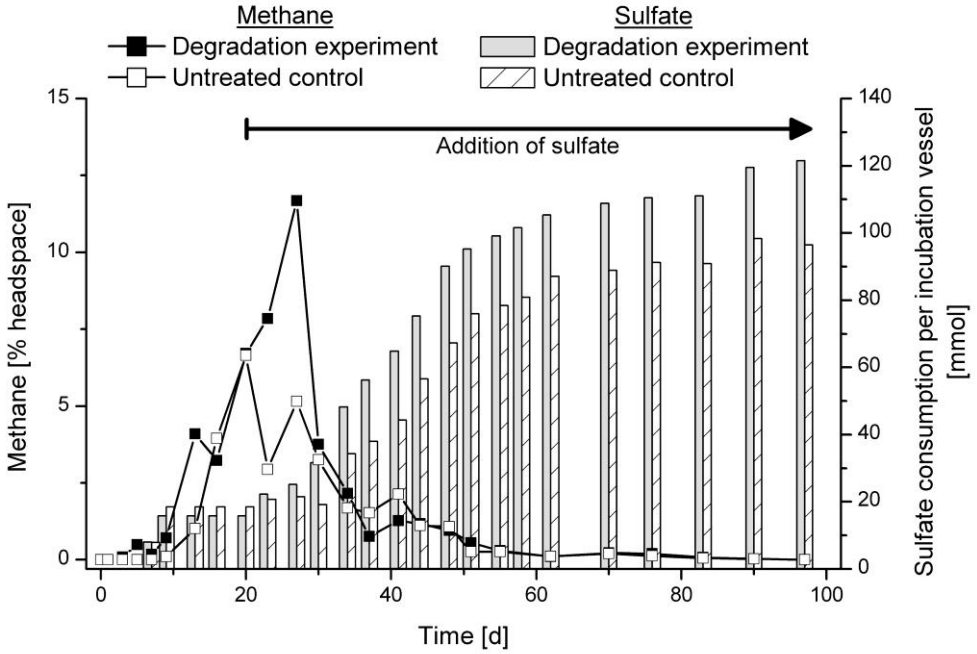

Figure 3.2: Methane production and cumulative sulfate consumption in the degradation experiment and the untreated control. Sulfate consumption for each time point was calculated by addition of measured day-to-day losses. Methane concentrations are given in percentage of incubation vessel headspace. After day 20 sulfate was refilled when the concentration decreased below 10 mM as indicated by the arrow.

The concentration of dissolved sulfide in the pore water remained relatively low (6 mM) until day 27. The maximum concentration of 38 mM was reached on day 76. Oxygen was never detected in any incubation vessel.

3.4.2. Degradation of intact polar lipids

The HPLC-MS chromatograms of the initial cell material of *Saccharomyces cerevisiae* and *Haloferax volcanii* showed characteristic signals for each organism (Fig. 3.3a and b).

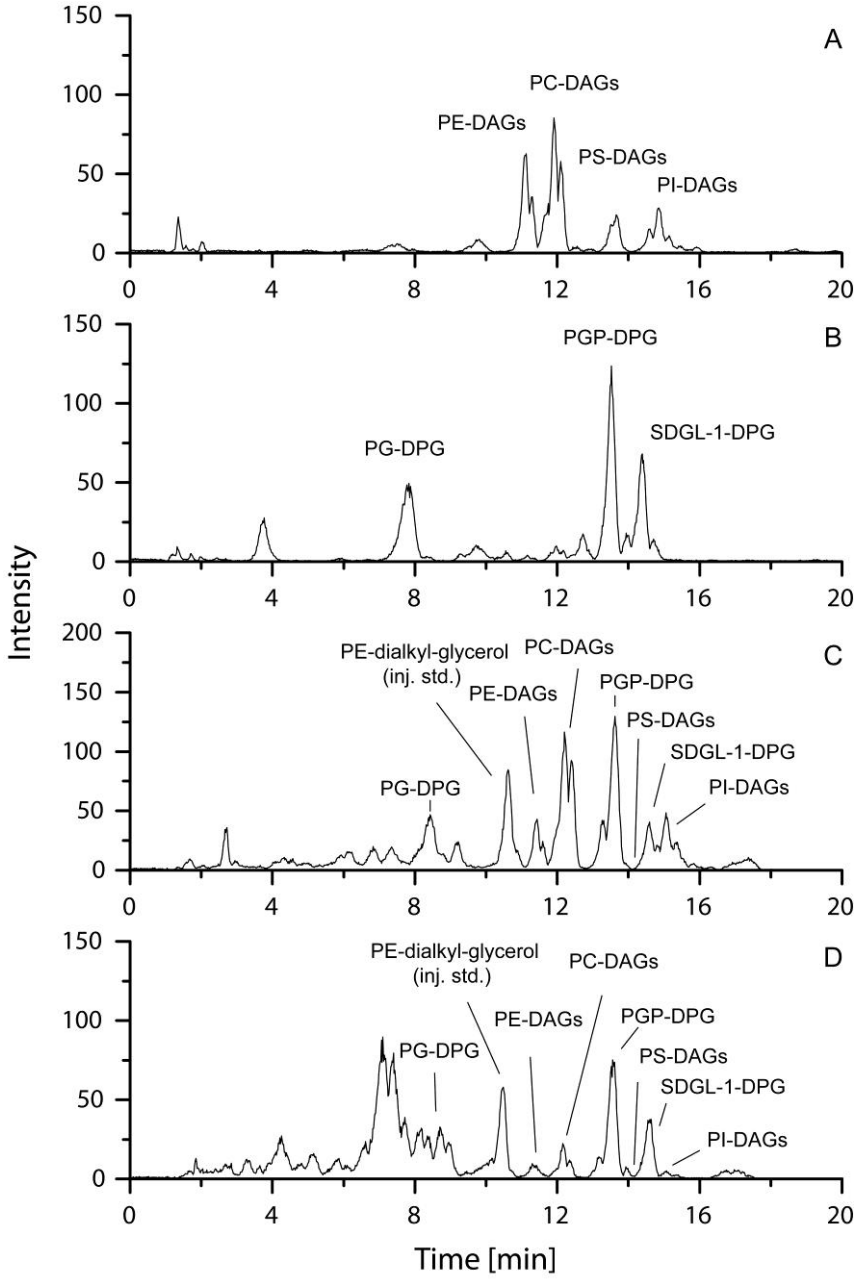

Figure 3.3: HPLC-ESI-MS base-peak intensity (BPI) chromatograms in negative ion-mode of A: total extract of *Saccharomyces cerevisiae*; B: total extract of *Haloferax volcanii*; C: total extract of the untreated control at day 0; D: total extract of the degradation experiment, sample taken at day 0. For abbreviations see Fig 3.1. Please note that the observed peak intensities cannot be directly transferred into concentrations.

The IPL composition of the microbial community in the untreated control is different from those found in the added cells and showed a high variability (Fig. 3.3c). The characteristic signals of the added cell material were easily detectable in the degradation experiment (Fig. 3.3d). After an incubation period of 97 day substantial amounts of ester-bound IPLs of *S.cerevisiae* were degraded, whereas ether-bound IPLs of *H. volcanii* were still present (Fig. 3.3e).

The amounts of ester-bound IPLs decreased in the course of the experiment whereas those of ether-bound IPLs remained stable (Figs. 3.4 - 3.6). The head groups had no visible influence on the observed degradation pattern. The amounts of ester-bound IPLs with PC and PI head groups did not show any significant change in the first days of the degradation experiment. Beginning at day 5, they decreased rapidly over 5 days followed by a phase of moderate loss until day 90. In case of phosphatidylethanolamine-diacylglycerol (PE-DAG) and phosphatidylserine-diacylglycerol (PS-DAG) highest amounts were found on day 5 (Figs. 3c and d). Subsequently, the signal decreased over the rest of the experiment.

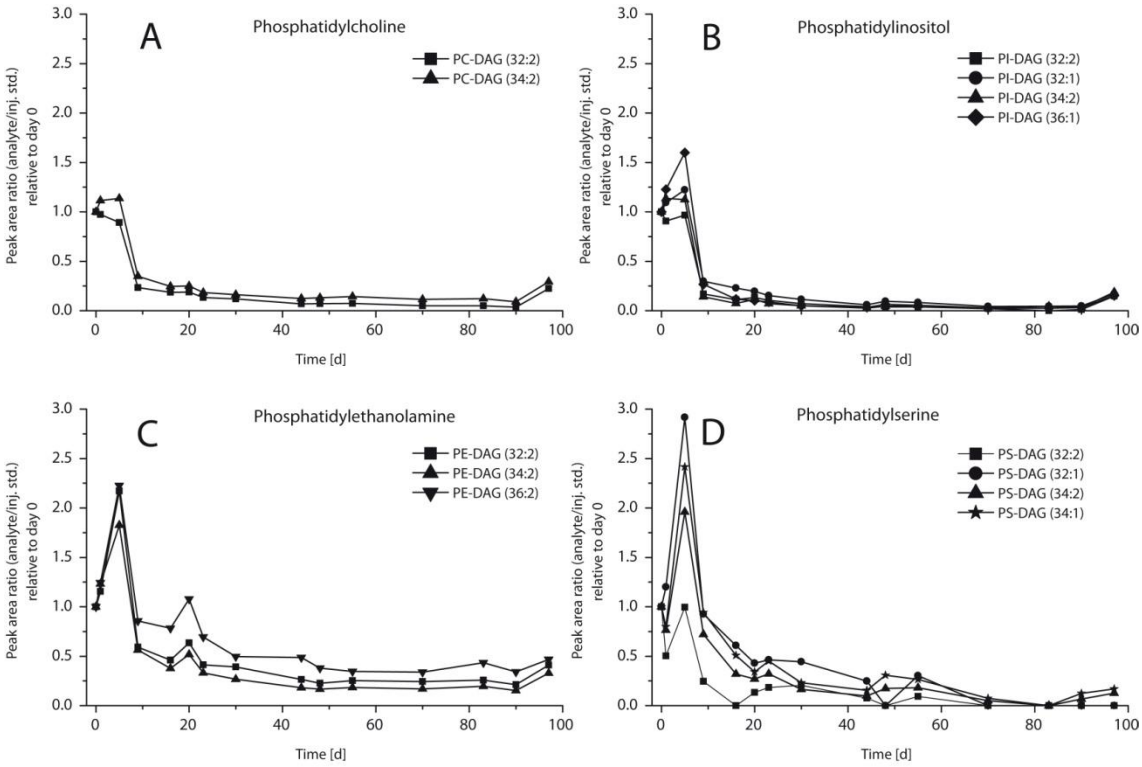

Figure 3.4: Relative concentrations of ester-bound IPLs with different head groups in the degradation experiment vs. time in days (normalized to day 0). Core lipid structures are given as sum of fatty acids (e.g. 32:2) where 32 represents the number of carbon atoms and 2 represents the number of double bonds in the core lipid structure. See Fig. 3.1 for abbreviations.

In contrast to this, the amounts of all ether-bound IPLs (Fig. 3.5) scattered but did not decrease significantly until the end of the degradation experiment. Ester- and ether-bound IPLs in the abiotic control showed behavior similar to each other with slightly decreasing values in

the course of the experiment (Fig. 3.6). The amounts of IPLs in the degradation experiment increased within the first days while the abiotic control did not show this effect. The archaeal ether lipids in the degradation experiment remained elevated by about 50 % relative to the abiotic control throughout the entire experiment.

Samples from the untreated control were taken in the same sampling intervals as in the degradation experiment. Most of the IPLs added to monitor the degradation were not detected in the untreated control. Exceptions were the IPLs PE-DAG 34:2 and PE-DAG 36:2, but compared to the corresponding PE-DAGs in the added biomass their total amount was low (less than 3 %). However, other IPLs such as sulfoquinovosyl diacylglycerol (SQ-DAG) and phosphatidylglycerol-diacylglcerol (PG-DAG) were identified in the untreated control. Additionally, PE-DAGs with side chains different from those in the degradation experiment were found (31:0, 31:1, 33:1, 33:2, 35:2). SQ-DAG was the most prominent IPL in the untreated control. It showed increasing abundance from day 0 to day 23 and then a decrease to the starting value at a moderate rate after a major drop between days 23 and 27. PE-DAG and PG-DAG showed an increase between day 0 and day 5 and returned to the starting values in the course of the experiment.

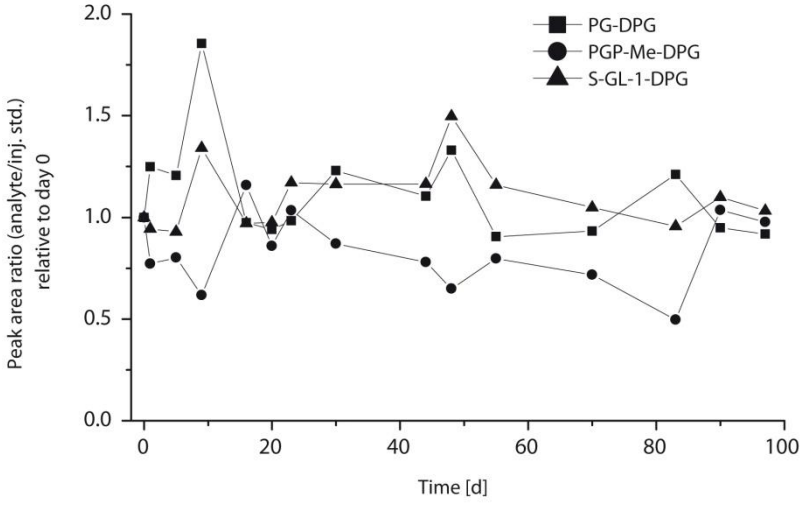

Figure 3.5: Relative concentrations of ether-bound IPLs with different head groups in the degradation experiment vs. time in days (normalized to day 0). Abbreviations: sulfono diglyco dialkylglycerol (S-GL-1-DPG, according to Sprott et al., 2003), diphytanylglycerol (PG-DPG) and diphytanylglycerol methylphosphate (PGP-Me- DPG).

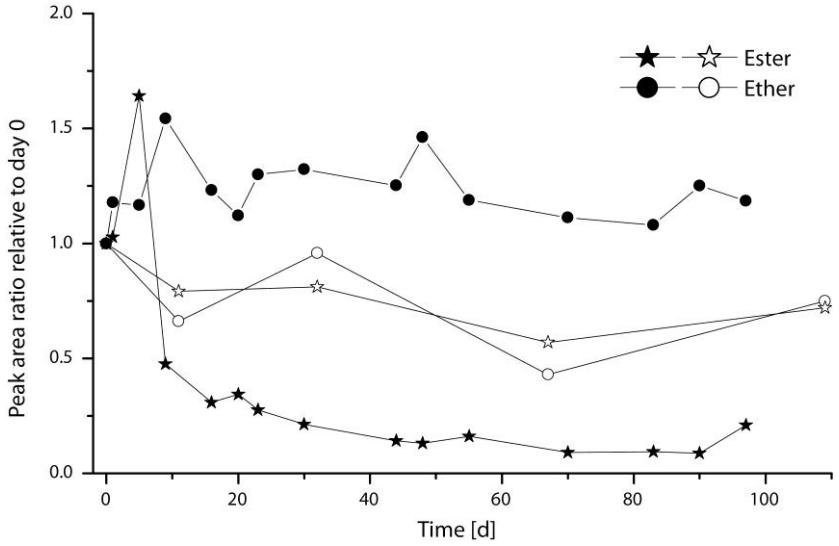

Figure 3.6: Sum of ester- and ether-bound IPLs in the degradation experiment and the abiotic control given as relative concentrations normalized to day 0. Closed symbols: degradation experiment; open symbols: abiotic control.

3.4.3. IPL degradation rates

The calculation of degradation rates for ester-bound IPLs resulted in a linear relation between log k' and log time (Fig. 3.7). The k'-values of ester-bound IPLs were higher than those of ether-bound IPLs reflecting the faster decreasing concentration of ester-bound IPLs (Fig. 3.6). Since the k'-values are plotted logarithmically, an increase of one unit represents ten times faster degradation. Due to the scattered amounts of ether-bound IPLs (Fig. 3.6), the relation for their kinetic degradation constants was less linear (Fig. 3.7).

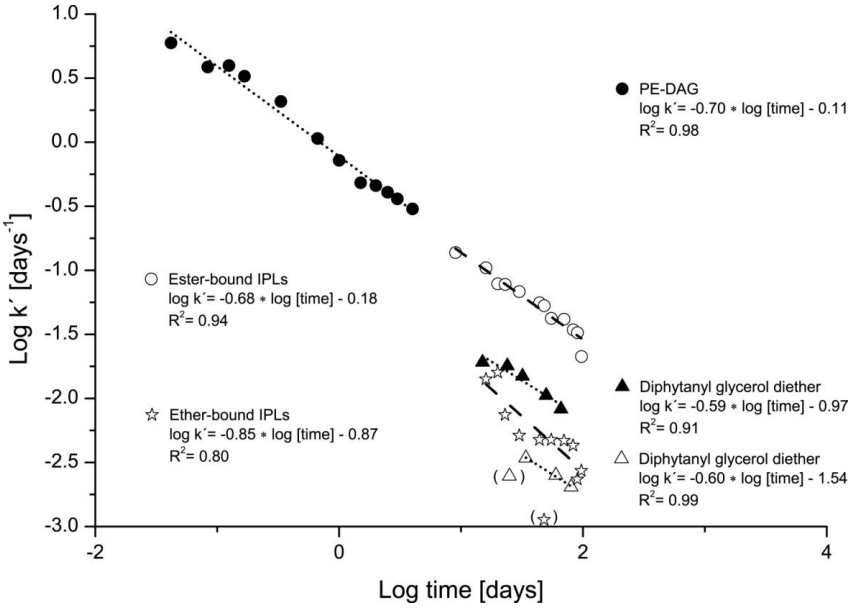

Figure 3.7: Log-log plot of calculated kinetic degradation constants of ester-bound and ether-bound IPLs (dashed lines) with time in combination with three incubation experiments from Harvey *et al.* (1986, dotted lines). Closed symbols indicate aerobic degradation and open symbols anaerobic degradation. The plot was prepared as described by Schouten *et al.* (2010).

3.4.4. Succession in microbial abundance, diversity and activity

The development of the microbial communities was monitored to obtain background information for the degradation of IPLs. The total cell counts of the degradation experiment and the untreated sediment showed the same trend and decreased only slightly during the experiment (Fig. 3.8). The total cell numbers of the degradation experiment were marginally higher than those of the untreated control. The numbers of bacterial 16S rRNA targets were one to two orders of magnitude higher than those of *Archaea*. Between days 7 and 16, the bacterial 16S rRNA copy numbers dropped significantly before returning to their previous values. The number of archaeal 16S rRNA copies showed a generally increasing trend. After day 20, the numbers of both bacterial and archaeal 16S rRNA targets remained relatively constant. The RNA content of the sediment ranged from 80 to 4800 ng cm^{-3}. Ravenschlag et al. (2000) determined an rRNA content of 0.9 to 1.4 fg rRNA per cell for two sulfate-reducing bacteria from surface sediments. These values were used to assess the total cell numbers of our study. The calculated values range between 8.9×10^7 and 3.4×10^9 cells cm^{-3}, which fits nicely to our total cell counts. The analysis of fermentation products showed no significant difference between the degradation experiment and the untreated control (Fig. 3.9). The bacterial community profiles of the degradation experiment and the untreated sediment looked similar to each other (Fig. 3.10). Initially, the community structure was highly diverse. This diversity decreased in the course of the experiment probably due to a diminishing substrate spectrum as indicated in Fig. 3.9.

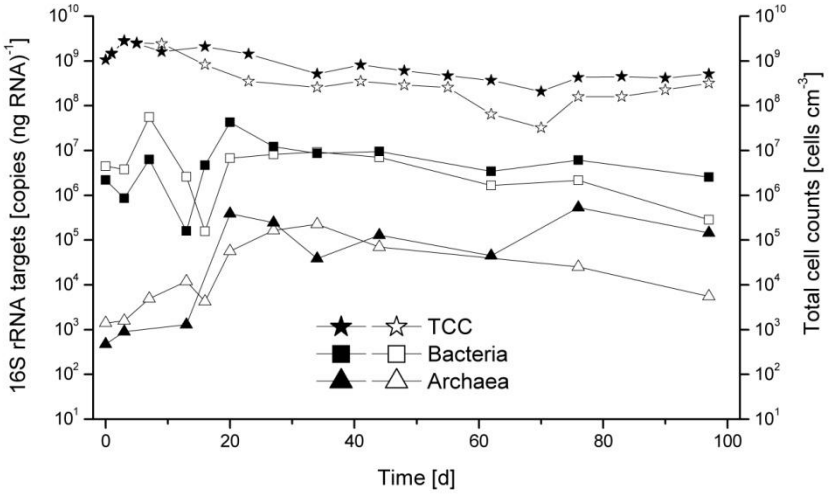

Figure 3.8: Bacterial and archaeal 16S rRNA copies and total cell counts (TCC) in the course of the experiment. The number of bacterial and archaeal 16S rRNA targets are given in copies per ng of extracted RNA, whereas the total cell counts are given in cells per cm^3 sediment. Closed symbols: degradation experiment; open symbols: untreated control.

The archaeal community pattern showed minor differences between the degradation experiment and the untreated control (Fig. 3.11). *H. volcanii* was only detected at the very first

sampling point in the degradation experiment. In the beginning of the experiment, when sulfate was still present, no methanogenic archaea were detected (Fig. 3.11). Only after depletion of sulfate the rRNA of methanogens was found. The presence and activity of these organisms were supported by methane production observed in the absence of sulfate. In all samples, the content of eukaryotic RNA was too low to obtain sufficient PCR products to prepare a DGGE with eukaryotic primers.

3.4.5. Total organic carbon

The carbon content was analyzed at five time points of the degradation experiment and the untreated control. The difference between the degradation experiment and the untreated control reflected the amount of organic matter that was added to the degradation experiment with the inactive cell material of *S. cerevisiae* and *H. volcanii*. We added 1.36 g of cell derived organic carbon which increased the TOC content of the natural sediment from 0.23 % C_{org} (4.9 g) to 0.29 % C_{org} (6.2 g). No pronounced difference was visible between the degradation experiment and the untreated control for all other parameters.

3.4.6. Effects of sediment-derived organic matter on microbial processes

The increasing amounts of fermentation products and total cell counts within the first week demonstrate a stimulation of microbial activity. This was likely caused by a temperature increase from 4 °C (storage temperature) to 20 °C in the laboratory. The addition of biomass caused a faster increase of methanogenesis and fermentation. The added biomass also resulted in slightly increased rates of sulfate consumption, methane production and fermentation (Figs. 3.2 and 3.9). Roughly 10 % of the cellular dry weight consists of lipids (Stouthamer, 1979) and the IPL content is even lower. Therefore, the absolute amount of IPLs that were added with the biomass of *H. volcanii* and *S. cerevisiae* should have been low compared to other organic compounds present in the sediment itself. Accordingly, the fermentation products do not only reflect IPL degradation but mainly degradation of the organic matter inherited from the natural sediment (Fig. 3.9).

3.5. Discussion

In this experiment, ester-bound bacteria-like IPLs were degraded faster than ether-bound archaeal IPLs while an influence of the structure and the bonding type of the head group was not detected.

3.5.1. Quality assessment of experimental design and data

Haloferax volcanii and *Saccharomyces cerevisiae* do not occur naturally in tidal-flat sediments. Thus, it might be suspected that the observed degradation rates of IPLs turn out to

be different from those of IPLs in the natural microbial community. However, the source of ether- and ester-bound IPLs should have no influence on the degradation rate since the chemical structures and the bonding types of the added material also occur naturally in IPLs found in Wadden Sea sediments (Rütters et al., 2001). Nevertheless, it is necessary to use cell material that is not indigenous to the sediment matrix to monitor the degradation of individual IPLs without confusing them with the inherited IPL inventory.

One could argue that the different degradation patterns observed for ether- and ester-bound IPLs are caused by a selective protection of ether-bound IPLs in intact *H. volcanii* cell material and on the other hand disrupted cells of *S. cerevisiae*. Membrane lipids in living organisms are protected by their surrounding cell walls. The outer surface of *Haloferax volcanii* consists of a hexagonally packed surface (*S*) layer glycoprotein (Sumper et al., 1990), whereas the cell wall of *Saccharomyces cerevisiae* consists mainly of glucan polymers, chitin and glycoproteins (Levin, 2005). Thus, the cell walls are likely to differ in stability. As archaeal S-layers are exceptionally stable, one might assume that the cells were still largely intact during the experiment and thus, the ether-bound lipids were not readily available for degradation. There are several arguments that this problem did not occur in our experimental set-up:

Although approximately 1×10^9 *H. volcanii* cells ml^{-1} were added, no difference in total cell counts was observed between the degradation experiment and the untreated control at the beginning of the experiment.

The amount of archaeal 16S rRNA of the untreated control was even lower than in the degradation experiment at the beginning of degradation.

The RNA of *H. volcanii* was only detected at the very first sampling point of the experiment. The rapid degradation of the RNA indicates disintegration of the *H. volcanii* cells. RNA-based community analyses are often used to determine the active part of a community since the RNA content of cells can be correlated with cellular activity (Lee & Kemp, 1994; Wagner, 1994). However, stable isotope probing (SIP) experiments have shown that the RNA is detectable for days even when the microbes are not active (Graue et al., 2011). In our experiment we found RNA of sulfate-reducing bacteria in the absence of sulfate, supporting the finding that RNA is not degraded immediately when these organisms are inactive (Fig. 3.10).

Most importantly, however, the glycoprotein cell wall of halophilic archaea of the order *Halobacteriales* requires a high NaCl concentration for stability and cells almost instantaneously lyse in the absence of salt as described by Mohr and Larson (2003) and Kushner (2001). *H. volcanii* additionally requires high concentrations of magnesium (Cohen et al., 1983).

As the *H. volcanii* cells were pasteurized in distilled water, it is unlikely that the cells remained intact.

Finally, cultivation attempts with pasteurized cells showed no growth which indicates that the *H. volcanii* cells were killed during the experiment. Thus, according to our line of argument it is very unlikely that the added biomass contained intact cells protecting IPLs from degradation.

If the degradative capacity of the microbial community is exceeded due to the high load of organic matter this would affect the degradation rates. However, high organic matter concentrations in Wadden Sea sediments are not unusual. They are caused e.g. by burial of algal blooms during storm events. In a study of Neira and Rackemann (1996) the degradation of algae biomass was investigated in situ. The amount of introduced total organic matter was 300 times higher than in our experiment. Nevertheless, the organic matter was degraded within two month. Even if the conditions of this study are not completely the same, we can assume that the degradative capacity in our study is not a limiting factor.

Comparing Figs. 3.4 and 3.5, different scattering patterns for ester- and ether-bound IPLs become apparent. The ester-bound IPLs showed a strong increase from day 0 to 7 only for PE-DAGs (Fig. 3.4c) and PS-DAGs (Fig. 3.4d). This effect was not observed for PC-DAGs, PI-DAGs and ether-bound IPLs (Figs. 3.4a, b and 3.5, respectively). Additionally, the analysis of ether-bound IPLs showed non-systematic scattering. Adsorption/desorption processes of IPLs to/from the sediment matrix may be an issue. Different head groups lead to significant differences in chemical properties such as solubility, polarity and acidity. Changes in pH and redox potential influence adsorption/desorption processes and due to different chemical properties this may affect the investigated IPLs unequally. Sediment inhomogeneity is also a possible explanation. We tried to minimize this effect by the design of the incubation vessel and intense mixing prior to every sampling. In addition to this, the sediment was resuspended by shaking directly before opening the incubation vessels in the anaerobic chamber. Other reasons for scattering IPL values may be varying extraction efficiencies or changing matrix effects during ESI ionization.

There are several possible explanations for the elevated amounts of archaeal ether lipids in the degradation experiment relative to the abiotic control (Fig. 3.6). The higher values for ether-bound IPLs may be caused by the presentation of data normalizing to day 0. If the sediment despite all caution was not completely homogenized during the first sampling, this affects all data points. Furthermore, the sediment of the abiotic control was autoclaved. This thermal treatment may have changed the structure of organic matter leading to a stronger adsorption. Another explanation may be that the microbial community in the degradation

experiment has a positive effect on the release of adsorbed IPLs leading to a high recovery of those molecules. Nevertheless, all mentioned difficulties do not have any impact on the general results of this study.

3.5.2. Influence of bonding types and structural moieties on IPL degradation

Ester-bound IPLs are generally not only degraded by the hydrolysis of head groups but also by the hydrolysis of fatty acid side chains (Matos & Pham-Thi, 2009). Since the degradation pattern and hence the stability of all investigated ester-bound bacteria-like IPLs was approximately the same (Figs. 3.4a-d), it has to be considered that both the hydrolysis of head groups or side-chains might be responsible for their degradation patterns. In case of the archaeal lipids two structurally different bonding types of head groups were present – one glycosidic (S-GL-1) and two phosphoester (PG-DPG and PGP-Me-DPG; Fig. 3.1) head groups. However, the same degradation patterns were observed for both binding types. In general, compared to glycosidic bonds, phosphoester bonds are chemically more labile, since phospho-esters can be hydrolyzed by acids and bases whereas glycosidic bonds can usually only be hydrolyzed by acids (Beyer, 1991). Correspondingly, Harvey *et al.* (1986) found that glycosidic ether lipids were more stable than phospholipids with ester-bound moieties. However, they investigated the degradation of a glycosidic ether-bound lipid and a phosphoester ester-bound lipid. Thus, the study of Harvey et al. (1986) cannot be used to answer the question which part of an IPL-molecule (head group or moiety bonding type) is responsible for the observed differential stability of bacterial and archaeal IPLs.

According to our results, we assume that the differences in chemical stability of IPLs play only a minor role during the degradation of IPLs, at least in the investigated system. Since not only the head groups may have an influence on IPL degradation, also the structurally different core lipids have to be considered as possible factor for the observed degradation patterns. In general, complex molecules like lipids and proteins are hydrolyzed by exo-or ectoenzymes which are released by prokaryotic cells (Cypionka, 2010). Therefore, we conclude that microbiological enzymatic processes are the driving force in IPL degradation as also suggested by White et al. (1979) and Harvey et al. (1986) rather than expected chemical stabilities alone.

3.5.3. IPL degradation rates

The kinetic degradation constants of ester-bound IPLs (Fig. 3.7) in the beginning of our experiment are in the same range as those at the end of aerobic degradation of PE-DAG in beach sediments observed by Harvey et al. (1986). Measured signal intensities in the degradation experiment on day 7 were as high as expected from the amount of added biomass. Therefore, significant degradation did not occur during the first 7 days. This means

that the kinetic degradation constants of ester-bound IPLs in our experiment were significantly lower than those determined by Harvey et al. (1986).

In case of ether-bound IPLs the kinetic degradation constants determined in our study lie between those calculated for the ether-bound IPL with a glycosidic head group under oxic and anoxic conditions. In all cases this is in accordance to the common knowledge that the turnover of organic matter is aerobically faster than anaerobically.

3.5.4. Microbial activity

Originally, the experiment was also planned as an enrichment culture for lipid-degrading bacteria. It turned out, however, that the added cell material had only little influence on the community structure and the metabolic activity. This had a positive side-effect, since the processes which originally occur in tidal flat sediments were not disturbed and superposed. Therefore, the selected set-up is suitable to reflect the natural IPL degradation in such sediments. The increasing total cell counts in the beginning of the experiment were also visible in the untreated control and thus were probably caused by stimulation due to mixing and the higher temperature (4 °C storage temperature and 20 °C during the experiment).

Approximately 10 % of the *Archaea* in Wadden Sea sediments are known to be methanogens (Wilms et al., 2007). They have a narrow substrate spectrum limited to simple molecules and are not known to degrade lipids. Probably, the main lipid degraders are *Bacteria* which are more abundant than *Archaea* in intertidal surface sediments (e.g. Beck et al., 2011). Anyhow, the physiological roles of most sedimentary archaea are not known (e.g. Teske & Sorensen, 2008) and therefore it cannot be excluded that they are also involved in the degradation of lipids.

Some studies indicate that *Archaea* use other enzymes than *Bacteria* to synthesize and degrade IPLs (Choquet et al., 1994; Daiyasu et al., 2005). The IPL degradation experiment was conducted with anoxic surface sediment which is dominated by bacteria affiliated to *Proteobacteria, Actinobacteria, Bacteriodetes, Firmicutes, Spirochaetes* and *Chloroflexi*, and archaea affiliated to *Methanosarcinales*, *Thermococcales, Methanomicrobiales* and *Methanobacteriales* (Wilms et al., 2006). The community structure of the deep biosphere, however, consists mainly of bacteria related to *Chloroflexi, Gammaproteobacteria* and JS1 candidate group and of archaea affiliated to the Miscellaneous Crenarchaeotic Group (MCG), Marine Benthic Group (MBG) and South African Goldmine Euryarchaeotal Group (SAGMEG) (Horsfield et al., 2006; Inagaki et al., 2006; Teske & Sorensen, 2008). As our results strongly suggest a microbial degradation of IPLs by enzymes, the community structure probably has a major influence on the degradation pattern. Thus, the observed degradation rates might be

different if the experiment is repeated with sediment from the deep biosphere or in sediments where sulfate-reducing bacteria are not stimulated.

The question whether ester-bound lipids are recycled and used for membrane synthesis by *Bacteria* cannot be answered by our experimental-setup. Enzymes are described for *Eukarya* and *Prokarya* that facilitate bidirectional ATP-independent flipping of polar lipids across cytoplasmic membranes (Sanyal & Menon, 2009). The incorporation of extrinsic cell building blocks is energetically useful for microorganisms because it is more efficient to recycle existing molecules than to break them down to smaller molecules to synthesize them "de-novo". Takano *et al.* (2010) added ^{13}C-labeled glucose to marine sediment and found that the ^{13}C was incorporated into the glycerol backbone of archaeal membrane lipids whereas the isoprenoid core lipids remained unlabeled. This indicates a recycling of comparable large membrane building blocks. To answer the question if IPLs can be recycled as intact molecules, stable-isotope probing (SIP) or ^{14}C-labeling would be suitable tools to follow the degradation and incorporation of IPLs and their resulting products.

3.5.5. DNA, RNA and intact polar lipids as biomarkers for living cells

DNA and IPLs are commonly used as biomarkers for living cells (e.g. Biddle et al., 2006; Wilms et al., 2006). Additionally, RNA is used to determine the active part of microbial communities (e.g. Griffiths et al., 2000). However, the successful application of these methods may be hampered by several aspects like varying extraction efficiencies, matrix effect and adsorption/desorption processes. The presence of DNA does neither prove the activity nor even the presence of the corresponding cells (Lorenz & Wackernagel, 1987; Josephson et al., 1993). In contrast, RNA is labile and the RNA content of cells can be correlated with cellular activity (Lee & Kemp, 1994; Wagner, 1994). However, when cells are inactive or have a very slow metabolism, the RNA content might not be sufficient for detection. The stability of ether-bound IPLs in our study suggests that IPLs also do not reflect exclusively the living community. This shows that none of the mentioned tools can be used without caution but a combination of these tools may help to get a more accurate picture.

3.6. Conclusions

The quantification of *Bacteria* and *Archaea* in the deep marine biosphere by IPL and FISH analysis led to the assumption that this habitat is dominated by *Archaea* (Biddle et al., 2006; Lipp et al., 2008). In contrast, catalyzed reporter deposition fluorescence in situ hybridization (CARD-FISH) and quantitative polymerase chain reaction (q-PCR) suggest a predominance of *Bacteria* (Schippers et al., 2005; Inagaki et al., 2006). Our experiment shows that these inter-pretations have to be considered with caution, since etherbound archaeal IPLs were clearly

more stable than esterbound bacteria-like IPLs over a period of 100 days. In nature, ether-bound IPLs may even be preserved for longer time periods since the enhanced temperature in the laboratory accelerated the degradation processes.

To give better insights into the degradation of IPLs in other environments like the deep biosphere, it is desirable to conduct degradation experiments reflecting the in situ conditions of those habitats. For the deep biosphere this would mean that the experiments must be conducted under high pressure and over a long period since microbial turnover of organic matter is low. Furthermore, the addition of high substrate concentration would lead to a distorted image of ongoing processes as the in situ available organic matter is recalcitrant.

On the other hand, IPL-based quantification may lead to an overestimation of archaeal cell counts as indicated by our study. Regarding the discussion above, molecular biological methods based on DNA or RNA can also be inaccurate. Thus, the abundances of *Archaea* and *Bacteria* obtained with both methods have to be interpreted with caution.

Acknowledgements

The authors are grateful to B. Kopke, B. Buchmann, H. Haase and P. Neumann for their technical assistance. Many thanks to Michael Seidel for sharing his experience in IPL degradation experiments that were conducted prior to this work. We thank K.-U. Hinrichs, A. Schippers and two anonymous referees for their constructive comments and suggestions. This work was financially supported by Deutsche Forschungsgemeinschaft (DFG) within the Research Group on “BioGeoChemistry of Tidal Flats” (FOR 432).

3.7. Supplementary material

3.7.1. Introduction

To provide background information on the microbial activity and community structure, the fermentation products as well as the bacterial and archaeal community profiles were determined.

3.7.2. Materials and methods

Fermentation products

Concentrations of fermentation products in the pore water were analyzed by high-performance liquid chromatography (Sykam, Fürstenfeldbruck, Germany) using an Aminex HPX-87H column (Biorad, München, Germany) at 60 °C. The eluent was 5 mM H_2SO_4 at 0.5 ml min^{-1}. Organic acids were detected by an UV-VIS detector (UVIS 204; Linear Instruments, Reno, USA) at 210 nm. Alcohols were detected by a refractive-index detector (Knauer, Berlin, Germany). Prior to injection, the pore water was filtered through a 2 µm filter.

Denaturing gradient gel electrophoresis (DGGE) analysis

Partial 16S rRNA were amplified using the OneStep RT-PCR Kit (Qiagen, Hilden, Germany) with bacterial primers GC-357f and 907r as given in Muyzer et al. (1995) and archaeal primers S D Arch-GC-0344-a-S-20 und 907r as described previously (Wilms et al., 2006). Each forward primer contained a GC-clamp (Muyzer et al., 1993). Thermal cycling included a reverse transcription step for 30 min at 50 °C, followed by an initial denaturation step for 15 min at 95 °C, 30 cycles of bacterial RNA amplification and 35 cycles of archaeal RNA amplification (30 s at 94 °C, 45 s at 57 °C for bacterial RNA amplification or 48 °C for archaeal RNA amplification, 60 s at 72 °C), and a terminal step (10 min at 72 °C). The PCR amplicons and loading buffer (40 % [wt/vol] glycerol, 60% [wt/vol] 1× tris acetate-EDTA [TAE], and bromphenol blue) were mixed in a ratio of 1:2. DGGE was carried out as described by Süß et al. (2004) using an INGENYphorU-2 system (Ingeny, Leiden, Netherlands) and a 6 % (wt/vol) polyacrylamide gel containing denaturant gradients of 50 to 70 % for separation of bacterial PCR products. For separation of archaeal PCR products a denaturant gradient of 30 to 80 % was prepared. The gels were stained for 2 h with 1×SYBR Gold (Molecular Probes, Leiden, Netherlands) in 1× tris-acetate-EDTA buffer and washed for 20 min in distilled water prior to UV transillumination.

Sequence analysis

Individual DNA bands were excised from the gel with sterile scalpels, and the DNA was eluted into 50 µl molecular-grade water (Eppendorf, Hamburg, Germany) by incubation at 4 °C.

For subsequent sequence analysis, PCR products of DGGE bands were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) and commercially sequenced by GATC Biotech (Konstanz, Germany). The partial 16S rRNA sequences of the DGGE bands were compared to those in GenBank using the BLAST function (Altschul et al., 1997). All partial 16S rRNA gene sequences obtained in this study have been deposited in the EMBL database under accession numbers HE585651 – HE585692.

3.7.3. Results and Discussion

Fermentation products

Fermentation products were analyzed to monitor the utilization of organic matter. The data show the net balance of fermentation products since they are produced and consumed simultaneously (Fig. 3.9). In the beginning of the experiment the short-chain fatty acids were produced faster than consumed resulting in increasing concentrations. The main fermentation product was acetate followed by propionate, butyrate and valerate. During the final phase of the experiment, the fermentation products were utilized at the same rate as they were produced or the consumption was faster than the formation. Ethanol was also produced but could not be quantified due to signal overlapping with those of other unknown organic compounds from the sediment matrix. The graphs for short-chain fatty acids in the degradation experiment and the untreated control shared the same characteristics. The accumulation of short-chain fatty acids tended to be slightly faster in the degradation experiment but overall the added cell material had only a minor influence on the shape of the production curves of short-chain fatty acids. This indicates that the experiment was not disturbed by the addition of biomass as external substrate.

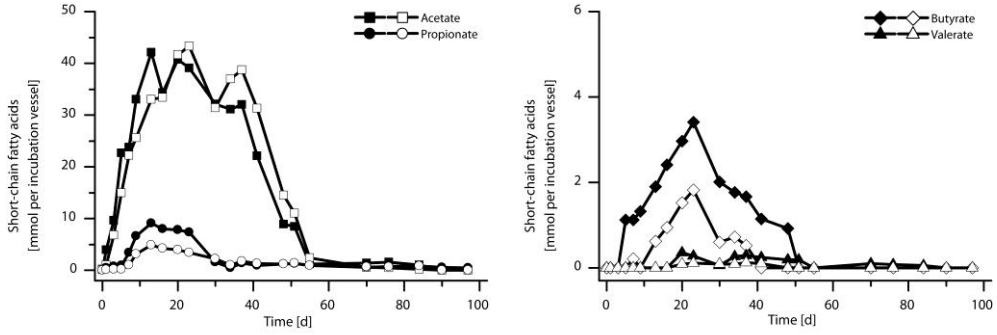

Figure 3.9: Pore water concentrations of acetate, propionate, butyrate and valerate in mmol per incubation vessel vs. time in days. Closed symbols: degradation experiment; open symbols: untreated control.

Community profiles

Sequencing of representative bands indicated the presence of different fermenting and sulfate-reducing bacteria (Fig. 3.10). The predominant organisms were relatives of *Pelobacter*

sp. and *Dethiosulfovibrio acidaminovorans*. These bacteria were highly abundant during the end phase of the experiment, when acetate and propionate were absent, indicating an enhanced activity in this period. A sequence of a Cytophaga fermentans-related organism was detected after one week, also hinting toward an increased activity of that organism from that time on. Additionally, this bacterium showed a higher abundance in samples from the degradation experiment. The abundance of other organisms, most of them being sulfate-reducing bacteria like a Desulfobacter psychrotolerans-related organism, appeared to be relatively constant over time, since sulfate was available almost during the entire experiment. Sulfur-oxidizing bacteria were only detected at the beginning of the experiment.

Surprisingly, chloroplast RNA of diatoms was found over the whole course of the experiment. Since no oxygen or light for algal growth were available in the experiment, the diatoms may have been inactive in a resting state. On the other hand, the RNA of chloroplasts may be exceptionally stable, because the RNA in this cell organelle is surrounded by four membranes (Kroth & Strotmann, 1999). In addition to this, the RNA of diatoms is protected by their rigid silica shell.

For both, the degradation experiment and the untreated control six bands of Archaea were detected throughout the whole experiment (Fig. 3.11). All sequences were closely related to either *Methanogenium frigidum* or *Methanogenium marinum*. The community patterns reflect the quantification of *Archaea* by qPCR: In the first week when the number of archaeal 16S rRNA gene targets was lowest, only very faint bands were detected in the DGGE profile.

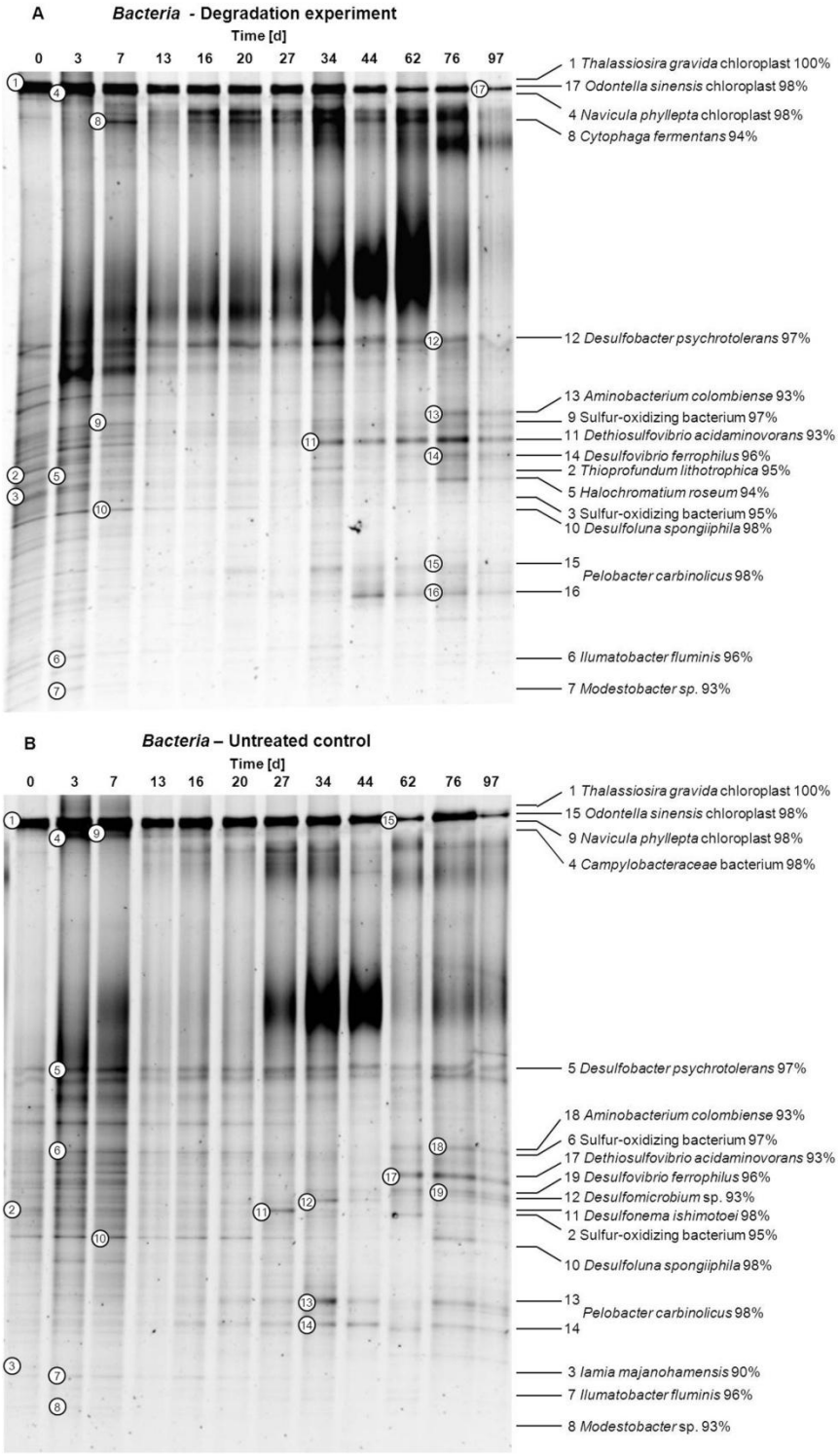

Figure 3.10: DGGE community profiles of Bacteria and closest cultivated relatives of the sequenced DGGE bands with similarity in % compared to data from the EMBL database. A: degradation experiment B: untreated control.

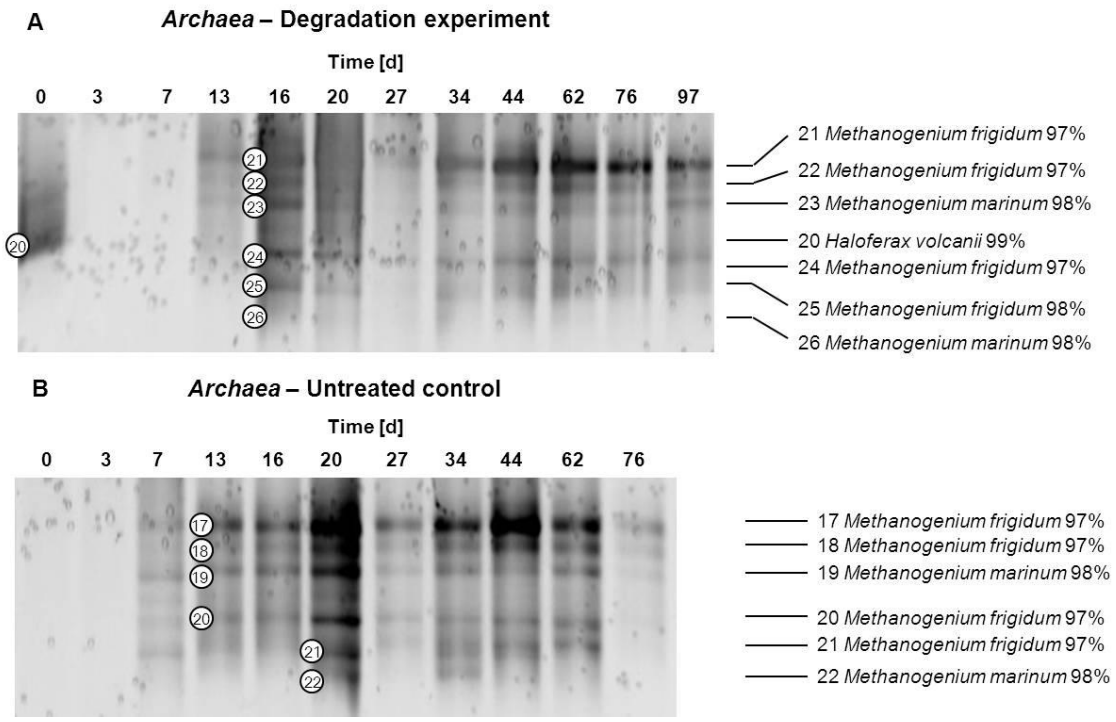

Figure 3.11: DGGE community profiles of Archaea and closest cultivated relatives of the sequenced DGGE bands with similarity in % compared to data from the EMBL database. A: degradation experiment B: untreated control.

Conclusion

The results of fermentation product and community analyses lead to the assumption that the added biomass did not significantly influence the microbial degradation and community structure in this study.

4. Adaptation of growth and membrane lipid composition of a piezothermophilic sulfate reducer from the deep subsurface as a response to temperature changes and *in-situ* pressure

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4.1. Abstract

Microbial life in deep marine subsurface is facing increasing temperatures and hydrostatic pressure with depths. In this study, we have examined growth characteristics and temperature-related adaptations of the *Desulfovibrio indonesiensis*-affiliated strain P23 to the *in-situ* pressure of 30 MPa since it was previously isolated at 20°C and atmospheric pressure from 61°C warm subsurface sediments. Faster growth was recorded at *in-situ* pressure and high temperature, while cell filamentation was induced by further compression. Complementary cellular lipid analyses revealed a two-step response of membrane viscosity to increasing temperatures with an exchange of unsaturated to saturated fatty acids and subsequent changes from branched to unbranched moieties. While temperature had a stronger effect on the degree of fatty acid saturation and restructuring of main phospholipids, pressure mainly affected branching and length of side chains. The detection of phosphate-free ornithine lipids points towards an adaptation to the low *in-situ* values of phosphate. Incorporation of ornithine lipids into the cell membrane was found to be temperature- rather than pressure-dependent. However, the simultaneous decrease of both factors to ambient laboratory conditions allowed the cultivation of our moderately thermophilic strain which in turn might be one key to a successful isolation of high-temperature and pressure adapted microorganisms from the subsurface.

4.2. Indroduction

The volume of world's oceans 200 m below sea level constitutes more than 95 % of all aquatic habitats (Michiels et al., 2008). Additionally, the subseafloor represents a large reservoir for prokaryotic life (Whitman et al., 1998; Kallmeyer et al., 2012) and even extends into the upper oceanic crust (Heberling et al., 2010; Orcutt et al., 2011). Taking the deep ocean and the marine subsurface into account, the majority of all prokaryotic cells on earth are facing high pressure conditions. Indigeneous microorganisms share the ability to thrive under extreme pressure and are classified according to their growth behaviour. While piezotolerant microorganisms grow best at atmospheric pressure of 0.1 MPa, piezophiles are defined to possess optimal growth rates at high pressure (Yayanos, 1995). Growth of piezosensitives is limited to pressures of up to 40-50 MPa, which is equivalent to depths of 4000-5000 meters (Abe & Horikoshi, 2001; Molina-Höppner et al., 2003). Obligat piezophiles require high pressure for growth (Yayanos et al., 1984).

Previous investigations on pressure adaptation of marine microorganisms were performed mainly on psychrophilic deep-sea bacteria (DeLong & Yayanos, 1985; Wirsen et al., 1986). While some data exist for thermophilic bacteria and hyperthermophilic archaea from hydrothermal vents (Jannasch et al., 1992; Alain et al., 2002), physiological data on mesophilic piezophiles are limited to a few isolates (Kaneko et al., 2000; Alazard et al., 2003; Khelaifia et al., 2011). Moreover, pressure studies on isolates from marine subsurface sediments are rare (Bale et al., 1997).

In our previous studies, we have isolated several sulfate reducers from up to 260 m deep subsurface sediments of the Juan de Fuca Ridge exhibiting a water depth of 2656 m, corresponding to an *in-situ* pressure of ~30 MPa (Fichtel et al., 2012). At this site, low-temperature hydrothermal fluids are diffusing from the underlying oceanic crust into the sediment column, resulting in a steep temperature gradient from 2°C to 62°C. Crustal fluids are introducing energy sources like sulfate (16 mM) from below, thus stimulating sulfate-reducing communities to thrive within this habitat (Engelen et al., 2008). Pure cultures were obtained under standard lab conditions, i.e. at atmospheric pressure and 20°C. The sulfate-reducing bacteria isolated from the deepest sediments above the basement solely belonged to the *Deltaproteobacteria*, namely one *Desulfotignum balticum* affiliated strain from 260 meters below seafloor (mbsf) and five strains related to *Desulfovibrio indonesiensis* from 240 to 260 mbsf. As members of the *Deltaproteobacteria* are not known to form any resting stages, they are supposed to belong to active microbial populations of the deep subsurface. Physiological characterization of the isolates revealed that *Dv. indonesiensis* affiliated strains turned out to be moderately thermophilic, growing from 10°C to 48°C, exhibiting chemoheterotrophic and –lithoautotrophic life-mode (Fichtel et al., 2012). Interestingly, the temperature range for growth did not reach *in-situ* temperatures of 56°C to 61°C. As temperature and pressure counteract on the cell membrane, an insufficient combination of both parameters might result in a disintegration of cells or an inhibition of cross-membrane processes (Mangelsdorf et al., 2005). Thus, the question arose wether incubation under *in-situ* pressure of ~30 MPa is inducing a shift in their temperature range.

This adaptation capacity is described in the homeoviscous theory (Macdonald, 1988; Somero, 1992). Organisms are able to regulate structure and organization of their cell membrane as a response to changes in temperature and pressure to maintain the membrane fluidity necessary for sustaining biological functions (DeLong & Yayanos, 1985; Delong & Yayanos, 1986; Wirsen et al., 1986; Yano et al., 1998). The reorganization influences the membrane lipid composition, the degree of saturation of membrane-bound fatty acids, as well as their chain length and branching.

In this study, several sulfate-reducing isolates from the deep marine subsurface are examined for growth under high pressure and various temperatures. We have chosen the *Dv. indonesiensis*-affiliated strain P23 to be analyzed representatively as the strain has been isolated from the deepest sediment sample and exhibited a relatively fast growth both, under high hydrostatic pressure and high temperatures. Microbial investigations such as the recording of specific growth curves were complemented by detailed lipid analysis to identify a cellular response to changing incubation conditions.

4.3. Material and Methods

4.3.1. Bacterial strains, their origin and growth conditions

Pure cultures of strictly anaerobic, sulfate-reducing bacteria used in this study were obtained from up to 260 m-deep subseafloor sediments. Samples were collected in the northeast Pacific at the Eastern Flank of the Juan de Fuca Ridge, Site U1301C (47°45.28'N, 127°45.80'W; water depth: 2656 m) during IODP Expedition 301 in 2004. Details of environmental conditions, sampling, contamination tests, sub-sampling and isolation procedures have been reported previously (Expedition 301 Scientists, 2005; Lever et al., 2006; Engelen et al., 2008; Fichtel et al., 2012). Enrichment and isolation of pure cultures were performed at ambient conditions, i.e. atmospheric pressure of ~0.1 MPa and 20°C. Culture media and cultivation procedures to obtain axenic cultures and the phylogenetic analysis have been described in detail by Fichtel et al. (2012). Strain P23 affiliated to *Desulfovibrio indonesiensis* (99 % 16S rRNA sequence similarity) was analyzed representatively for pressure and temperature adaptation in more detail.

For comparison, five additional isolates from the same sampling site, affiliated to *Dv. indonesiensis* (strains P12, P34), *Dv. aespoeensis* (P20), *Desulfotignum balticum* (P18), *Desulfosporosinus orientis* (P26), and the type strain of *Dv. indonesiensis* (Ind1^T, DSM 1512) were taken as references. All strains were pre-cultured to early stationary phase at atmospheric pressure and 25°C to 35°C in sulfate-containing (28 mM) artificial-seawater media that has originally been used for isolation (Fichtel et al., 2012). Lactate (10 mM) or betaine (5 mM) was used as carbon source.

Growth was routinely followed by photometrical determination of sulfide in form of colloidal CuS at 480 nm (Cord-Ruwisch, 1985) and of cell protein concentrations at 595 nm (Bradford, 1976) as well as by visual inspection of the cells using phase-contrast microscopy. Transmission-electron microscopy (TEM) of strain P12 was performed as described by Fichtel et al. (2012).

4.3.2. Pressure incubations

All pure cultures were generally examined whether they are able to grow under pressure (10 and 40 MPa). Bacterial growth experiments were performed in 'high-pressure steel vessels' (High Pressure Equipment Company - HiP, Linden, PA, USA). Inoculations were done in 60 or 70 ml serum bottles containing freshly prepared culture media and were afterwards sealed with rubber stoppers and crimp caps. Pre-cultures (4 % of final volume) were injected and bottles were completely filled with the respective media while gas bubbles were carefully eliminated by the usage of second syringe. Three serum bottles were placed inside a pre-heated pressure vessel filled with distilled water. Samples were set under hydrostatic pressure by means of a hand operated 'high pressure generator' (model 81-5.75-10, HiP) using distilled water as hydraulic fluid. For subsampling, the

vessel was carefully depressurized (~1 min) through a valve. The triplicate set of bottles was sub-sampled for growth analyses as quickly as possible (15-30 min), refilled with media and again compressed within a few minutes. Pressurized samples were incubated between one and sixteen days depending on growth behaviour. Growth at hydrostatic pressure was defined to be positive after two independent successful experiments. Growth at 0.1 MPa was assessed by using the same general protocol except pressurization. In general, all assays were carried out in triplicates and repeated at least twice.

4.3.3. Hydrostatic pressure effects on growth of *Dv. indonesiensis* strain P23

To record detailed growth curves, strain P23 was incubated at 20°C and 45°C, both at atmospheric pressure and *in-situ* pressure of 30 MPa. Sulfide content was determined immediately after decompression. For measurement of protein concentration of whole sample series, 4 ml-aliquots were transferred to sterile 15 ml-tubes and frozen until further processing. After sub-sampling, serum bottles were quickly refilled with substrate-free media and set under the respective test conditions. Exponential growth rates as a function of pressure and temperature were calculated according to Yayanos et al. (1995) and Kato et al. (1995) from 3-5 data points along the logarithmic portion of sulfide and protein production curves using linear regression analysis.

Additionally, the effect of different hydrostatic pressure on growth properties was determined at 0.1, 10, 20, 26, 30 and 40 MPa at 20°C and 45°C. Assays at 20°C were incubated for five days, whereas assays at 45°C were stopped after 36 hours. Growth response was characterized by comparing the amount of sulfide and protein formed during the time of incubation.

4.3.4. The upper temperature limit for growth of strain P23 at different pressures

In preliminary experiments, growth was tested in the range of 45°C to 62°C at 20, 26, and 30 MPa. Specific growth curves were finally recorded in parallels with slowly increasing temperatures from 45°C to 52°C at both, 0.1 and 20 MPa. Cultures grown at 45°C were allowed to adapt to higher temperatures for nine hours before incubation at 48°C. Temperature was raised again to 50°C after 12 h and to 52°C after 36 h of incubation. Pressure vessels were decompressed for growth analyses as described above at the end of each temperature-step. After sub-sampling, fresh medium was used to refill the serum bottles to circumvent substrate limitation.

4.3.5. Cultivation and extraction for lipid analysis

For determination of whole cellular fatty acids and intact polar lipids (IPL) strain P23 was grown as described above at 20, 35 and 45°C at both, atmospheric and *in-situ* pressure, in total culture volumes of 1.5-2.2 L. To obtain enough cell material, all pressure incubations were performed in parallels of up to 30 serum bottles using several pressure cylinders. To avoid skewing of our data due to growth phase differences (Hamamoto et al., 1994; Allen et al., 1999), cells of each experiment

were immediately harvested at late exponential growth phase, separated from the cultivation medium by centrifugation at 4°C, and stored at -20°C until further analysis.

Total lipids were obtained by ultrasonic extraction from each washed cell pellet following a modified Bligh & Dyer procedure (Sturt et al., 2004) as described by Logemann et al. (2011). The lipid extracts were combined and evaporated to dryness under nitrogen at room temperature, stored at -20°C and analyzed by combined coupled gas chromatography mass spectrometry (GC-MS) and coupled high performance liquid chromatography mass spectrometry (LC-MS).

4.3.6. Whole cell fatty acid analysis by gas chromatography and mass spectrometry

The following described technical procedures were adapted from Rütters et al. (2002). Aliquots of the total lipid extracts were transesterified with trimethylsulfonium-hydroxide as described by Müller et al. (1990). Fatty acid-methyl esters were quantified by using a gaschromatograph (7890A GC-System Agilent Technologies, Santa Clara, CA, USA) equipped with a flame-ionization detector (FID) and a capillary column (DB-5HT, length 30 m, ID 0.25 mm, 0.1 µm film thickness; J&W Scientific, Folsom, CA, USA). Identification was performed on a GC-MS using a HP 5890 Series II gas chromatograph (Hewlett Packard, Waldbronn, Germany) also equipped with a DB-5HT-column and coupled to a Finnigan MAT SSQ 710B mass spectrometer (Finnigan-Thermoquest, San Jose, CA, USA). Helium with a constant pressure of 12 psi was used for both systems. Oven temperature was raised from 60°C (isothermal for 2 min) to 360°C at a rate of 3°C min^{-1} and held for 5 min. Mass spectra were collected in full scan mode (*m/z* 50-650, ionization energy 70 eV and 230°C source temperature). Mass spectrometric investigations were used to confirm the results obtained with GC-FID. Fatty acids were identified by comparison of the retention times with those of known standards (Bacterial Acid Methyl Esters CP Mix; Supelco, Bellefonte, PA, USA).

4.3.7. Analysis of intact polar lipids

Intact polar lipids (IPLs) were analyzed from an aliquot of each cell extract using LC electrospray ionization (ESI) MS in the negative-ion-mode as described by Logemann et al. (2011). MS/MS spectra and full scan mass spectra (*m/z* 100-2000) were used for identification of head groups, diacylglycerols (DAG) or acyl/ether glycerol (AEG) core lipids and fatty acid side chain fragments. Quantification was achieved by using an external multipoint calibration via compound mass trace areas. Phosphatidic acid (PA), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), and phosphatidylinositol (PI) (all from Avanti Polar Lipids, Alabaster, USA; Matreya, Pleasant Gap, USA; Sigma Aldrich, München, Germany, and Lipid Products, Redhill, UK) were used as standard compounds representative for different IPL classes. Due to the lack of commercially available standards for ornithine lipids (OL) or unknown polar lipids, quantification of OL was done with the calibration curve for PG. Unknown compounds were quantified by using the average signal response of all standards at every concentration.

4.4. Results

4.4.1. Growth of sulfate-reducing strains under high hydrostatic pressure

All sulfate-reducing *Deltaproteobacteria* isolated from the deep ocean with a water depth of 2656 m and sediment layers of 1.3 mbsf and 240-260 mbsf exhibited growth under elevated hydrostatic pressure. This was also true for the type strain of *Desulfovibrio indonesiensis* (Ind1^T), which was originally isolated from a corroding ship at the sea-surface (Feio et al., 1998). However, the combination of higher pressure (40 MPa) and high temperature (45°C) severely affected general cytological functions of *Dv. indonesiensis* P23, which was also observed for the closely related strain P12. As monitored by phase-contrast, cell motility was highly reduced and cells appeared to be filamentous (Figs. 4.1 A,B). Cell division producing vibrio-shaped cells seemed to be incomplete and highly elongated cells of straight shape or cells associated in long chains were observed by transmission electron microscopy (Figs. 4.1 C,D). Phase-contrast micrographs of cultures grown at 45°C revealed that the cell length of strain P23 varied from 1-1.7 µm at atmospheric pressure and increased to 7.5-23 µm at 40 MPa. As a consequence, growth in further experiments was determined via sulfide formation and by measuring of protein production rather than cell counting.

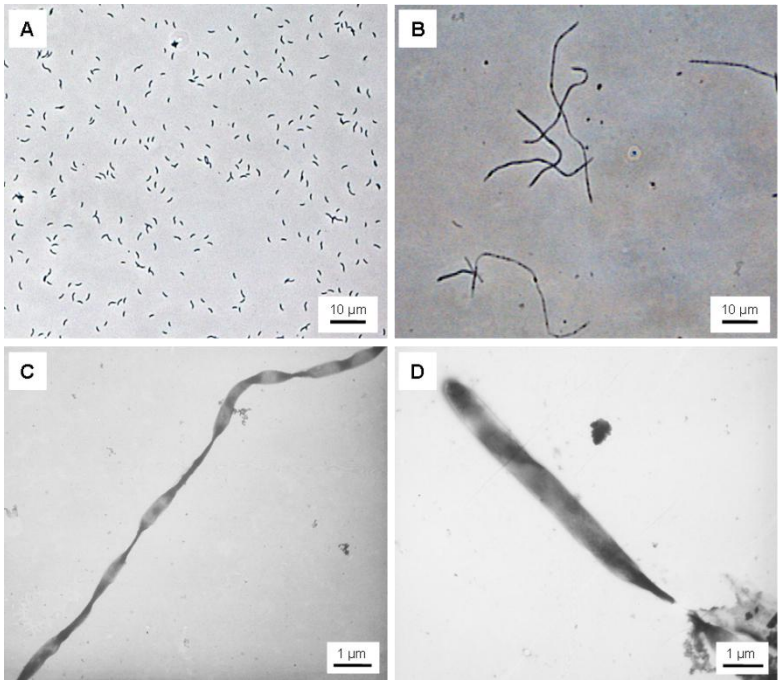

Fig. 4.1: Microscopic images of cell morphology obtained by phase-contrast of *Desulfovibrio indonesiensis* affiliated strains grown at A: 0.1 MPa and 45°C, B: 40 MPa and 45°C, C and D transmission-electron microscope images.

4.4.2. Specific growth rates of *Dv. indonesiensis* strain P23

Preliminary experiments were performed to identify the effect of various hydrostatic pressures (0.1, 10, 20, 26, 30 and 40 MPa) of low and high temperatures on the growth behaviour of strain P23 (Fig. 4.2). While at 20°C increasing pressure diminished the biomass formation, growth was

accelerated at 45°C and pressures between 10 and 30 MPa, as indicated by reaching higher protein contents at a given time-point.

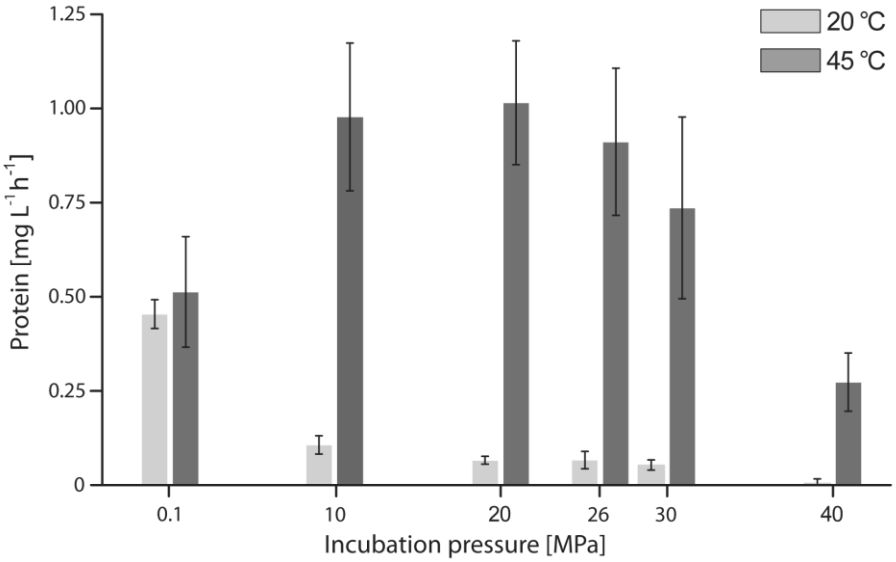

Figure 4.2: Growth of *Desulfovibrio indonesiensis*-affiliated strain P23 at 20 and 45°C under pressures of 0.1 to 40 MPa. Growth is shown as protein gain per hour, after 120 hours of pressure-incubation at 20°C and 36 h at 45°C, respectively. Assays at 0.1 MPa represent outgrown cultures. The standard deviation of five cultivation assays is indicated by error bars.

To confirm these findings, specific growth rates were determined as a function of pressure and temperature. Therefore, strain P23 was grown to early stationary phase at 20°C and 45°C both at atmospheric conditions and 30 MPa (Fig. 4.3).

Based on protein production, growth at 20°C was two times faster under 0.1 MPa than under high-pressure conditions. The exponential growth rate μ was calculated as 0.74 d^{-1} at atmospheric pressure and 0.38 d^{-1} at high pressure. In contrast, growth rates obtained at 45°C under high-pressure conditions (2.45 d^{-1}) were nearly comparable with those at atmospheric pressure (2.38 d^{-1}). A similar trend was determined via sulfide measurements. Comparing all rates, fastest growth was found at 45°C and 30 MPa, indicating the stimulation of growth by both, high temperature and *in-situ* pressure.

Highest protein yields were obtained at combinations of low temperature and atmospheric pressure (62 mg L^{-1}) as well as high temperature and *in-situ* pressure (53 mg L^{-1}). Interestingly, the opposite combination of low temperature and high pressure revealed a similarly diminished yield as high temperature and low pressure of 39 mg L^{-1} and 38 mg L^{-1}, respectively.

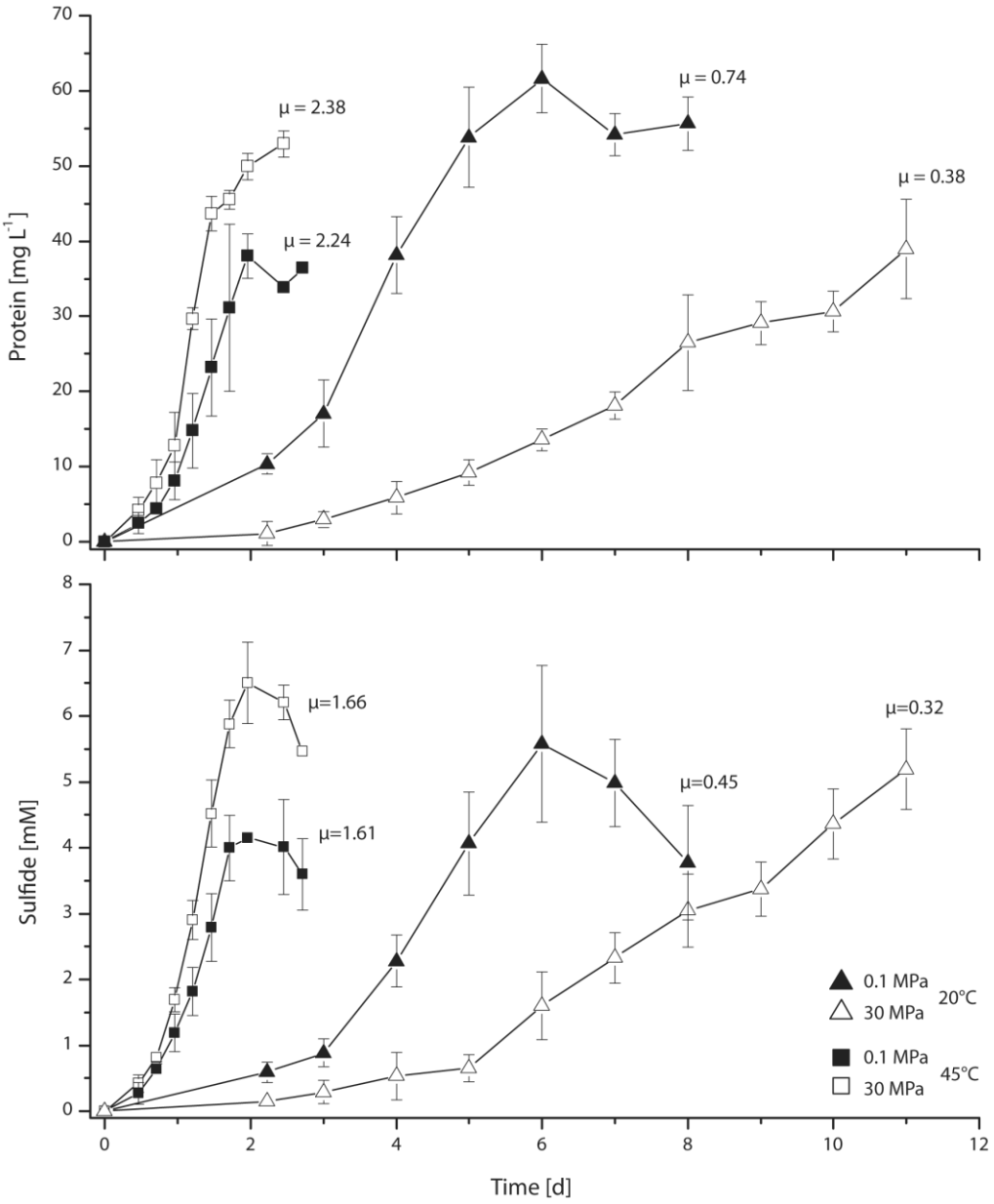

Fig. 4.3: Growth curves and rates μ [d^{-1}] of strain P23 grown at atmospheric (0.1 MPa) and high hydrostatic pressure (30 MPa) both at 20 °C and 45 °C. Values were calculated from photometrical measurements of protein and sulfide. The standard deviation of three cultivation assays is indicated by error bars.

4.4.3. Pressure-induced shift of the maximum growth temperature

During a cultivation experiment to determine the upper temperature of growth at elevated hydrostatic pressure (20 MPa), cells were allowed to slowly adapt to increasing temperatures. Under these conditions, the previously determined maximum growth temperature of 48°C at atmospheric pressure could be shifted to 50°C. At temperatures higher than 50°C, neither an ongoing protein production nor increasing sulfide formation was observed. This was proven in further test series that were inoculated with cells freshly grown at 50°C and 20 MPa. The upper temperature limit for

growth was again indicated by the observation of highly elongated, non-motile or deformed cells in comparison to the respective assays from 0.1 MPa.

4.4.4. Changes in whole cell fatty acid composition as a response to increasing temperatures at *in-situ* pressure

The majority of whole cell fatty acids (FA) of strain P23 was branched and accounted for up to 79 % under atmospheric pressure (Table 4.1). Regardless of growth temperatures and pressures, major components were *iso*- and *anteiso*-branched 15:0 and 18:0 FA, which is already known for the type strain (Feio et al., 1998).

Table 4.1: Whole cell fatty acid composition of *Dv. indonesiensis*-affiliated strain P23 grown at three different temperatures at constant atmospheric and high hydrostatic pressure. Fatty acids are commonly designated by number of carbon atoms to the number of double bonds (carbon number:number of double bonds). *Iso*- and *anteiso*-branching refer to the prefixes "*i*" and "*ai*", respectively, while "*n*" symbolizes straight chains.

Fatty acid	0.1 MPa			30 MPa		
	20°C	35°C	45°C	20°C	35°C	45°C
14:0	2.1	2.6	2.8	4.8	5.1	6.8
15:0	0.2	0.3	0.5	0.6	0.5	0.6
16:0	4.2	4.1	6.7	7.2	4.3	5.9
17:0						0.2
18:0	10.3	8.5	14.7	14.3	11.9	11.7
19:0						0.6
20:0	0.5	2.9	3.2	1.3	5.9	9.2
i-14:0	6.4	4.2	3.0	5.2	5.4	6.4
i-15:0	16.2	24.9	24.5	11.1	15.9	18.4
ai-15:0	39.3	45.1	37.0	33.5	43.3	35.0
i-16:0	2.0	1.0	0.7	1.6	1.1	1.0
i-17:0	0.4	0.4	0.5	0.3	0.2	0.0
i-18:0	1.3	1.1	1.0	1.0	0.8	0.6
i-19:0	0.3	1.7	3.0	0.2	0.6	0.5
ai-19:0			0.4			
i-20:0		0.6	0.4		0.3	0.6
i-15:1	1.6			2.1	0.5	
16:1		0.3	0.6			0.4
i-17:1	1.6			0.9		
ai-17:1	0.4			0.6		
18:1	8.2	1.0	0.9	7.1	1.9	0.8
i-19:1	2.1	0.3		1.5		
20:1	2.9	1.1		6.8	2.4	1.3
Σ Saturated (%)	83	97	98	81	95	97
Σ n-Saturated (%)	17	18	28	28	28	35
Σ *i*-Saturated (%)	27	34	33	19	24	27
Σ *ai*-Saturated (%)	39	45	37	34	43	35
Σ Unsaturated (%)	17	3	2	19	5	3
Σ Branched (%)	72	79	71	58	68	63
Σ Unbranched (%)	28	21	29	42	32	37

Concerning the degree of unsaturation, only monounsaturated fatty acids were detected. During incubations at high pressure, strain P23 showed strongly elevated levels of *n*-saturated fatty acids in comparison to cells grown at atmospheric conditions (Table 4.1 and Fig. 4.4). Under both pressure conditions, a two-step response was detected for increasing temperatures. First, the relative amount of unsaturated FA decreased strongly. Second, at higher temperatures, levels of *n*-saturated FA increased at the expense of branched saturated FA. Comparing temperature-dependent incubations under atmospheric and under *in-situ* pressure, pressure did not effect the ratio of saturated to unsaturated FA but led to elevated initial values of straight chained FA. Additionally, relative proportions of longer chained fatty acids were substantially elevated under high pressure conditions only (Table 4.1).

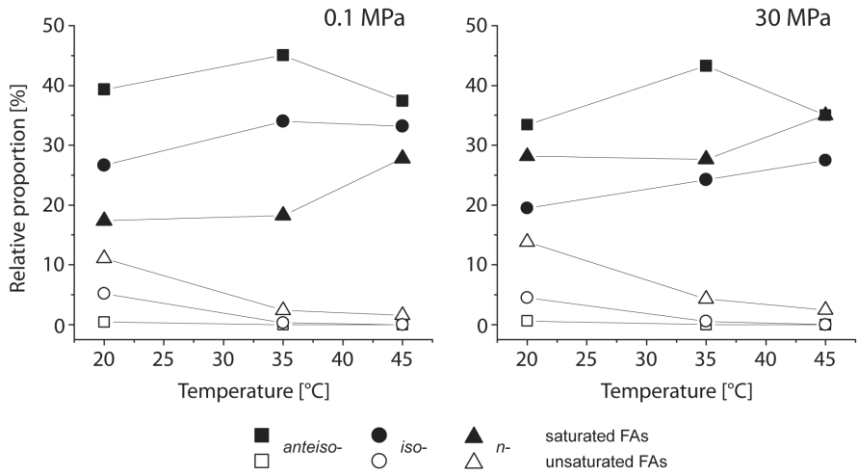

Figure 4.4: Fatty acid types (in % of whole cell fatty acids) in strain P23 grown at different temperatures and pressures.

4.4.5. Relative distribution of main IPLs depending on temperature and pressure

Under all conditions tested, strain P23 possessed two classes of intact polar lipids: phospholipids (phosphatidylglycerol, PG; phosphatidylethanolamine, PE; phosphatidic acid, PA) and phosphorus- and glycerol-free ornithine-containing lipids (OL, see Fig. 4.6 for molecular structues). Phospholipids were detected either as diacylglycerols (DAG) or acyl-ether glycerols (AEG) as identified by MS/MS experiments (Table 4.2). Four further IPLs with unidentified head groups might represent yet unknown phospholipids as they possessed DAG and AEG as core lipids too (Figs. 5.1 and 5.5).

Comparing the cell response to different pressure and temperature conditions, the sum of phospholipids dominated over ornithine lipids except for the set-up at low pressure and high temperature (Table 4.2). While the amount of OL increased with raising temperatures, the relative proportions of phospholipids decreased. For the phospholipid composition, main shifts with increasing temperatures were found for diacyl-phosphatidylglycerol (PG-DAG) and acyl-ether-phosphatidylglycerol (PG-AEG). While levels of PG-DAG dominated over PG-AEG at low temperature, an opposite ratio was found at higher temperatures. The values of total unknown IPLs (Σ Un1-4)

showed a similar response to temperature changes like PG-DAG. Generally, the effect of increasing temperature on the IPL composition at high pressure was most pronounced between 20°C and 35°C.

Table 4.2: Major polar lipid types (in % of total IPLs, intact polar lipids) in strain P23 grown at three different temperatures at atmospheric and high hydrostatic pressure.

IPLs	0.1 MPa			30 MPa		
	20°C	35°C	45°C	20°C	35°C	45°C
OL	26.1	32.2	45.2	15.7	31.3	28.1
Σ Phospholipids	54.1	50.7	41.5	66.8	53.9	56.5
PA-DAG	1.9	0.9	0.5	1.6	0.8	1.6
PE-DAG	22.9	20.2	18.5	23.7	20.1	22.3
PG-DAG	17.2	10.1	6.5	25.3	12.8	13.2
PG-AEG	12.1	19.5	16.0	16.2	20.2	19.4
Σ Un1-4	19.9	17.1	13.4	17.6	14.8	15.6

Abbreviations: OL = ornithine lipid, PE = phosphatidylethanolamine, PG = phosphatidylglycerol, PA = phosphatidic acid; Core lipids: DAG = diacyl glycerol, AEG = Acyl-ether glycerol, Un1-4 = four unidentified lipids, probably yet unknown phospholipid species.

4.4.6. Variability of IPLs side chains according to changes in cultivation pressure and temperature

In general, MS/MS-spectra indicated that the majority of analyzed IPLs contained a fatty acid with 15 carbon atoms (data not shown). Polyunsaturated fatty acids were never detected. Both findings are in good accordance with the whole cell fatty acid analysis (Table 4.1). In case of IPLs with an AEG-core, the C_{15} FA was the only fatty acid component. Side chain combinations of PE and PG were quite similar with either C_{14}- or C_{15}-FA and C_{15}- to C_{20}-FA at *sn*-2 position. Interestingly, two unknown IPLs (Un-1-DAG and Un-2-DAG) contained a fairly long C_{21}-C_{23}-fatty acyl chain. OL e.g. contained either 14:0 or 15:0 FA together with 3-hydroxy 16:0 20:0 as the second FA. Focusing on a single mass of an intact polar lipid without MS/MS-experiments, several possibilities for the combination of ester or ether-linked moieties arise. To simplify our data we have used the radyl value, comprising the total carbon number of both side chains. Radyl values varied between 28-37 in PG, 30-36 in PE, and 29-35 in OL. The radyl value pattern for PE and PG was dominated by 33 carbon atoms, resulting from high proportions of C_{15} and C_{18}-fatty acids (Fig. 4.5).

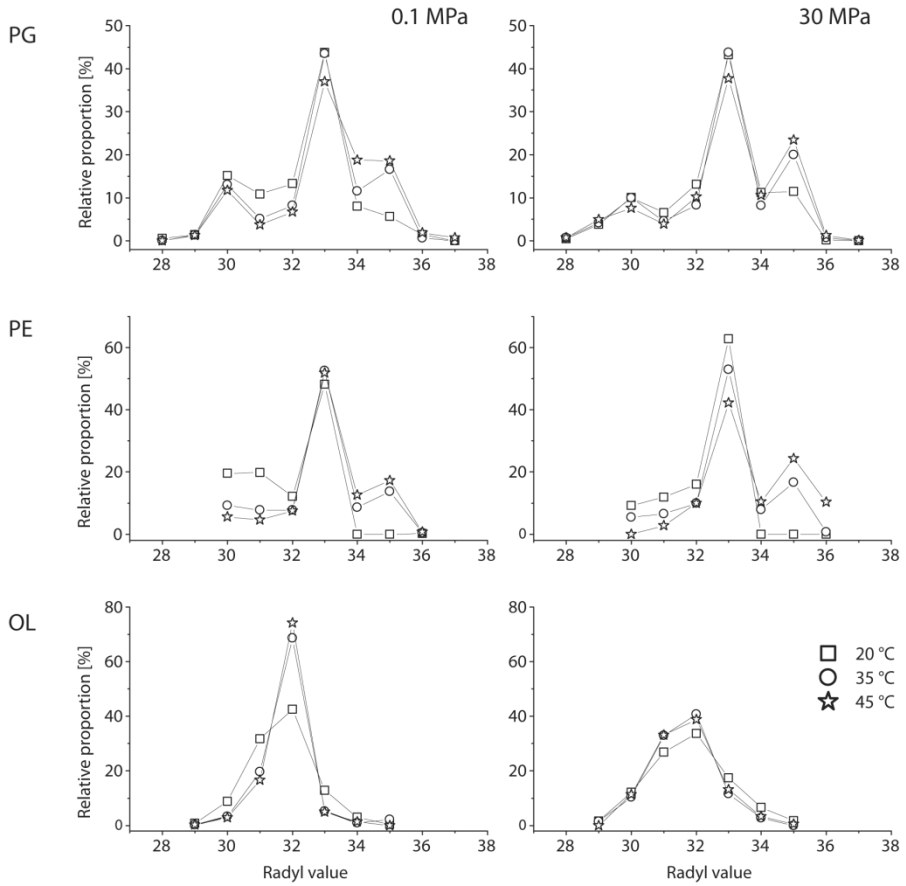

Figure 4.5: IPL inventory of strain P23 depending on different incubation conditions. Changes in fatty acyl side chain length are expressed by radyl values, the combined number of carbon atoms of the two fatty acid substituents of one IPL type. PG = phosphatidylglycerol, PE = phosphatidyl-ethanolamine, OL = ornithine lipid. PG data is based on the sum of PG-DAG and PG-AEG.

Pressure and temperature effects on cell membrane compounds are reflected in changes of the abundance, saturation and carbon-number distribution of fatty acyl side chains in the three major polar lipids. For PE and PG, higher incubation temperatures induced an increase in longer side chains on the expense of shorter ones. Additionally, a systematic decrease of the most abundant molecular species with temperature was visible for PE at high pressure incubations. Most strikingly, the radyl value pattern of OL was not affected by temperature during high pressure incubations. Here, the distribution patterns were nearly identical and were similar to that obtained at 20°C and 0.1 MPa (Fig. 4.5). In contrast, greatest changes were found at atmospheric pressure and high temperatures with a dramatically increase of the relative proportion of the most dominant OL with a radyl value of 32 carbon atoms.

At atmospheric pressure, levels of unsaturated side chains in all major IPLs were highest at 20°C and decreased strongly with raising incubation temperatures. At 30 MPa the ratios of unsaturated to saturated IPLs were predominantly higher than at 0.1 MPa, with remarkable changes of OL and PE-DAG lipids. While the latter contained the largest proportion of unsaturated FA side chains, PG possessed the greatest diversity of side chains.

In general, we found that the degree of saturation in PE lipids was affected by pressure and temperature but not their relative proportion. In contrast, the PG pool showed a temperature-induced restructuring with an internal shift from PG-DAG to PG-AEG.

4.5. Discussion

Reflecting the near *in-situ* conditions, our isolate P23 grew best at high pressure and high temperature. The cell response to a temperature rise was much more pronounced than to elevated pressure. According to the homeoviscous theory, the simultaneous decrease of both growth factors allowed the cultivation of our moderately piezothermophilic isolates from the warm deep subsurface.

4.5.1. High pressure experiments reveal the piezothermophilic nature of strain P23

The degree of piezophily is strongly dependent on the incubation temperature (Zobell & Johnson, 1949; Kato et al., 1995). In our previous study, the sulfate-reducing *Desulfovibrio* strain P23 was found to have an untypical upper growth temperature of 48°C (Fichtel et al., 2012). A high pressure tolerance should demonstrate that microorganisms of the deep biosphere are well-adapted to their pressurized subsurface habitat and that they belong to the active part of deeply buried microbial communities (Bale et al., 1997). In this study, we could demonstrate that strain P23 is able to grow under hydrostatic pressure of up to 40 MPa (*in-situ* pressure ~30 MPa), even after cultivation at atmospheric pressure for more than three years. Strain P23 obviously has not lost its piezophilic properties which might be a common feature of pressurized marine deep-sea organisms that were sampled and isolated under decompressed conditions (Zobell & Johnson, 1949).

Strain P23 showed a shift from piezosensitive to piezophilic behaviour when grown at higher temperatures. Results of growth experiments allow a pressure-based classification of strain P23 depending on its temperature range for growth. Under standard conditions, strain P23 is a piezosensitive moderately thermophile (Yayanos, 1995; Molina-Höppner et al., 2003). At 20°C our strain was capable of growing at both, atmospheric and elevated hydrostatic pressure with fastest growth rates at 0.1 MPa. In contrast, at higher temperatures, growth was even accelerated by high hydrostatic pressure in the broad range from 10 to 30 MPa. The maximum growth temperature could be elevated to 50°C only under pressure. Under these conditions strain P23 is considered to be moderately piezothermophilic (Yayanos, 1995; Kato & Bartlett, 1997), reflecting its adaptation to the *in-situ* conditions present in its original warm subsurface habitat. However, an increase of growth temperature does not always necessarily improve the piezotolerance of typically atmosphere-adapted microorganisms. For instance, in a study on lactic acid bacteria, higher temperatures did not stimulate microbial growth under elevated pressure (Molina-Höppner et al., 2003). The authors speculated that unlike many pressure-adapted species they might be unable to mount a specific pressure response in order to maintain membrane fluidity.

During growth at conditions above *in-situ* pressure, the cell morphology of our isolates changed. With increasing pressure and temperatures, cells became more elongated and cell division was inhibited, indicating a typical stress response. Cell filamentation seems to be a characteristic pressure-related phenomenon in mesophilic bacteria (Zobell & Cobet, 1964; Lauro & Bartlett, 2008). Pressure is believed to have a direct inhibitory effect on FtsZ ring formation, which is a prerequisite for membrane construction during cell division (Molina-Höppner et al., 2003; Ishii et al., 2004). Filament formation might also be mediated via a pressure-triggered SOS response involving the *RecD* protein which is essential for DNA recombination and repair (Bidle & Bartlett, 1999; Aertsen et al., 2004).

4.5.2. Whole cell fatty acid data are confirmed and complemented by IPL analysis

While the whole cell fatty acids analysis provides a fast overview on all cellular fatty acids, the analysis of intact polar lipids directly targets membrane building-blocks. The results of both methods can be combined as fatty acids extracted from the membrane fraction are very similar to those extracted from whole cells (Pluschke & Overath, 1981; Allen et al., 1999; Kaneko et al., 2000).

According to the theory of homeoviscous adaptation of membrane lipids (Sinensky, 1974; Somero, 1992), it was expected that increasing growth temperature mainly results in higher levels of saturated fatty acids to maintain membrane integrity. For strain P23, this adaptation was detected in both - GC-FID and LC-MS - investigations confirming previous studies on a variety of organisms (DeLong & Yayanos, 1985). Moreover, the cell response of strain P23 to changes in temperature apparently occurred stepwise. After changing the saturation level, as a subsequent response to higher incubation temperatures strain P23 decreased its membrane fluidity level by exchanging branched FA with straight-chained FA. This is most pronounced for *anteiso*-branched FA, as they have lower melting points compared to *iso*-branched FA which have similar effects like saturation (Zhang & Rock, 2008). For strain P23, elevated initial proportions of straight chained fatty acids under *in-situ* pressure and 20°C were independent from the degree of saturation and indicate that pressure mostly diminished the branching of FA. This is in contrast to investigations on other piezotolerant deep-sea bacteria that mainly respond to pressure in changing the saturation degree of whole cell fatty acids.

Another factor for membrane fluidity is the number of double bonds of unsaturated fatty acids. While polyunsaturated fatty acids have been found in many piezophilic deep-sea bacteria, this regulatory capacity seems to be limited to psychrophilic microorganisms (Delong & Yayanos, 1986; Wirsen et al., 1986; Kamimura et al., 1993; Yano et al., 1998). As strain P23 derives from a warm deep-sea habitat, polyunsaturated fatty acids might only be incorporated when growth temperatures fall below 20°C.

4.5.3. High proportions of ornithine-containing lipids might reflect phosphate-limitation within the original habitat

Ornithine-containing lipids are major membrane constituents in strain P23, as already described for other *Desulfovibrio* species (Makula & Finnerty, 1975; Seidel, 2009). The presence of ornithine lipids was found to be negatively correlated with available amounts of phosphate present in the medium used for cultivation (Geiger et al., 1999; Weissenmayer et al., 2002). Therefore, the assumption was made, that in case of phosphate limitation bacteria can exchange phosphate-containing membrane lipids by OL or others such as sulphoquinovosyl diacylglycerol (SQDG) or diacylglycerol trimethylhomoserine (DGTS). Although phosphate was not a limiting nutrient in our cultivation medium, strain P23 was isolated from phosphate-depleted sediment layers (Engelen et al., 2008). In this environment, the ability to produce phosphate-free membrane building blocks gives an important advantage over other microorganisms that are not capable of this feature. Thus, it is rational that this adaptation has originally developed in the deep biosphere and was not lost when strain P23 was cultivated in phosphate-rich media.

Furthermore, our study indicates that OL might not only be a substitute for other phosphate-containing membrane lipids. The cells grown at atmospheric pressure respond to temperature changes with higher relative amounts of OL within the membrane and chain-length variations. This temperature dependence proofs previous findings by Seidel et al. (2009) who found elevated proportions of OL with increasing incubation temperatures for other *Desulfovibrio* strains. The authors suggested that changes in lipid composition of the cytoplasmic membrane may rather be important for the presence and performance of membrane-bound enzymes and do not necessarily influence its viscosity. This is supported by our results as we could neither find the expected decrease with temperature in high pressure incubations nor any structural changes as an adaptation of membrane viscosity to high pressure.

4.5.4. Simultaneous decrease of pressure and temperature favours the cultivation of piezomesophiles from deep subsurface sediments

To mimic environmental conditions, most enrichment cultures are incubated at *in-situ* temperatures. Even for the cultivation of deep-sea microorganisms, pressure is normally not taken into account. This might be due to the high technical expenditure during sampling, storage and microbial analyses. While all our enrichments from IODP Site U1301 that were performed at *in-situ* temperatures and atmospheric pressure did not result in pure cultures, a great variety of isolates were gained at ambient laboratory conditions (Fichtel et al., 2012). This might be explained by opposed effects of pressure and temperature on general cell functions and the capability of microorganisms in adapting to these variations. Therefore, a key for a successful isolation of piezomesophilic and other high-temperature adapted prokaryotes from the subsurface under atmospheric pressure might be the decrease of the cultivation temperature below the *in-situ*

temperature. For the marine subsurface, this might be feasible for the isolation of mesophilic archaea which are of special interest as they were found to dominate respective clone libraries without any cultivated representative (Teske & Sorensen, 2008). For piezopsychrophiles in turn, this approach is not feasible as *in-situ* conditions of e.g. 40 MPa and 2°C, the average values at the seafloor, would require an incubation temperature of -2°C to -6°C at atmospheric pressure (Chong & Cossins, 1983; Bartlett, 2002; Wang et al., 2009). However, previous cultivations from cold deep-sea habitats have obtained isolates with a broad adaptation capability to changes in temperature and pressure. On the other hand, pressure above *in-situ* values might be advantageous for cultivating piezopsychrophiles at elevated temperatures which generally accelerates growth (Kato et al., 1995).

In case of strain P23, applying the *in-situ* pressure resulted in growth at 50°C but not at the respective *in-situ* temperature of 61°C which might be explained by the effect of pressure on abiotic factors such as the solubility of gases. Strain P23 turned out to be a chemolithoautotrophic sulfate reducer as it was able to grow on hydrogen and CO_2 as sole energy and carbon sources (Fichtel et al., 2012). In the present study, pressure incubations under autotrophic conditions were not performed as this would have required a completely different technical equipment allowing cultivation with a gaseous headspace. On the other hand, pressure is an inevitable factor for isolating obligate piezophiles (Kato et al., 1996) or investigating the microbial utilization of gases or hydrocarbons in the subsurface, including field studies on gas and oil reservoirs or CO_2-sequestration facilities.

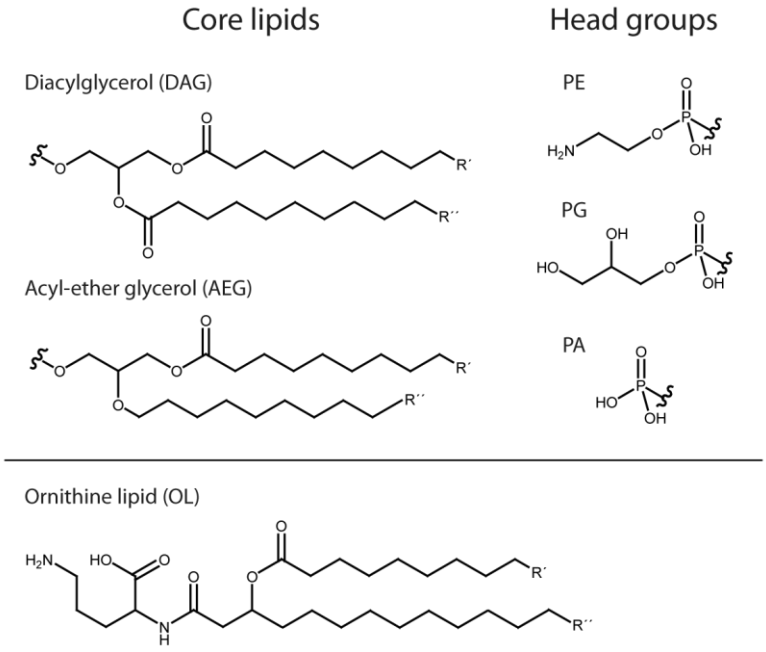

Fig. 4.6: Molecular structure of major IPLs present in *Desulfovibrio indonesiensis*-like strain P23. Abbreviations: PE, phosphatidyl-ethanolamine; PG, phosphatidylglycerol; PA, phosphatidic acid; R', R'', alkyl moieties.

Acknowledgments

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5. Unknown intact polar lipids found in *Desulfovibrio indonensiensis*

During the investigation of IPLs from *Desulfovibrio indonensiensis* four compounds were found, which to the best of my knowledge, have never been described before. When the lipid extracts of *Desulfovibrio indonensiensis* were analyzed under the HPLC conditions described in chapter 4, two of these, named Un-1 and Un-2, elute between 4 and 6 minutes and before all other quantified compounds. Additionally, two further unknown compounds, named Un-3 and Un-4, were found in all investigated samples from *Desulfovibrio indonensiensis* at retention times between 13 and 18 minutes.

5.1. Unknown compounds Un-1 and Un-2

Un-1 and Un-2 are mainly present as saturated compounds but small amounts of unsaturated analogs were also detected. Ion chromatograms of the molecular masses of Un-1 and Un-2 display two peaks. As shown by Rütters et al. (2001), this chromatographic behavior is typical for IPLs with diacyl glycerol (DAG) and acyl-ether glycerol (AEG) core lipids. IPLs with DAG and AEG core lipids differ in their molecular masses by 0.032 Da, and thus can only be distinguished with a high-resolution mass spectrometer. These components are present in *Desulfovibrio indonensiensis* as homologous series with fully saturated and small amounts of unsaturated allyl moieties, such as diacyl glycerols (DAGs) or mixed acyl-ether glycerols (AEGs).

Complicating their interpretation, Un-1 lipids and representatives of Un-2 (saturated and unsaturated) show identical retention times for the same type of core lipids (DAG or AEG). Due to the unclear structure of the Un-1 and Un-2 head groups and biological role of the parent compounds, their role and appearance was not discussed in detail in the previous chapter. Spectra from MS/MS experiments show fragmentation patterns of Un-1 and Un-2, typical for intact polar lipids (Fig. 5.1). The appearance of signals at *m/z* 241.2 and 311.3 in spectra a and b (Fig. 5.1) indicate the presence of two ester-bound fatty acids (15:0 and 20:0) attached to a diacyl glycerol (DAG). In the spectra c to e there is only one fragment at *m/z* 241.2. This indicates the presence of a glycerol backbone with one ester-bound (15:0 FA) and one ether-bound side chain (O-21:0, O-20:0 and O-19:0), called acyl-ether glycerol (AEG) core lipid. MS/MS experiments (Fig. 5.1) suggest that the majority of analyzed IPLs possess one fatty acid with 15 carbon atoms, which is in good accordance with the analysis of whole cell fatty acids by GC-FID (Table 5.1).

The small peak at *m/z* 153.3, very well visible in Figs. 5.1a and b but also present in all other spectra, is usually associated with the fragmentation and conversion product of glycerol in combination with phosphate (Pulfer & Murphy, 2003). Un-1 and Un-2 lose their head groups

very easily and therefore the molecular ion was not detected in the MS/MS experiments. As shown, Un-1 (head group mass 162 Da; Fig. 5.1d) and Un-2 (head group mass 164 Da; Fig. 5.1a, c and d) only differ by two mass units in their head group. This is a striking feature and was never reported before for membrane lipids. Unfortunately, the exact molecular structure of the head groups of these compounds could not be revealed. However, the comparison of the fragmentation patterns of the core lipid of Un-2 (Fig. 5.1b) recorded in parallel to spectrum a in the same run and at the same retention time, clearly shows that the fragments at *m/z* 116.9, 118.9, 120.9, 162.9 and 164.9 can be assigned to head group break down products of Un-2.

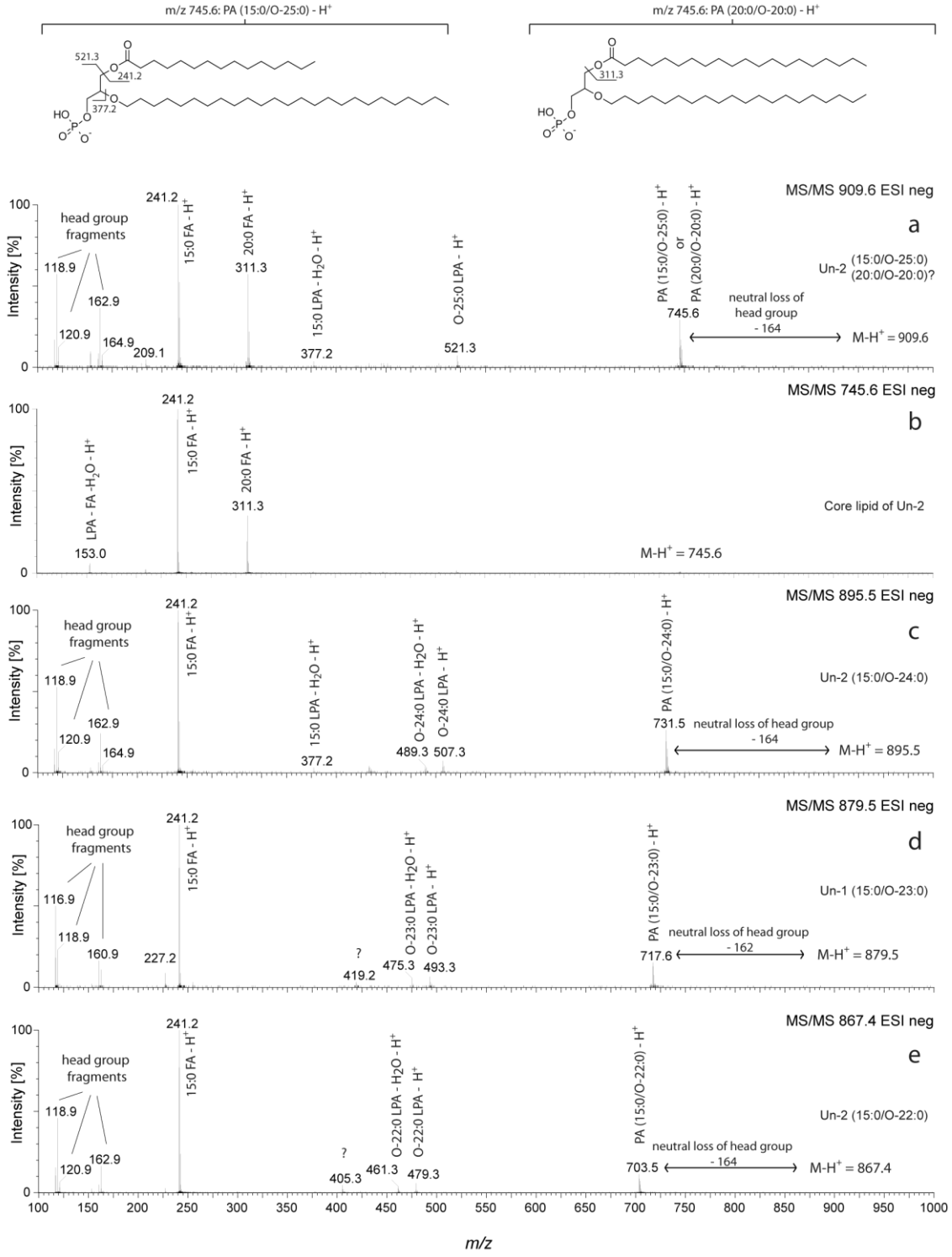

Figure 5.1: MS/MS spectra and interpretation of fragmentation patterns of unknown compounds Un-1 (spectrum d), Un-2 (spectra a, c and e) and core lipid of Un-2 (spectrum b). Spectra were recorded in ESI negative ion-mode. Abbreviations: LPA, lyso phosphatidyl acid; FA, fatty acid.

Transferring the findings for Un-2 to the spectrum of Un-1 (Fig. 5.1d), the presence of low weight fragments, such as *m/z* 116.9, 118.9, 120.9, 160.9 and 162.9, unveils that Un-1 and Un-2 must be structurally very similar. Interestingly, the difference between the fragments *m/z* 116.9 to *m/z* 160.9, *m/z* 118.9 to *m/z* 162.9 (Un-1, Fig. 5.1d) and *m/z* 118.9 to *m/z* 162.2 as

well as *m/z* 120.9 to *m/z* 164.9 (Un-2, Figs. 5.1a, c, e) is 44 Da, indicating the loss of a similar fragment which could be N_2O, C_2H_4O or C_3H_8 but most likely CO_2.

Therefore, the sole difference between Un-1 and Un-2 is the heavier head group, which differs by two mass units (Fig 5.1). Typically, bacteria incorporate double bonds in their lipids to reduce the membrane fluidity due to changing environmental parameters (Bright-Gaertner & Proulx, 1972; De Mendoza & Cronan Jr, 1983). The incorporation of a double bond lowers the molecular mass by two mass units. Without exception, double bonds have only been reported for the unpolar acyl or ether moieties. The appearance of a double bond in the head group section of membrane lipids was never observed before. However, the head group structures of Un-1 and Un-2 remain elusive for the moment.

5.2. Changing proportions of Un-1 and Un-2 lipids

The elution patterns of the main representatives of Un-1 and Un-2 lipids shown in Fig. 6.2 indicate temperature dependence in the distribution of DAG and AEG core lipids. For both investigated pressures the proportion of AEG core lipids increases with higher incubation pressure, clearly visible for the Un-1 mass traces *m/z* 881.5 and *m/z* 867.5 and Un-2 mass traces *m/z* 879.5 and *m/z* 865.5, respectively. Changes in Un-1 are always also visible for Un-2. Comparing all mass traces of Un-1 and Un-2 from different incubation conditions (Fig. 5.2) higher relative proportions of lower-molecular-weight representatives of Un-1 and Un-2 can be observed for ambient and high pressure incubations. While the latter observation can be easily explained with the theory of homoviscous adaptation, an increase in core lipids with mixed acyl-ether side chains was never reported before. In Fig. 5.3 the ratio of AEG to DAG core lipids is plotted versus the incubation temperature at the two investigated distinct pressures. As already indicated in Fig. 5.2, in Fig. 5.3 it becomes obvious that the proportion of AEG core lipid in Un-1 and Un-2 is mainly controlled by the incubation temperature of *Desulfovibrio indonensiensis*. An increased incubation pressure has a diminishing effect on the production of AEG core lipids. A higher stability of AEG lipids over DAG lipids may be an explanation for this behavior. However, further investigations are necessary to find out more about this fascinating observation.

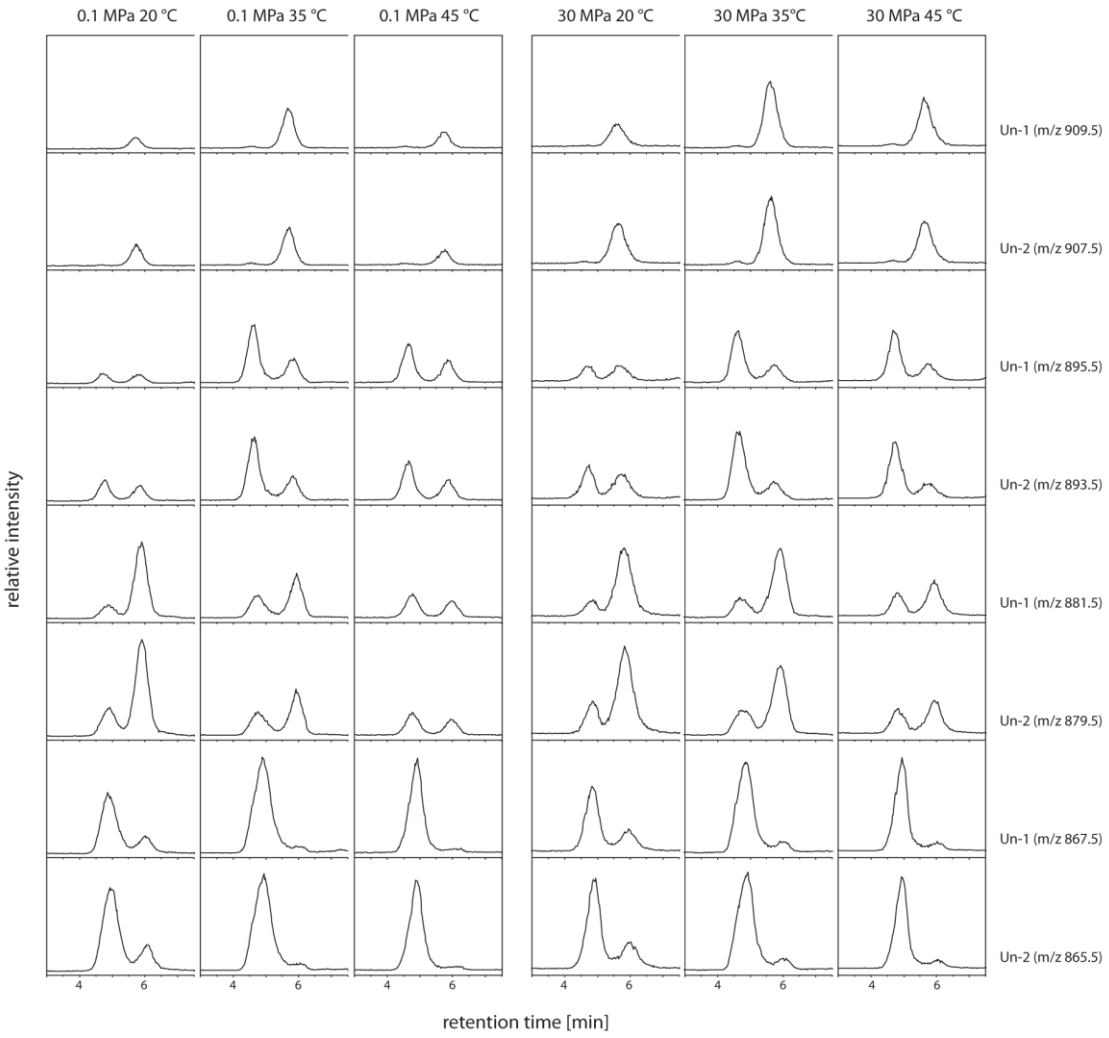

Figure 5.2: LC-MS mass-chromatograms of Un-1 and Un-2 obtained from different incubations of *Desulfovibrio indonensiensis* recorded in negative ESI-mode. Peaks with earlier retention times represent lipids with mixed acyl-ether-glycerol (AEG) core lipids, followed by lipids with diacyl-glycerol (DAG) core lipids. Signal intensities can be compared within each sample, but not between the different incubation conditions.

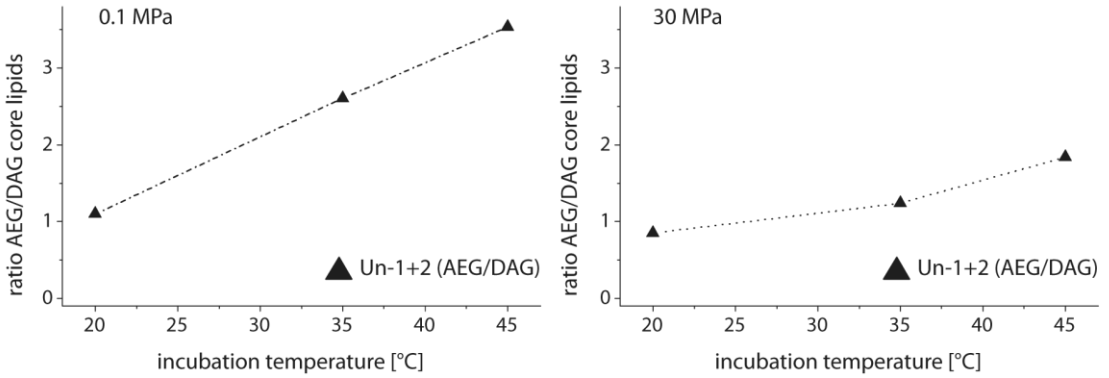

Figure 5.3: Ratios of the summed Un-1 and Un-2 AEG IPLs to the summed Un-1 and Un-2 DAG IPLs at different incubation temperatures and pressures.

5.3. Unknown compounds Un-3 and Un-4

In addition to the described IPLs Un-1 and Un-2 two other unknown IPLs, subsequently named Un-3 and Un-4, were found in the lipid pool of *Desulfovibrio indonensie*nsis. These compounds have retention times between 13 and 18 minutes. In comparison with all other IPLs found in this organism, the elution patterns of these compounds are complex. For most mass traces of Un-3 and Un-4 up to five peaks were detected in the LC-MS chromatograms (Fig. 5.4). Due to the instrumental limitations of the mass spectrometer used, it was not possible to identify these peaks more specifically than Un-3-DAG, Un-3-AEG, Un-4-DAG or Un-4-AEG.

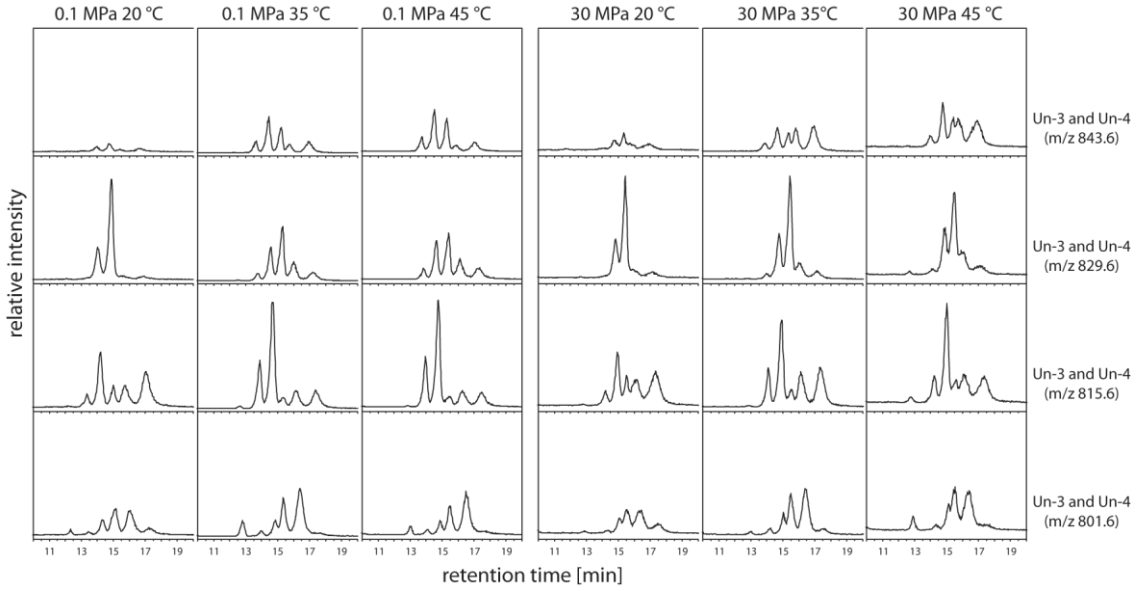

Figure 5.4: LC-MS mass-chromatograms of Un-3 and Un-4 obtained from different incubations of *Desulfovibrio indonensiensis* recorded in negative ESI mode. Signal intensities can be compared within each sample, but not between the different incubation conditions.

Looking at the results of the MS/MS experiments reveals that Un-3 and Un-4 share the same masses of their molecular ions (Fig. 5.5). As obvious from a comparison of Figs. 5.5a and b, most of the fragments occur in both spectra. All fragments in the mass range from *m/z* 400 to *m/z* 470 are formed by lyso phosphatidyl glycerol (LPA) fragments, either by loss of a proton or by loss of water (H_2O) and a proton. The structure and typical fragmentation sites of PA are indicated for better illustration (Fig. 5.5a). In both spectra, the peak at *m/z* 647.5 is formed by a negatively charged phosphatidyl acid (PA) fragment. However, the molecular ion in Fig. 5.5a is 14 u heavier than the respective ion in Fig. 5.5b. This mass difference is typically associated with an additional CH_2 unit inserted somewhere in the head group structure. Therefore, two different head groups are proposed with an identical core lipid bound to the masses of *m/z* 154 (Un-3) and *m/z* 168 (Un-4) (Figs. 5.5, b and a). Since the MS/MS spectrum of Un-4 shows a fragment with *m/z* 110.9 (Fig. 5.5a) which is not visible in the spectrum of Un-3 (Fig.

5.5b), it is possible that Un-3 and Un-4 have completely different chemical head group structures.

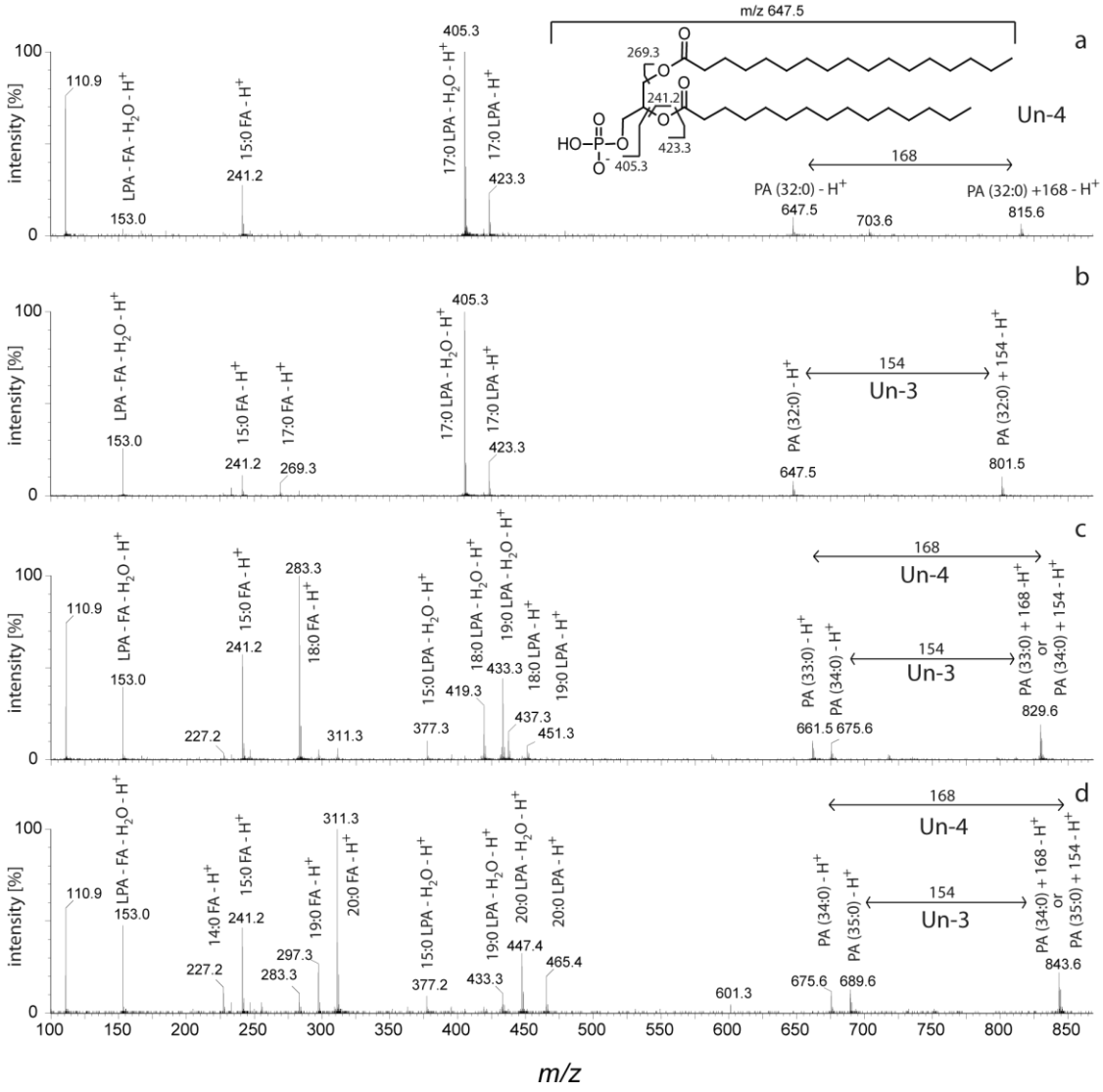

Figure 5.5: Spectra from MS/MS experiments recorded in ESI neg-mode with Un-3 (b, c and d) and Un-4 (a, c and d). Abbreviations: LPA, lyso phosphatidyl glycerol; FA, fatty acid.

Due to coelution of Un-3 and Un-4 it was not possible to do separate MS/MS experiments for Un-3 and Un-4. Thus, in some cases the fragmentation patterns of Un-3 and Un-4 were recorded simultaneously, resulting in a mixture of both fragmentation patterns (Figs. 5.5 c, d). When the fragmentation patterns from Figs. 5.5a and 5.5b are transferred to the spectra c and d, it is obvious that these spectra originate from two intact polar lipids with the same molecular ion, but different head groups.

In parallel to Un-1 and Un-2, which were described before, also Un-3 and Un-4 differ from each other in the head group region of the molecule. Unfurtunately, it was neither possible to reveal the molecular structure of the Un-3 and Un-4 head groups nor to define potential roles of these new membrane lipids.

6. Intact polar lipids of halophilic archaea and bacteria from hypersaline Lake Tyrrell, Victoria, Australia

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6.1. Abstract[1]

We investigated the core and intact polar lipids (IPLs) in a natural, evaporating brine pool at Lake Tyrrell in outback Victoria using LC-ESI-MS and GC-FID/MS methods. The most abundant known archaeal IPLs were PGP-Me-DPG, PGS-DPG, PGP-Me-PSG, S-DGD, S_2-DGD and PG-DPG. Also abundant, but reported for the first time from hypersaline systems, are the inositol-based IPLs PI-DPG and PIP-DPG as well as an unknown archaeal IPL carrying two sugar moieties. Based on quantitative core lipid data, 74% of the membrane lipids are derived from archaea and 24% from bacteria. Steroids of hypersaline eukaryotes were below detection limits.

The detected IPLs can be broadly correlated with phylogenetic data extracted from the community genome of the brine pool using EMIRGE technology. According to EMIRGE, Lake Tyrrell is dominated by hypersaline archaea of the genera *Halosarcina, Haloquadratum walsbyi, Haloarcula and Halobacterium*, including high relative abundances of Nanohaloarchaea, a new group of Haloarchaea recently detected in Lake Tyrrell. The Nanohaloarchaea are potential sources of two IPLs hitherto unknown from extreme halophiles, phosphatidylinositol (PI-DPG) and phosphatidylinositolphosphate diphytanylglycerol (PIP-DPG).

6.2. Introduction

Lake Tyrrell is an ephemeral salt lake situated in the Murray Basin in southeast Australia. It covers an area >150 km³ and represents a playa without surface outflow. The geological setting and hydrology of Lake Tyrrell have been well characterized and described in several studies (e.g. Herczeg et al., 1992; Long et al., 1992; Lyons et al., 1995). Water depth varies from 0.5 m in winter to dryness in summer when a halite crust forms on the lake floor. The salt concentration in winter is usually >250 g L^{-1} and halite re-solution and precipitation occurs throughout the year (Macumber, 1992). According to Jones et al. (1994), the solutes in the water of Lake Tyrrell are thalassohaline, i.e. have a pronounced marine signature, although the geology of the catchment also influences the chemical inventory of the lake water.

[1] Currently detailed data on community structure is missing in this manuscript. This information will be added and discussed upon manuscript submission.

Hypersaline microbial communities in salt lakes have been investigated for several decades, and publications go back to the 1930s (Wilansky, 1936). In the past 25 years, these systems have become subject of particularly intense research (e.g. Kates, 1977; Upasani et al., 1994; Oren & Rodriguez-Valera, 2001; Ochsenreiter et al., 2002; Stiehl et al., 2005; Maturrano et al., 2006). In many salt lakes at halite saturation level, the planktonic microbial community appears to be quite similar. In most systems, halotolerant unicellular green algae of the genus *Dunaliella* are the dominant or sole primary producers in the water column (Oren, 2005; Bardavid et al., 2008; Oren, 2011) providing the bulk of primary product utilized by heterotrophic archaea and bacteria. The most abundant representative of these two domains frequently are the archaeon *Haloquadratum walsbyi* and the bacterium *Salinibacter ruber* (e.g. Anton et al., 1999; Anton et al., 2000).

In previous studies, hypersaline microbial communities were identified by microscopy and rDNA surveys (e.g. Burns et al., 2004; Oh et al., 2010) and to some extend by elucidation of the intact polar lipid (IPL) repertoire (Litchfield et al., 2000; Oren & Rodriguez-Valera, 2001; Lattanzio et al., 2002). IPL surveys, although taxonomically less specific, have the advantage of being relatively cheap, fast and potentially quantitative. However, all previous IPL studies on hypersaline systems near salt saturation level were performed using thin layer chromatography (TLC) techniques (Litchfield et al., 2000; Oren & Rodriguez-Valera, 2001; Lattanzio et al., 2002), which are largely qualitative, have high detection thresholds and significant uncertainties in compound identification when compared to mass spectrometry (MS) based methodologies. Here we provide the first study of IPLs and core lipids from a planktonic hypersaline microbial community based on high-performance liquid chromatography - mass spectrometry (LC-MS) and gas chromatography - mass spectrometry (GC-MS) technology. The lipid inventory is compared to genus-level community genomic data to assess the taxonomic value of this approach.

6.3. Material and Methods

6.3.1. Sampling

Lake Tyrrell is located in the Murray Basin in outback Victoria, Australia. Water samples were collected on 10 January 2010 in a small natural brine pool that became separated from the water in the lake within the Cheetham salt works at the western perimeter of Lake Tyrrell. The floor of the pool was covered by a thick halite crust. In the field, water was transferred into sterilized 20 L plastic containers using a sterilized hand water pump. After transfer to the field laboratory (30 min) the water for lipid analysis was immediately filtered over pre-combusted glass fiber disc filters (GFF) without binder (Millipore APFF14250, Ø 142 mm, 0.7 µm pore size, 90% porosity) using a peristaltic pump. Filter samples were collected in duplicates. Water was filtered until clogging of the GFF (≈2 L). The loaded filters were packed into combusted aluminum foil, transported on dry ice and stored in the laboratory at -35°C until analysis in March 2010.

6.3.2. Ion analysis

Water samples were analyzed for inorganic cations and anions. For cation analysis, a Varian Vista AX CCD Simultaneous ICP-AES (Varian, Palo Alto, CA, USA) instrument was employed. Anion analysis was carried out on a Dionex Ion Chromotograph Series 4500i (Dionex, Sunnayvale, CA, USA).

6.3.3. Extraction of lipids

One quarter of each filter was used for lipid extraction using a modified Bligh and Dyer procedure (Sturt et al., 2004). For the first three extraction steps, the extraction solvent consisted of methanol, dichloromethane and phosphate buffer (8.7 g K_2HPO_4 per liter water) in a composition of 2 : 1 : 0.8 (v:v:v). For the following three extraction steps an aqueous solution of trichloroacetic acid (50 g l^{-1} CCl_3COOH) was used for the Bligh-Dyer mixture instead of the phosphate buffer. After sonication for 10 min and centrifugation for 4 min the supernatants were combined in a separatory funnel. Dichloromethane and water were added to the mixture to achieve phase separation at a final methanol/dichloromethane/buffer ratio of 1 : 1 : 0.8. After removing the organic phase containing the IPLs, the aqueous phase was extracted two more times with dichloromethane.

All organic solvents were GC Resolv1 or Optima1 grade (Mallinckrodt, Phillipsburg, NJ, USA); deionized water was obtained from a MilliQ1 system (Millipore, Billerica, MA, USA). To reduce the loss of intact polar lipids prior to analysis via LC-MS, no separation or cleaning of the extracts was performed.

6.3.4. HPLC analysis of intact polar lipids

All extracts were dissolved in eluent A, and phosphatidylethanolamine glycerol dialkylether (O-PE, Avanti Polar Lipids, Inc., Alabaster, AL, USA) with side chains containing 16 carbon atoms each) was added as injection standard. Phospholipids were analyzed on an HPLC instrument (Agilent 1100 Series, Agilent Technologies, Santa Clara, CA, United States) coupled to an LCQ DECA XP ion-trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA) equipped with an electrospray ionization source (ESI). HPLC separation was achieved, as described by Rütters et al. (2001), on a diol phase (LiChrospher100 Diol 5 µ, CS - Chromatographie Service GmbH, Langerwehe, Germany) using a 125 x 3 mm column with 20 mm guard column filled with the same material. A flow rate of 0.2 ml min^{-1} was employed with the following solvent gradient: 1 min 100 % A, increasing over 20 min to 35 % A, 65 % B using a linear gradient, followed by 40 min of reconditioning. Eluent A was a mixture of *n*-hexane, isopropanol, formic acid, ammonia (25 % solution in water) (79:20:1.2:0.04 by volume), eluent B was isopropanol, water, formic acid, ammonia (25 % solution in water) (88:10:1.2:0.04 by volume). The optimal mass spectrometer settings were determined by direct injection of the injection standard O-PE. For negative-ion-mode the mass spectrometer was set to a spray voltage of 3 kV, sheath gas flow of 30 (arbitrary units), capillary voltage of -45 V and a capillary temperature of 220°C. For positive-ion-mode we observed highest ion intensities with a spray voltage of 3 kV, sheath gas flow of 20 (arbitrary units), capillary voltage of +40 V and a capillary temperature of 200°C. MS/MS experiments were done in the dependent-scan mode, i.e. the most intense quasi-molecular ion species of each full scan was automatically isolated and fragmented up to MS^3. Helium was used as collision gas (relative collision energy: 30 – 60 %, depending on compound). Mass spectra (full scan and MS/MS) were used for compound identification and determination. Confirmation of identified compounds was achieved by determination of accurate masses with a high resolution LC-ESI-MS system (2695 separations module coupled to Micromass Q-TOF micro, Waters, Milford, MA, USA) without any HPLC separation. Because of the lack of commercially available standards for most of the identified intact polar lipids, we performed a semi quantitative analysis using peak areas of mass traces of all major compounds.

6.3.5. Saponification, derivatisation and gas chromatography-mass spectrometry (GC-MS)

Aliquots of filter quarters, containing the biomass of approximately 500 ml lake water, were saponified in 10 ml methanol:aqueous potassium hydroxide solution (4.45 mol l^{-1}) 80:20 (v/v) at 80°C for 5 h. The neutral lipids were extracted from the aqueous solution by liquid/liquid extraction with *n*-hexane in three steps. Acidic compounds, including fatty acids, were

obtained in the same way after acidification with HCl to pH 2-3. The released fatty acids were transformed into trimethylsilyl (TMS) ether derivates using N,O-bis-trimethylsilyl-trifluoroacetamide (BSTFA). The TMS and methyl-ester derivates were identified by GC-MS in full scan mode using an Agilent 6890N gas chromatograph (Agilent Technologies, Santa Clara, CA, United States) equipped with a DB-5 column (60 m, 0.25 mm, 0.25 µm film thickness, Agilent J&W Agilent Technologies, Santa Clara, CA, United States) with helium as carrier gas (constant flow). The GC oven was programmed from 40°C (2 min) to 315°C at a rate of 4°C min^{-1}, followed by an isothermal phase of 50 min. The injector temperature was programmed from 40°C (5 s) to 315°C (60 s) at 10°C s^{-1}. The gas chromatograph was coupled to an AutoSpec Premier sector field mass spectrometer (Micromass MS Technologies, Manchester, UK, mass range: 55 - 600 u) run at an electron impact ionization energy (EI) of 70 eV and a source temperature of 260°C. Compounds were identified using retention times and mass spectra in comparison with commercially available standards. Quantification of saponified whole cell lipids was achieved on an Agilent GC-FID system (Agilent 6890, Agilent Technologies, Santa Clara, CA, United States) using a high temperature capillary column (J&W Agilent Technologies, Santa Clara, CA, United States, DB-5HT, 30 m length, 0.25 mm inner diameter, 0.1 µm film thickness) with helium as carrier gas (constant flow). The GC oven was programmed from 60 °C (2 min) to 350 °C at a rate of 4 °C min^{-1}, followed by an isothermal phase of 20 min. The injector temperature was programmed from 60 °C (5 s) to 350°C (60 s) at 10 °C s^{-1}. Behenic acid methyl ester was used as internal standard.

6.4. Results

6.4.1. Lake water chemistry

The concentrations of major cations and anions in the sampled brine pool at Lake Tyrrell are given in Table 6.1. The water has a clear thalassohaline signature (Jones et al., 1994), i.e. it has ion ratios similar to marine systems.

Table 6.1: Ion concentrations in the evaporating brine pool at Lake Tyrrell.

Ion	Concentration in mmol L^{-1}
Na^+	3370
Mg^{2+}	380
K^+	31.2
Ca^{2+}	7.98
B^{3+}	0.24
Li^+	0.16
Sr^{2+}	0.12
Mn^{2+}	0.004
Cl^-	4670
SO_4^{2-}	120
Br^-	5.85

In general, during evaporation of marine waters, solutes precipitate in the sequence calcium carbonate ($CaCO_3$), calcium sulfate ($CaSO_4$), halite (NaCl) and $MgSO_4$. By comparison with ion concentrations and the typical progression of precipitation in salterns (Herrmann et al., 1973), we determined that precipitation of gypsum was ongoing and almost completed at the time of sampling. Water samples were also analyzed for the ions of aluminum, cobalt, chromium, copper, molybdenum, vanadium and iron as well as fluoride, phosphate and nitrate. However, these ions were below detection limits. Due to its low depth and continuous wind mixing, it can be assumed that the water column of the separated brine pool was fully oxygenated.

6.4.2. Core lipids at Lake Tyrrell

Hydrolysis of aliquots of the Bligh and Dyer extracts resulted in relatively simple distributions of fatty acids (FAs) and alcohols (Fig. 6.1).

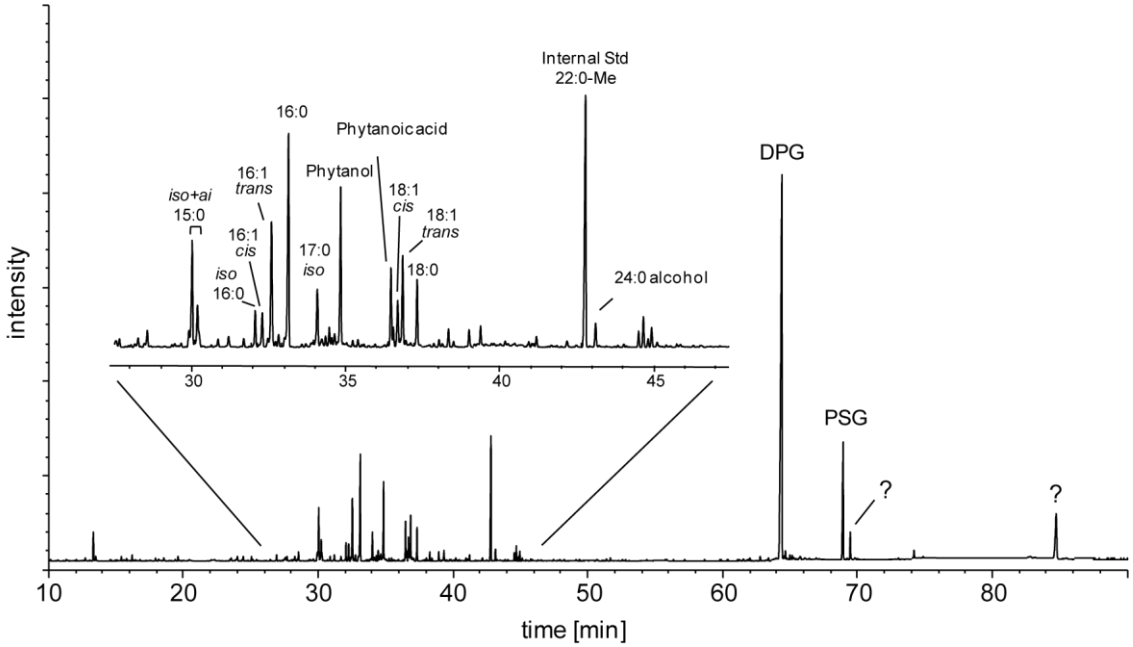

Figure 6.1: GC-FID chromatogram of lipids derived from alkaline hydrolysis of planktonic microorganisms in Lake Tyrrell. Abbriviations: *iso* (*iso* FA), *ai* (*anteiso* FA), *cis* (*cis* FA), *trans* (*trans* FA).

Isoprenoidal building blocks of archaeal cytoplasm membranes with 43 (diphytanyl glycerol, DPG) and 48 (phytanylsesterterpanyl glycerol, PSG) carbon atoms show the highest abundances (Table 6.2). The presence of these core lipids was expected as they are common in hypersaline environments (Kates, 1977).

Table 6.2: Concentrations of fatty acids, alcohols and archaeal core lipids released by alkaline hydrolysis**.**

Identified compounds	**Concentration[µg l^{-1}]**
iso-$C_{15:0}$FA	3.9
anteiso-$C_{15:0}$FA	1.8
iso-$C_{16:0}$ FA	0.6
cis-$C_{16:1}$ FA	1.0
trans-$C_{16:1}$ FA	5.4
$C_{16:0}$ FA	8.6
iso-$C_{17:0}$	2.2
phytanol	4.7
phytanoicacid	1.6
cis-$C_{18:1}$FA	1.9
trans-$C_{18:1}$FA	3.2
$C_{18:0}$ FA	1.6
$C_{17:0}$ 2-OH FA	0.2
$C_{24:0}$alcohol	0.2
DPG corelipid	66.2
PGS corelipid	13.7
Total core lipids	116.9

Phytanol and phytanoic acid are also relatively abundant. In contrast to archaeal core lipids, fatty acids (FAs) of bacterial or eukaryotic origin play a secondary role.

The most abundant FA is *n*-$C_{16:0}$ followed by *trans*-$C_{16:1}$, *iso*-$C_{15:0}$, *trans*-$C_{18:1}$ and *n*-$C_{18:0}$. As in most environments, FAs with even-numbered carbon numbers dominate, and saturated FAs are more abundant than their unsaturated counterparts. Sterols and other lipids diagnostic of eukaryotes were below detection limits.

6.4.3. IPL classes present in the water of Lake Tyrrell

Table 6.3 summarizes the IPLs detected in Bligh & Dyer extracts of Lake Tyrrell water filtrate using LC-ESI-MS in positive and negative ion-modes. Comparing the chromatograms of the two ionization modes, significant differences in ionization efficiencies become apparent (Fig. 6.2).

Table 6.3: IPL classes in Lake Tyrrell and major HPLC-MS characteristics.

IPL compound classes	Major ions (ESI)		Likely source	Abundance[1]
	neg	pos		
Archaeal lipids				
PG-DPG	805.7 (M - H)	807.7 (M + H)	archaeal	+
PGS-DPG	885.6 (M - H)	887.6/ (M + H) 904.7 (M + NH_3 + H)	archaeal	+++
PI-DPG	893.7 (M - H)	895.7 (M + H)	archaeal	+
PGP-Me-DPG	899.7 (M - H)	901.7 (M + H) 918.7 (M + NH_3 + H)	archaeal	++++
PGP-Me-PSG	969.7 (M - H)	971.7 (M + H) 988.8 (M + NH_3 + H)	archaeal	++
PIP-DPG	973.7 (M - H)	975.7 (M + H)	archaeal	++
S-DGD	1055.7 (M - H)	1074.8 (M + NH_3 + H) 1091.8 (M + 2 x NH_3 + H)	archaeal	+
S_2-DGD	1135.7 (M - H)	1154.8 (M + NH_3 + H) 1171.8 (M + 2 x NH_3 + H)	archaeal	+
Unknown	1201.8 (M - H)	1140.5 (M -SO_3? + NH_3 + H) 1220.8 (M + NH_3 + H) 1237.8 (M + 2 x NH_3 + H)	archaeal	+++
Bacterial lipids				
SL	646.4 (M - H)	648.4 (M + H) 665.5 (M + NH_3 + H)	bacterial	+
SL	660.4 (M - H)	663.4 (M + H) 678679.5 (M + NH_3 + H)	bacterial	++
SL	674.4 (M - H)	674.5 (M + H) 693.5 (M + NH_3 + H)	bacterial	+
PC-DAG	802.6 (M - CH_3 + $HCOO^-$)	758.5 (M + H)	bacterial or eukaryotic	++

[1] Due to the lack of commercial available standards an absolute quantification of IPLs was not possible. Therefore, we estimated the abundances tentatively by a rating system based on signal intensities. Abbreviations: PG-DPG, phosphatidylglycerol-diphytanylglycerol; PGS-DPG, phosphatidylglycerolsulfate-diphytanylglycerol; PI-DPG, phosphatidylinositol-diphytanylglycerol; PGP-Me-DPG, phosphatidylglycerolmethylphosphate-diphytanylglycerol; PGP-Me-PSG, phosphatidylglycerolmethylphosphate-phytanylsesterterpanylglycerol; PIP-DPG, phosphatidyl-inositolphosphate-diphytanylglycerol; SDGD, sulfated diglyco-diphytanylglycerol; S_2DGD, bissulfatdiglyco-diphytanylglycerol; Unknown, proposed structure see Fig. 6.4; SL, sulfonolipid; PC-DAG, phosphatidylcholine-diacylglycerol.

For example, lipids with PC and PGS head groups have significantly higher signal intensities in negative-ion-mode, while S-DGD and the injection standard O-PE have higher ionization

efficiencies in positive-ion-mode. This demonstrates that the ionization of IPLs is highly compound specific and makes quantification without suitable standards speculative.

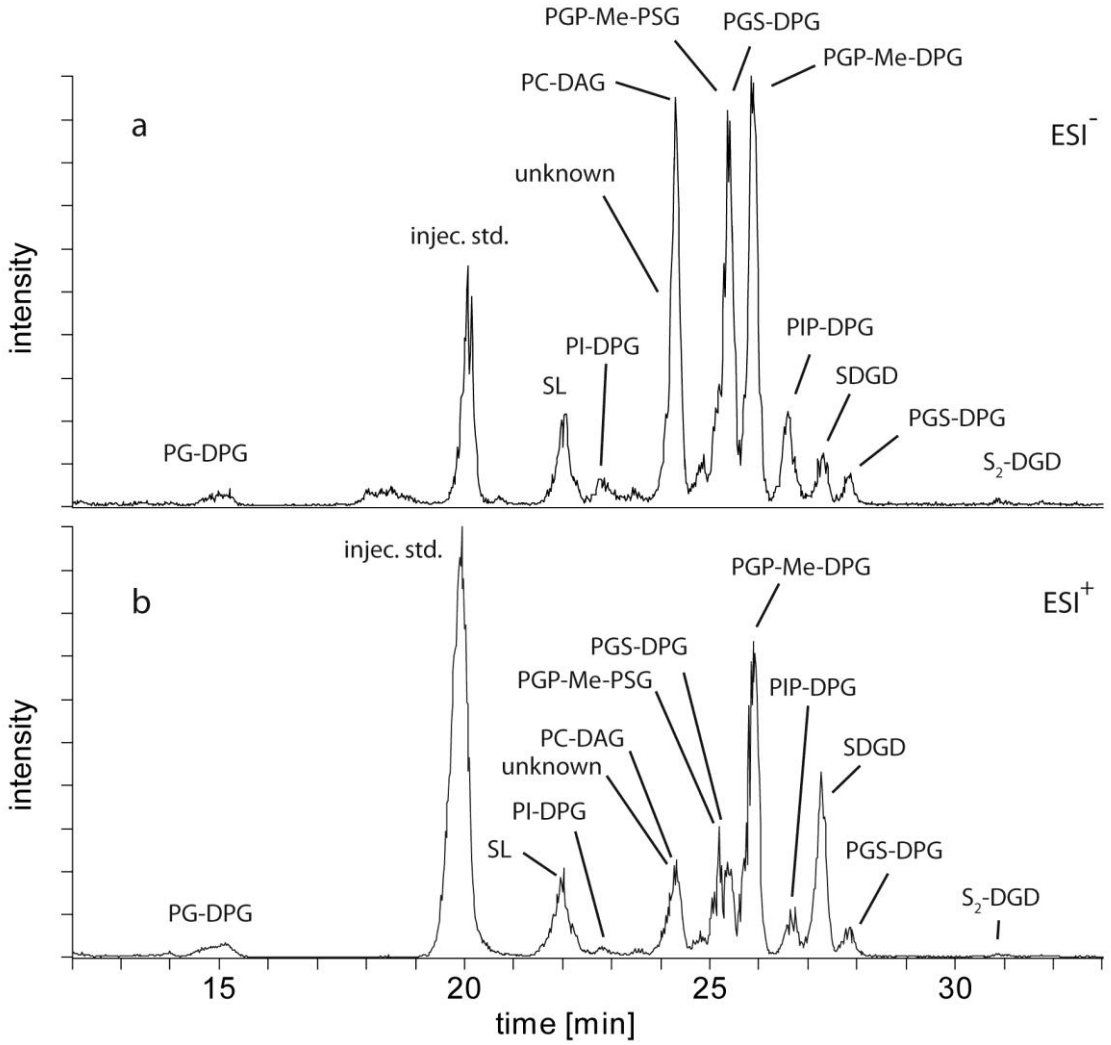

Figure 6.2: LC-ESI-MS chromatograms of IPLs from Lake Tyrrell brine recorded in negative-ion-mode (top) and positive-ion-mode (bottom). For abbriviations see Table 6.3 legend.

Compared to extracts from most other environments, the diversity of IPL classes in Lake Tyrrell is low. The major IPL classes found were bacterial sulfonolipids (SL) and phosphatidylcholinediacylglycerol (PC-DAG), and archaeal phosphatidylglyceroldiphytanylglycerol (PG-DPG), phosphatidylglycerolmethylphosphatediphytanylglycerol (PGP-Me-DPG), phosphatidylglycerolmethylphosphatephytanylsesterterpanylglycerol (PGP-Me-PSG), phosphatidylglycerolsulfatediphytanylglycerol (PGS-DPG), sulfate diglycosyl-diphytanylglycerol (S-DGD), bissulfatediglycosyl-diphytanylglycerol (S_2-DGD), phosphatidylinositoldiphytanylglycerol (PI-DPG), phosphatidylinositolphosphate-diphytanylglycerol (PIP-DPG) and an unknown archaeal lipid. The archaeal core lipid DPG occurred with five different head groups while the core lipid PSG exclusively carried the head group PGP-Me (Table 6.3).

6.4.4. Unusual intact polar lipids PI and PIP

The mass spectra (a) and (b) in Fig. 6.3 belong to two archeal PI and PIP diphytanyl glycerols that have, to our knowledge, not been reported in hypersaline environments before. The fragment at *m/z* 241.0 occurs in both spectra and indicates an inositol phosphate (IP) head group (Pulfer and Murphy, 2003), while the fragment at *m/z* 731.7 can be related to phosphatidylacid DPG. Thus, the quasi-molecular ion at *m/z* 893.7 in (a) suggests PI-DPG as the correct structure.

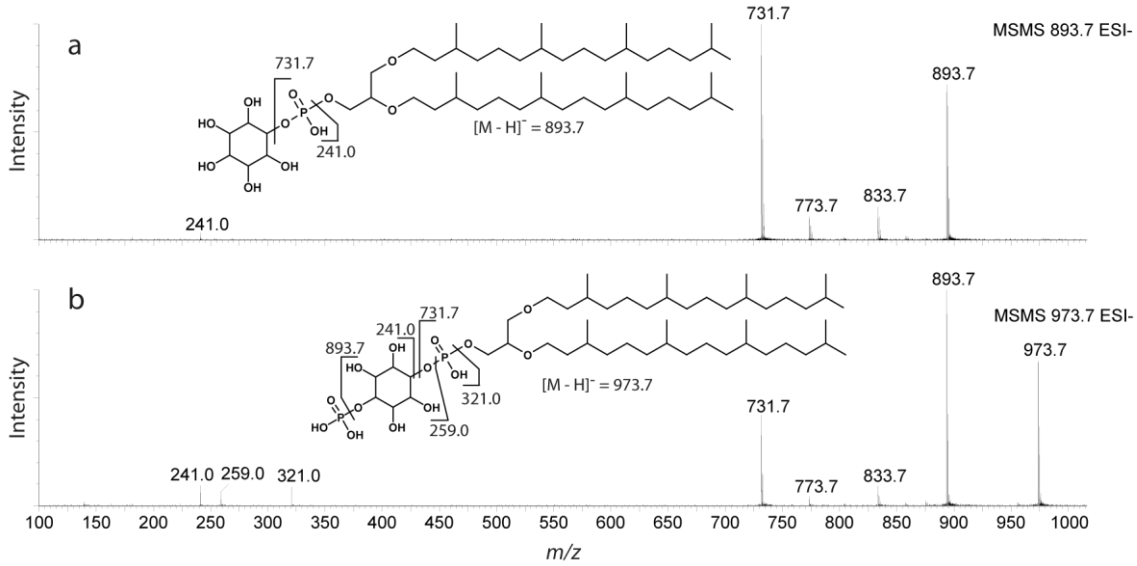

Figure 6.3: ESI-MS/MS spectra and potential molecular structures of two for hypersaline systems unknown compounds A and B (quasi-molecular masses in ESI-neg: 893.7 and 973.7, respectively). Stereochemistry of the sugar moieties is uncertain.

Comparing the two spectra, the ions at *m/z* of 259.0, 321.0 and 973.7 are only present in spectrum (b). The fragment at *m/z* 321.0 is most likely generated by an additional phosphate group (80 u) attached to the inositol head group. Correspondingly, the mass of the quasi-molecular ion of (b) (*m/z* 973.7) is also elevated by 80 u in comparison to (a), and the fragment at *m/z* 259.0 is consistent with the loss of an inositol head group carrying an additional terminal phosphate group. Because of the similarity of spectra (a) and (b), we suggest the second compound to be PIP-DPG (Fig 6.3b).

Unknown archaeal IPL

The LC-MS chromatograms (Fig. 6.2) contain an unknown IPL that occurrs in the evaporating brine pool in significant concentrations. Its MS/MS spectrum in positive-ion-mode is shown in Fig. 6.4. Although the exact structure remains uncertain, there are several diagnostic peaks that suggest an archaeal core lipid with at least two sugar moieties.

When analyzed with the LCQ DECA XP ion-trap mass spectrometer, the main ions for the unknown compound were at *m/z* 1199.8 and 1201.8 $[M-H]^-$ in negative-ion-mode, and at

m/z 1235.8 and 1237.8 $[M + 2NH_3 + H]^+$ in positive-ion-mode (using ammonium formate to enhance ionization efficiency; Pulfer and Murphy, 2003).

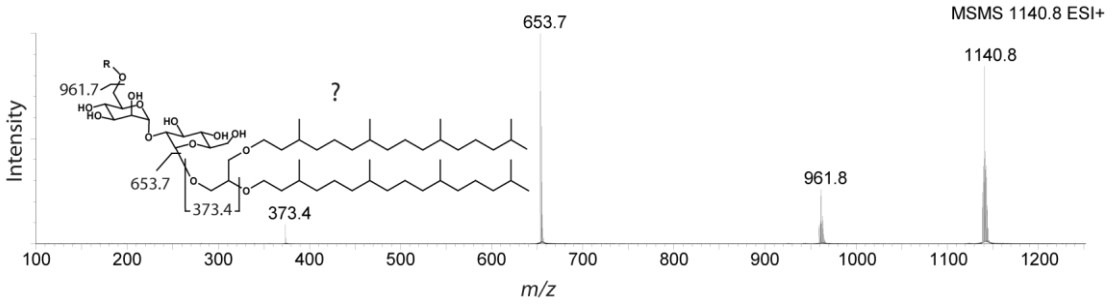

Figure 6.4: MS/MS spectrum of ion *m/z* 1140.8 from Lake Tyrrell water filtrate recorded in positive-ion-mode. The structure only serves as a model, the exact substituents and the stereochemistry are uncertain.

The presence of two molecular ions two mass units apart indicates the presence of a second compound with one double bond. Using the Micromass Q-TOF mass spectrometer, we also observed two quasi-molecular ions (*m/z* 1199.8 and 1201.8) in negative-ion-mode. However, in positive-ion-mode the main ions were at *m/z* 1138.8, 1140.8, and *m/z* 1218.8, 1220.8 $[M + NH_3 + H]^+$. Fragment *m/z* 1140.8 probably relates to $[M - 80 + NH_4]^+$, where the loss of 80 mass units is likely correlated with the elimination of a terminal phosphate (PO_3H) or sulfate (SO_3) moiety (Fredrickson et al., 1989). In hypersaline environments, the presence of a sulfate group is more likely than phosphate (Table 6.4).

Further MS/MS analysis of *m/z* 1140.8 in positive-ion-mode yielded *m/z* 653.7 and *m/z* 373.4, fragments that can be attributed to archaeol and its breakdown product with one phytanyl side chain (Fig. 6.4). Moreover, the strong signal at *m/z* 961.8 is consistent with a typical archaeal DPG core lipid that carries two sugar moieties. Unknown only remains substituent R (Fig. 6.4) that carries the sulfate (or phosphate) group and that must have a mass of 161.1 u.

6.5. Discussion

6.5.1. Core Lipids

The fatty acids detected in the hydrolyzed water filtrates (Fig. 6.1, Table 6.2) may be derived from bacterial and eukaryotic sources. According to Heidelberg et al. (2013) who investigated the same set of samples *Colpodella* dominated the microbial eukaryotic assemblage in the water for both 18S rDNA clone libraries and microscope counts. 18S rDNA surveys suggest that mainly eukaryotic ciliates of the genus *Colpodella* (87 % 18S rDNA sequences) were present in Lake Tyrrell at the time of sampling in January 2010. The green alga *Dunaliella* accounted to only 3 % of the found 18S rDNA sequences. However, as eukaryotic biomarkers like sterols were below detection limit, eukaryotes probably did

account for only a small proportion to the total planktonic biomass present in this hypersaline ecosystem. Therefore, the FAs in the LakeTyrrell extracts were probably nearly quantitatively derived from bacteria.

Core lipid concentrations can be used to estimate the relative abundances of major taxonomic groups (Simon & Azam, 1989; Lipp et al., 2008). Comparing the GC-FID response of bacterial fatty acids and archaeal DPG and PSG in the Lake Tyrrell brine, the ratio of archaea to bacteria is approximately 2:1. This proportion is in accordance with PCR-derived abundances of archaea (73%) and bacteria (27%) in the brine of a solar saltern in Majorca (Anton et al., 2000). The results underscore the relative importance of bacteria in salt-saturated aquatic environments.

6.5.2. IPL classes in Lake Tyrrell and their potential sources

To determine whether IPLs from Lake Tyrrell can be related to the microbial community composition, we conducted an extensive literature review of IPL-data and physiological properties of Halobacteriaceae (Table 6.4). The table can generally be used to explore correlations between IPL composition, phylogenetic position and physiology.

Table 6.4: Literature survey of halophilic archaea mainly found in the water column of hypersaline habitats. For molecule abbreviations and structures see Fig. 6.5.

Genus	Species	Identified IPLs																Properties									Literature
		PA	PG	PGP-Me	PGS	DGD-1 = x[1)]	DGD-2 = x[2)]	S-DGD-1 = x[1)]	S-DGD-3 = x[2)]	S-GL-2	S_2-DGD-1	TGD-1 = x[1)]	TGD-2 = x[2)]	S-TGD-1	S-TeGD	CL	DPG	PSG	motile	aerobic growth	anaerobic growth	chemo-organotrophic	heterotrophic	gas vacuoles	data by TLC	data HPLC-MS	
	cibarius		x	x															x	x							(Roh et al., 2010)
Haladaptatus	*paucihalophilus*		x	x	x															x		x					(Savage et al., 2007)
	litoreus		x	x	x			x[1)]												x		x					(Cui et al., 2010f)
Halalkalicoccus	*tibetensis*		x	x	-												x	x	-	x	-	x		-	x		(Xue et al., 2005)
Haloarchaeobius	*iranensis*		x	x	x														x	x	-				x		(Makhdoumi-Kakhki et al., 2012)
Haloarcula	*amylolytica*		x	x	x														x	x	x	x					(Yang et al., 2007)
	argentinensis		x	x	x	x[2)]						x[2)]							x	x		x					(Ihara et al., 1997)
	marismortui		x	x[#)]	x	x[1)]						x[2)]							-	x		x			x		(Oren & Ventosa, 1996)
	quadrata		x	x[#)]	x							x[2)]							x	x	x	x			x		(Oren et al., 1999)
	salaria		x	x	x	x						x[2)]							-	x	-	x			x		(Namwong et al., 2011)
	tradensis		x	x	x	x						x[2)]							-	x	-	x			x		(Namwong et al., 2011)
Halobacterium	*jilantaiense*		x	x	x							x[1)]		x	x		x			x		x					(Yang et al., 2006)
	noricense		x	x	x							x[1)]			x		x		x	x	x	x					(Gruber et al., 2004)
	piscisalsi		x	x	x							x[1)]		x	x		x		x	x	x						(Yachai et al., 2008)
	salinarum		x	x	x							x[1)]		x	x		x		x	x	x	x			x		(Gruber et al., 2004)
Halobaculum	*gomorrense*		x	x	-			x[1)]											x	x	-	x		-	x		(Oren et al., 1995)
Halobellus	*clavatus*		x	x	x			x[1)]											x	x	-		x		x		(Cui et al., 2011c)
	limi		x	x	x	x[1)]		x[1)]									x		x	x	-				x		(Cui et al., 2012)
	salinus		x	x	x	x[1)]		x[1)]									x		x	x	-				x		(Cui et al., 2012)
Halobiforma	*haloterrestris*		x	x													x	x	x	x					x		(Hezayen et al., 2002)
	lacisalsi		x	x													x	x	x	x	x	x		-	x		(Xu et al., 2005c)
	nitratireducens		x	x													x	x	x	x	x	x		-	x		(Hezayen et al., 2002)
Halococcus	*dombrowskii*		x	x				x[1)]									x	x		x							(Stan-Lotter et al., 2002)
	hamelinensis		x	x				x[1)]												x							(Goh et al., 2006)
	qingdaonensis		x	x				x[1)]												x							(Wang et al., 2007)
	salifodinae		x	x[#)]				x									x	x	-	x					x		(Denner et al., 1994)
	thailandensis		x	x				x[1)]									x	x		x							(Namwong et al., 2007)
Haloferax	*alexandrinus*		x	x				x[1)]									x			x		x					(Asker & Ohta, 2002)
	denitificans	-	x	x[#)]		x		x[1)]									x	-	-	x	x			-	x		(Tindall et al., 1989)
	elongans		x	x		x[1)]		x[1)]												x		x					(Allen et al., 2008)
	larsenii		x	x				x[1)]											x	x	x	x					(Xu et al., 2007a)
	lucentense		x	x		x[1)]		x[1)]									x		x	x		x			x		(Gutiérrez et al., 2002)
	mediterranei		x	x[#)]		x[1)]		x[1)]																	x		(Oren et al., 1996)
	mucosum		x	x		x[1)]		x[1)]												x		x					(Allen et al., 2008)
	prahovense		x	x				x[1)]												x		x					(Enache et al., 2007)
	sulfurifontis		x	x				x[1)]									x			x	x	x					(Elshahed et al., 2004)
	volcanii		x	x[#)]		x[1)]		x[1)]									x			x		x			x		(Oren et al., 1996)
	volcanii	x	x	x				x[1)]		x						x				x		x					(Sprott et al., 2003)

Table 6.4: continued.

Genus	Species	Identified IPLs														Properties								Literature
		PA	PG	PGP-Me	PGS	DGD-1 = $x^{1)}$ / DGD-2 = $x^{2)}$	S-DGD-1 = $x^{1)}$ / S-DGD-3 = $x^{2)}$	S-GL-2	S_2-DGD-1	TGD-1 = $x^{1)}$ / TGD-2 = $x^{2)}$	S-TGD-1	S-TeGD	CL	DPG	PSG	motile	aerobic growth	anaerobic growth	chemo-organotrophic	heterotrophic	gas vacuoles	data by TLC	data HPLC-MS	
Halogeometricum	*borinquense*		x	x	-											x	x	x	x		-	x		(Montalvo-Rodriguez et al., 1998)
	rufum		x	x		$x^{1)}$	$x^{1)}$									x	x		x			x		(Cui et al., 2010g)
Halogranum	*amylolyticum*		x	x	x	$x^{1)}$	$x^{1)}$										x		x					(Cui et al., 2011d)
	gelatinilyticum		x	x	x	$x^{1)}$	$x^{1)}$									x	x		x					(Cui et al., 2011d)
	rubrum		x	x		$x^{1)}$	$x^{1)}$									x	x			x				(Cui et al., 2010c)
	salarium		x	x		$x^{1)}$	$x^{1)}$									x	x		x			x		(Kim et al., 2011)
Halolamina	*pelagica*		x	x	x	$x^{1)}$	$x^{1)}$			$x^{1)}$							x			x				(Cui et al., 2011a)
Halomarina	*oriensis*		x	x						$x^{2)}$				x	x		x		x					(Inoue et al., 2011)
Halomicrobium	*katesii*		x	x			$x^{1)}$										x							(Kharroub et al., 2008)
	mukohataei	x	x	x	x		$x^{1)}$										x	x	x					(Oren et al., 2002)
	zhouii		x	x	x	x	x									x	x	-	x			x		(Yang & Cui, 2012)
Halonotius	*pteroides*		x	x			$x^{1)}$										x			x				(Burns et al., 2010a)
Halopelagius	*inordinatus*		x	x		$x^{1)}$	$x^{1)}$									x	x			x				(Cui et al., 2010e)
Halopenitus	*persicus*		x	x	-											-	x	-	x			x		(Amoozegar et al., 2012)
Halopiger	*aswanensis*		x	x					x					x	x	x	x							(Hezayen et al., 2010)
	xanaduensis		x	x					x					x	x		x							(Gutiérrez et al., 2007)
Haloplanus	*aerogenes*		x	x	x		$x^{1)}$										x		x		x	x		(Cui et al., 2011b)
	natans		x	x	x		$x^{1)}$										x		x		x			(Bardavid et al., 2007)
	vescus		x	x	x		$x^{1)}$										x				x			(Cui et al., 2010a)
Haloquadratum	*walsbyi*		x	x			$x^{1)}$							x			x		x					(Burns et al., 2007)
Halorhabdus	*tiamatea*		x	x		$x^{2)}$				$x^{1)}$								x						(Antunes et al., 2008)
	utahensis		x	x						$x^{1)}$	x			x		x	x	x						(Wainø et al., 2000)
Halorientalis	*regularis*		x	x			$x^{1)}$		x		x					x	x	-	x			x		(Cui et al., 2011c)
Halorubrum	*aidingense*		x	x	x									x		x	x		x					(Cui et al., 2006b)
	alkaliphilum		x	x										x	x	x	x	x	x		-	x		(Feng et al., 2005)
	arcis		x	x	x		$x^{1)}$							x		x	x		x					(Xu et al., 2007b)
	aquaticum		x	x			x									-	x	-	x		-	x		(Gutiérrez et al., 2011)
	californiense		x	x	x		$x^{1)}$							x		x	x		x					(Pesenti et al., 2008)
	chaoviator		x	x	x		$x^{1)}$									x	x		x					(Mancinelli et al., 2009)
	cibi		x	x			$x^{1)}$									x								(Roh & Bae, 2009)
	coriense				x		x																	(Oren & Ventosa, 1996)
	distributum				x		x																	(Oren & Ventosa, 1996)
	ejinorense		x	x													x		x					(Castillo et al., 2007a)
	ezzemoulense		x	x	x		$x^{1)}$							x		x	x		x					(Kharroub et al., 2006)
	kocurii		x	x			$x^{1)}$							x			x		x					(Gutiérrez et al., 2008b)
	lacusprofundi		x	$x^{\#)}$	x		x							x		-	x	-	x		-	x		(McGenity & Grant, 1995)
	lipolyticum		x	x	x		$x^{1)}$							x		x	x		x					(Cui et al., 2006b)
	litoreum		x	x	x		x									x	x	-	x		-	x		(Cui et al., 2007a)
	luteum		x	x												x	x		x					(Hu et al., 2008)
	orientale		x	x			x							x	-	x	x		x		-	x		(Castillo et al., 2006b)

Table 6.4: continued.

Genus	Species	Identified IPLs																	Properties								Literature
		PA	PG	PGP-Me	PGS	DGD-1 = $x^{1)}$	DGD-2 = $x^{2)}$	S-DGD-1 = $x^{1)}$	S-DGD-3 = $x^{2)}$	S-GL-2	S_2-DGD-1	TGD-1 = $x^{1)}$	TGD-2 = $x^{2)}$	S-TGD-1	S-TeGD	CL	DPG	PSG	motile	aerobic growth	anaerobic growth	chemo-organotrophic	heterotrophic	gas vacuoles	data by TLC	data HPLC-MS	
Halorubrum	*saccharovorum*		x	x	x				$x^{2)}$										x					-	x		(McGenity & Grant, 1995)
	sodomense		x	$x^{\#)}$	x			x									x		x	x	-	x		-	x		(McGenity & Grant, 1995)
	tebenquichense		x	x					$x^{2)}$											x		x					(Lizama et al., 2002)
	trapanicum		x	$x^{\#)}$	x			x									x	-	-	x	-	x		-	x		(McGenity & Grant, 1995)
	terrestre		x	x				$x^{1)}$									x		x	x		x					(Ventosa et al., 2004)
	tibetense		x	x													x	x	-	x	-	x		-	x		(Fan et al., 2004)
	vacuolatum		x	x	-			-									x	x	-	x	-	x		-	x		(Kamekura et al., 1997)
	xinjiangense		x	x	x			x									x		-	x	-	x		-	x		(Feng et al., 2004)
Halorussus	*rarus*		x	x	x	$x^{1)}$	$x^{2)}$	$x^{1)}$				$x^{1)}$		x					x	x	-	x			x		(Cui et al., 2010d)
Halosarcina	*limi*		x	x				$x^{1)}$			x								x	x		x					(Cui et al., 2010b)
	pallida		x	x		$x^{1)}$		$x^{1)}$												x		x					(Savage et al., 2008)
Halosimplex	*carlsbadense*		x	x							x	x			x					x		x					(Vreeland et al., 2002)
Halostagnicola	*alkaliphila*		x	x													x	x	x	x	x	x					(Nagaoka et al., 2011)
	kamekurae		x	x															x	x	x	x					(Nagaoka et al., 2010)
	larsenii		x	x													x	x	-	x	x				x		(Castillo et al., 2006c)
Haloterrigena	*daquingensis*		x	x							x									x		x					(Wang et al., 2010)
	hispanica		x	x				$x^{1)}$									x	x		x		x					(Romano et al., 2007)
	jeotgali		x	x							x									x	x						(Roh et al., 2009)
	limicola		x	x							x						x	x	x		x	x					(Cui et al., 2006c)
	longa		x	x							x						x	x		x		x					(Cui et al., 2006c)
	saccharevitans		x	x							x						x	x		x	x						(Xu et al., 2005a)
	salina		x	x							x						x	x		x		x					(Gutiérrez et al., 2008a)
	thermotolerans		x	x							x						x	x		x		x					(Montalvo-Rodriguez et al., 2000)
	turkmenica		x	x							x						x	x									(Ventosa et al., 1999)
Halovivax	*asiaticus*		x	x													x	x	-	x	-			-	x		(Castillo et al., 2006a)
	ruber		x	x																x							(Castillo et al., 2007b)
Halovenus	*aranensis*		x	x															-	x	-	x			x		(Makhdoumi-Kakhki et al., 2012)
Natrialba	*asiatica*		x	x							x						x	x	x	x		x			x		(Hezayen et al., 2001)
	aegyptiaca		x	x							x						x	x	x	x		x			x		(Hezayen et al., 2001)
	chahannaoensis		x	x													x	x	-	x	-			-	x		(Xu et al., 2001)
	hulunbeirensis		x	x													x	x	-	x	-			-	x		(Xu et al., 2001)
	magadii		x	x													x	x								x	(Qiu et al., 1998)
	taiwanensis		x	x							x						x	x	x	x					x		(Hezayen et al., 2001)
Natrinema	*altunense*		x	x	x												x	x	x	x	x	x			x		(Xu et al., 2005b)
	ejinorense		x	x							x						x	x	-	x	-	x			x		(Castillo et al., 2006d)
	gari		x	x	x												x	x	x	x	x	x	x		x		(Tapingkae et al., 2008)
	pallidum		x	x	x												x	x		x		x			x		(McGenity et al., 1998)
	pellirubrum		x	x	x												x	x		x		x			x		(McGenity et al., 1998)
	salaciae		x	x	x						x								-	x	x	x			x		(Albuquerque et al., 2012)
	versiforme		x	x	x												x	x	-	x	x	x		-	x		(Xin et al., 2000)

Table 6.4: continued.

Genus	Species	Identified IPLs																	Properties								Literature
		PA	PG	PGP-Me	PGS	DGD-1 = $x^{1)}$	DGD-2 = $x^{2)}$	S-DGD-1 = $x^{1)}$	S-DGD-3 = $x^{2)}$	S-GL-2	S_2-DGD-1	TGD-1 = $x^{1)}$	TGD-2 = $x^{2)}$	S-TGD-1	S-TeGD	CL	DPG	PSG	motile	aerobic growth	anaerobic growth	chemo-organotrophic	heterotrophic	gas vacuoles	data by TLC	data HPLC-MS	
Natronoarchaeum	*mannanilytium*		x	x							x								-	x	-	x		-	x		(Shimane et al., 2010)
Natronobacterium	*nitratireducens*		x	x													x	x	x	x	x	x			x		(Xin et al., 2001)
Natronococcus	*amylolyticus*		x	x													x	x	-	x	x	x		-	x		(Kanai et al., 1995)
	jeotgali		x	x															-	x	-	x		-	x		(Roh et al., 2007)
Natronolimnobius	*baerhuensis*		x	x													x	x	x	x		x		-	x		(Itoh et al., 2005)
	innermongolicus																x	x	-	x		x		-	x		(Itoh et al., 2005)
Natronomonas	*pharaonis*	x	x	x													x	x		x		x			x		(Kamekura et al., 1997)
	moolapensis	x	x	x													x	x	x	x	-	x		x	x		(Burns et al., 2010b)
Natronorubrum	*aibiense*		x	x								x					x	x	-	x	-	x			x		(Cui et al., 2006a)
	bangense		x	x													x	x	-	x	-	x			x		(Xu et al., 1999)
	sediminis		x	x													x	x	-	x		x			x		(Gutiérrez et al., 2010)
	sulfidifaciens		x	x															x	x		x			x		(Cui et al., 2007b)
	tibetense		x	x													x	x	-	x	-	x			x		(Xu et al., 1999)
Salarchaeum	*japonicum*		x	x				$x^{1)}$											x	x	x	x			x		(Shimane et al., 2011)

More specifically, Table 6.5 compares the IPL inventory of Lake Tyrrell with IPL data of cultivated hypersaline bacteria and archaea detected in the lake using metagenome-based EMIRGE technology (Miller et al., 2011). Overall, the inventory of IPLs of the cultivated strains is in good agreement with the IPLs found in the lake (Table 6.5). Some IPLs detected in cultures (DGD, TGD, S-TGD and S-TeGD) were not found in Lake Tyrrell, maybe because growth conditions were significantly different or other strains were involved. Conversely, PI-DPG and PIP-DPG were found in Lake Tyrrell although they are not known from any cultivated strain of halophilic Archaea (Table 6.4). As discussed in the following section on individual IPLs of Lake Tyrrell, these lipids may derive from a major uncultivated archaeal group.

Table 6.5: Comparison of IPLs in Lake Tyrrell with the published IPL inventories of cultivated halophilic archaea and bacteria.

identified Genera (EMIRGE)		*Halosarcina*	*Haloquadratum walsbyi*	*Haloarcula*	*Halobacterium*	*Salinibacter ruber*	Lake Tyrrell
described strains		2	1	6	4	1	
IPLs present	SL	-	-	-	-	x	x
	PC-DAG	-	-	-	-	x	x
	PG	x	x	x	x	-	x
	PGP-Me	x	x	x	x	-	x
	PGS	-	-	x	x	-	x
	SDGD-1	x	x	-	-	-	x*)
	DGD-1	(x)	-	(x)	-	-	-
	DGD-2	-	-	(x)	-	-	-
	TGD-1	-	-	-	x	-	-
	TGD-2	-	-	(x)	-	-	
	S-TGD	-	-	-	(x)	-	-
	S-TeGD	-	-	-	x	-	-
	S_2-DGD	-	-	-	-	-	x
	DPG	x	x	?	x	-	x
	PSG	?	-	?	-	-	x
Salt requirement		1.7-5.1 M NaCl min. 1 mM Mg^{2+}	2.4 M-sat. NaCl Mg^{2+} not needed	2-5 M NaCl 5-100 mM Mg^{2+} strain dependent	2.1-5.2 M NaCl strain dependent 0.1 - 1 M Mg^{2+}	150-300 g L^{-1} total salts	3.3 - 3.4 M NaCl 38 - 45 mM Mg^{2+}
Chemo-organotroph		both strains	x	all strains	*H. jilantaiense* *H. noricense* *H. salinarum*	x	
Anaerobic growth		-	-	*Har. amylolytica* *Har. quadrata*	all but *piscisalsi*	-	
References		Savage (2008) Cui (2010)	Burns (2007)	Yang (2007) Ihara (1997) Oren (1996) Oren (1999) Namwong (2011)	Yang (2006) Gruber (2004) Yachai (2008)	Anton (2002) Corcelli (2004)	this study

'?': no data available
(x): lipid data only known for some strains
x*): SDGD-1, SDGD-3 or SDGD-5 were not distinguished in this work
For compound abbreviations see Table 6.3 or Figure 6.5.

6.6. Archaeal IPLs

PG and PGP-Me

The archaeal membrane lipids with the head groups PG and PGP-Me occur in all genera of *Halobacteriaceae* (Kamekura & Kates, 1999; Oren et al., 2009) (Table 6.4). PGP-Me plays an important role in membrane stabilization at highest salinities (Tenchov et al., 2006). The stabilizing effect is caused by steric repulsion that prevents close approach of the two layers of the cytoplasmic membrane and is attributed to the large polar head group of PGP-Me. Interestingly, in Lake Tyrrell PGP-Me was the only head group of the phytanylsesterterpanyl (PSG) core lipids. Thus, it is possible that the C_{25} sesterterpanyls also contribute to membrane stability at highest salinities.

PGS

Phospholipids with the PGS head group are found in selected haloarchaeal genera. They occur in all investigated species of *Haloarcula, Halobacterium, Halobellus, Halolamina* and in some species of *Haloadaptus, Halogranum*, *Halomicrobium, Haloplanus, Halorubrum, Halorussus and Natrinema* (Table 6.4). According to our data base, the PGS head group appears to be diagnostic for these genera. However, apart from its role as a membrane constituent, the exact function of this lipid remains unknown.

S-DGD

Sulfur-containing diglycolipids (S-DGDs) are widely distributed in the family Halobacteriaceae. They occur in three different varieties: S-DGD-1 (1-O-[α-D-mannose-(6´-SO_3H)-(1´-2´)-α-D-glucose]-2,3-di-O-phytanyl-*sn*-glycerol) is the major glycolipid of the genus *Haloferax* (Kushwaha et al., 1982), while S-DGD-3 (1-O-[α-D-mannose-(2´-SO_3H)-(1`-4`)-α-D-glucose]-2,3-di-O-phytanyl-*sn*-glycerol) or S-DGD-5 (1-O-[α-D-mannose-(2´-SO_3H)-(1´-2)-α-D-glucose]-2,3-di-O-phytanyl-*sn*-glycerol) have only been found in representatives of the genus *Halorubrum* (Tindall, 1990; Trincone et al., 1990; 1993). However, the presence of SDGD-1 is not limited to the genus *Haloferax*, it was also found in members of at least 18 further genera (Table 6.4). However, the position of the sulfate group in S-DGDs and the exact configuration of sugar linkages appear to contain some taxonomic information and may help distinguish between genera (Table 6.5). The potential of S-DGSs as chemotaxonomic makers has yet to be developed as the position of the sulfate and sugar moieties are difficult to establish and are not commonly reported.

S_2-DGD

The bis-sulfated diglycolipid S_2-DGD found in Lake Tyrrell was first detected in the non-alkaliphilic halophilic strain 172 by Matsubara et al. (1994), later classified as *Natrialba asiatica* (Kamekura and Dyallsmith, 1995). S_2-DGD is less often reported than PG, PGP-Me or S-DGD-1. It was found in the Genera *Halopiger, Halorientalis, Halosarcina, Halosimplex, Haloterrigena Natrialba, Natrinema* and *Natronoarchaeum* (Table 6.4). Only one isomer of S_2-DGD is known (2,3-diphytanyl- or phytanyl-seterterpenyl-1-[2,6-$(HSO_3)_2$-α-Man *p*-1 2-Glc*p*]-*sn*-glycerol). As S_2-DGD is relatively uncommon, it has potential as a marker for the above genera.

PI and PIP and unknown IPL

IPLs with PI and PIP head groups are produced by some Archaea and were, for instance, found in the aerobic hyperthermophilic archaeon *Aeropyrumpernix* K1 (Morii et al., 1999). However, as Table 6.4 reveals, these IPLs have never been observed before in hypersaline ecosystems. Using LC-MS-based techniques, PI-DPG, PIP-DPG and the unknown IPL shown in Fig. 6.4 proved to be relatively abundant in Lake Tyrrell brine, and they may be equally

common in other hypersaline ecosystems. Although there is currently no taxonomic information for these IPLs, we speculate they may derive from *Nanohaloarchaea. Nanohaloarchaea*, which are abundant in Lake Tyrrell, were detected for the first time using community genomics and *de novo* sequence assembly (Narasingarao et al., 2012).

6.6.1. Bacterial IPLs

Sulfonolipid (SL)

To date, the identified sulfonolipids (or acylhalocapnines) with *m/z* 646, 660 and 674 in ESI neg have only been found in the hypersaline bacteria *Salinibacter ruber* (Corcelli et al., 2004; Corcelli, 2009) and *Salisaeta longa* (Baronio et al., 2010). The salt requirement for these two hypersaline bacteria is different. Whereas *S. longa* needs moderate sodium chloride concentrations (5-20%, w/v), *S. ruber* prefers hypersaline systems with concentrations of 15 to 30% (Anton et al., 2002; Vaisman & Oren, 2009) confirming *S. ruber* as the most likely source for SL in Lake Tyrrell.

PC-DAG

Intact polar lipids with phosphatidylcholine head groups in combination with diacyl core lipids are commonly abundant in eukaryotes and many bacteria and do not contain further phylogenetic information. Since eukaryotic sterols are below detection limits in the Lake Tyrrell filtrates, PC-DAG is most likely derived from *S. ruber* (Corcelli et al., 2004) and minor halophilic bacteria.

6.6.2. Comparison of IPLs in Lake Tyrrell with other hypersaline habitats

Table 6.6 compares IPLs from Lake Tyrrell with the lipids from four other hypersaline settings (Litchfield et al., 2000; Oren & Rodriguez-Valera, 2001; Lattanzio et al., 2002). Despite an intense literature search, these appear to be the only publications presenting IPLs of planktonic hypersaline microbial communities. Apart from IPL data on salterns in Spain, California and Italy, all three publications include corresponding information from a saltern near Eilat in Israel sampled at different dates and locations.

The IPL data shown in Table 6.6 originate from ponds with different salinities. Where multiple datasets were available (Litchfield et al., 2000), IPL data from samples with salinities closest to Lake Tyrrell were chosen. All studies used similar Bligh and Dyer methods for lipid extraction and TLC for lipid identification. Additionally, Lattanzio et al. (2002) used ESI-MS analysis with direct injection to support TLC results.

Lake Tyrrell and the other four hypersaline environments all contained the IPLs S-DGD, PG-DPG and PGS-DPG (Table 6.6). Also present in all investigated environments were archaeal lipids with the PGP-Me head group, although the method employed by Oren and Rodriguez-

Valera (2001) and Litchfield et al. (2000) did not allow to distinguish between the diphytanylglycerol (DPG) and phytanylsesterterpanylglycerol (PSG) cores. The presence of IPLs with the PG and PGP-Me head groups in all environments is not surprising as these IPLs have been detected in all cultivated halophilic archaea (Table 6.4). S_2-DGD found in Lake Tyrrell was also reported by Oren and Rodriguez-Valera (2001) in the salterns in Spain and Eilat, Israel. Surprisingly, S_2-DGD was not found in the Eilat samples collected by Litchfield et al. (2000) and Lattanzio et al. (2002) chronologically before and after the study of Oren and Rodriguez-Valera (2001).

Table 6.6. Comparison of the IPL inventory of Lake Tyrrell with that in other hypersaline environments.

intact polar lipids	This study	Oren and Rodriguez-Valera (2001)		Litchfield et al. (2000)		Lattanzio et al. (2002)	
	Lake Tyrrell, Victory, Australia	Santa Pola, Allicante, Spain	Eilat, Israel	Newark, California, USA	Eilat, Israel	Margherita di Savoia. Italy	Eilat, Israel
Salt (g L^{-1})	266	370	350-360	150-220	170-220	n.d.	n.d.
SL	x						
PC-DAG	x						
PG-DPG	x	x	x	x	x	x	x
PGP-Me-DPG	x	x[a)]	x[a)]	x[a)]	x[a)]	x	x
PGP-Me-PSG	x					-	-
PGS-DPG	x	x	x	x	x	x	x
S-DGD	x	x	x	x	x	x	x
S_2-DGD	x	x	x				
PI-DPG	x						
PIP-DPG	x						
Unknown	x						
BGP[b)]	-					x	x
GlyC[c)]	-					x	-

n.d.: not determined
[a)] no differentiation between DPG and PSG
[b)] BGP = bisphosphatidylglycerol
[c)] GlyC = glycocardiolipin
For compound abbreviation see Table 7.3 or Fig 7.5.

An archaeal analogue (CL) to bacterial cardiolipinbisphosphatidyl-glycerol (BGP) was identified by Lattantzio et al. (2002) in the saltern ponds in Italy and Israel, and a glycocardiolipin (GlyC) was exclusively found in Italy in minor concentrations. These lipids are not reported in the studies of Oren and Rodriguez-Valera (2001) and Litchfield et al. (2000), possibly because they used less sensitive techniques. However, even with the most sensitive LC-ESI-MS techniques employed in this study, and upon injection of highly concentrated extracts, we were not able to find these compounds in Lake Tyrrell.

Eukaryotic or bacterial PC-DAG and bacterial SL were only found in Lake Tyrrell but not in the other salterns (Table 6.6). However, these lipids may have been overlooked as bacteria

were not considered to play a major role in salt saturated systems (e.g. Oren, 1994) until 2002, when (Anton et al.) recognized the quantitative importance of the hypersaline bacterium *Salinibacter ruber*.

In summary, although IPL information on salt lakes is still sparse and methodologically heterogeneous, the investigated five systems show substantial similarities. However, as this is the first study of IPLs in a salt lake using LC-MS-based techniques, more comparable studies are required to establish global similarities and differences in this type of habitat.

6.7. Conclusion

The IPLs of the microbial community of Lake Tyrrell appear to be typical for an ecosystem at salt saturation level. Based on quantitative core lipid data, the brine is dominated by Archaea (74%) and includes a substantial proportion of bacteria (26%). However, eukaryotic sterols were below detection limits. Two archaeal IPLs with inositol-bearing head groups as well as an unknown archaeol with at least two sugar moieties were detected in a hypersaline ecosystem for the first time.

This is the first study to investigate IPLs of a salt-saturated microbial ecosystem using sensitive LC-MS technology. A tabulated compilation of all known IPLs in Halobacteriaceae reveals that these compounds have potential as broad taxonomic markers, but that a larger, global data collection based on LC-MS technology is required that investigates cytoplasmic membrane lipids in more detail and includes minor IPLs from environmental samples as well as cultured representatives.

While commonly applied TLC analyses are cost effective for the fast identification of major known membrane components, this method easily leads to the misidentification of IPLs such as PGP for PGP-Me. TLC is also limited in its separation power (Lattanzio et al. 2002) and usually not suitable for the identification of unknown lipids. For the identification of unknown IPLs and minor components, a combination of TLC with mass spectrometry (TLC-MS) or LC-MS are required. Particularly a detailed investigation of the stereochemistry of sugar-containing head groups like DGD, S-DGD, TGD or TeGD has the potential to improve chemotaxonomic resolution. In this way, taxonomy based on IPLs could become a fast and cheap tool to screen and compare the composition of hypersaline ecosystems and to control and verify results of genome based data. Once injection standards become available, IPLs also have the potential to yield quantitative data for groups of organisms. Moreover, the carbon and hydrogen isotopic compositions of individual IPLs may facilitate investigation of carbon cycling and the hydrology of salt lakes.

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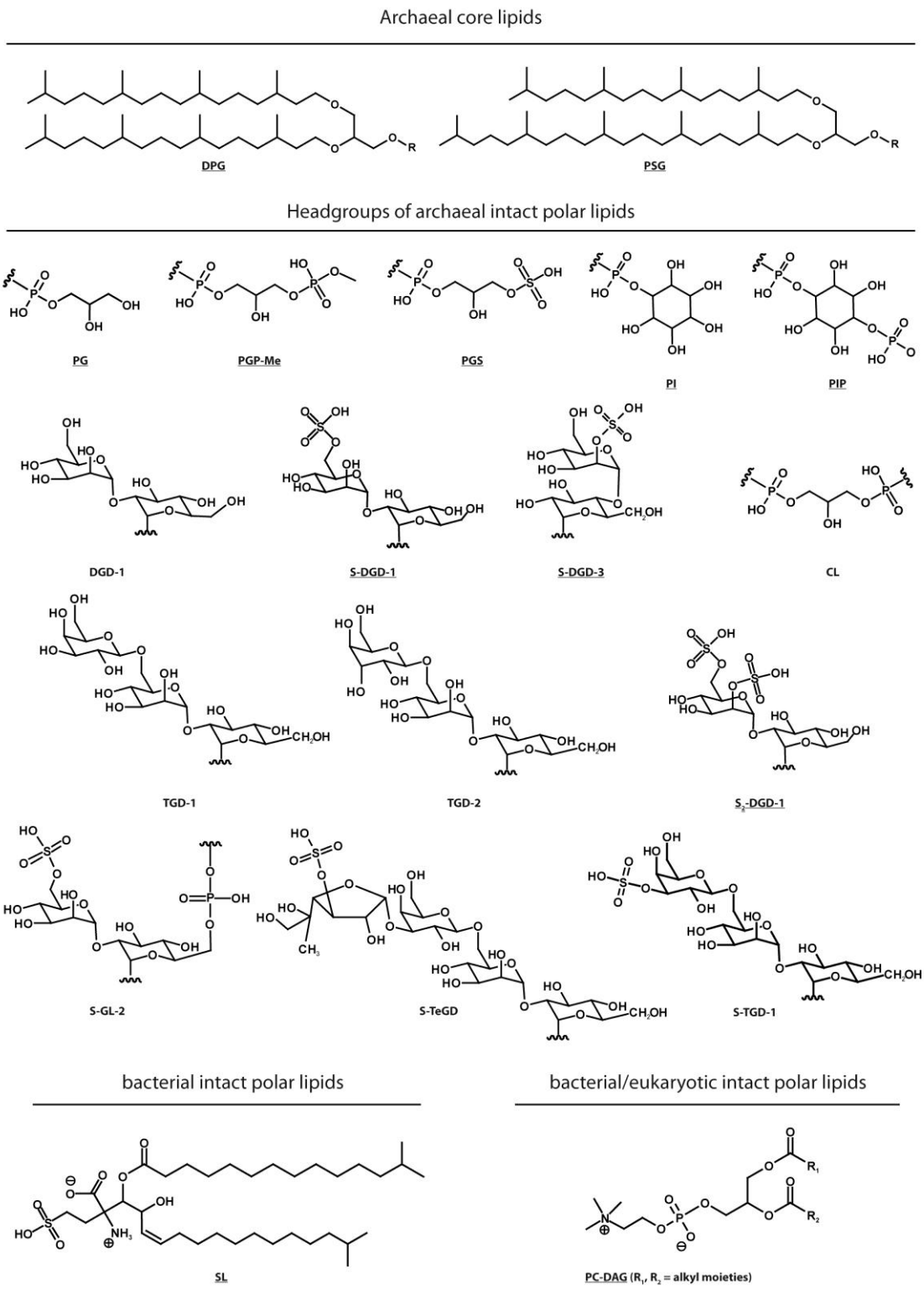

Figure 6.5: Molecular structures of IPL head groups and core lipids found in hypersaline environments. Structures with underscored abbreviations were identified in Lake Tyrrell. Core lipid abbreviations: DPG, diphytanol glycerol; Head groups: PSG, phytanolsesterterpanyl glycerol; PG, phosphatidylglycerol; PGP-Me, phosphatidylglycerolphosphate methyl ester; PGS, phosphatidylglycerolsulfate; PI, phosphatidylinositol; PIP, phosphatidylglycerolphosphate; DGD, diglyco; CL, archaeal cardiolipin SDGD, sulfated diglycodiphytanyl; TGD, triglyco, S_2DGD, bis-sulfated diglycodiphytanyl; S-GL-2, sulfated diglycosyl archaeol phosphoarchaeol; S-TeGD, sulfated tetraglyco; S-TGD-1, sulfated triglyco; Bacterial lipids: SL, sulfonolipid; PC-DAG, phosphatidylcholine-diacylglycerol.

7. Gesamtbetrachtung und Ausblick

Diese Arbeit lässt sich bezüglich der Untersuchung von intakten polaren Lipiden thematisch in drei große Bereiche gliedern: 1. Die chemisch-technischen Aspekte von IPL-Untersuchungen; 2. Die Anpassung der Cytoplasmamembran bei veränderten Umweltbedingungen; 3. IPL als Biomarker für bestimmte Mikroorganismen oder Mikroorganismengruppen.

Die größten Herausforderungen bei der IPL-Analyse sind die sehr unterschiedlichen chemischen Eigenschaften der Zielanalyten, welche hauptsächlich durch die polare Kopfgruppe bestimmt werden. Die chemischen Unterschiede wirken sich zum einen auf die Wiederfindungen bei der säulenchromatographischen Auftrennung von Lipidextrakten aus, zum anderen haben die unterschiedlichen chemischen Eigenschaften von intakten polaren Lipiden auch Auswirkungen auf die Quantifizierung dieser Substanzen. Aus den im Rahmen dieser Arbeit gewonnenen Ergebnissen wird deutlich, dass IPL mit den Kopfgruppen PA, PC, Lyso-PC, PS und PI bei der säulenchromatographischen Auftrennung sehr viel leichter verloren gehen und empfindlicher auf methodische Veränderungen reagieren. Im Gegensatz dazu verhalten sich Cardiolipin (CL) und IPL mit den Kopfgruppen PG, PE, DGDG und GDGT vergleichsweise unproblematisch. Eine Verbesserung der Wiederfindungen und damit eine Reduzierung der Nachweisgrenze lässt sich zwar für einzelne intakte polare Lipide optimieren, dies geschieht allerdings immer auf Kosten der Wiederfindung anderer Lipide. Die Zugabe von Wasser zum Elutionsmittel hat den deutlichsten Effekt auf die Wiederfindung aller Lipide. Der hohe Zeitbedarf für die Aufarbeitung von Proben, die mit Anteilen von Wasser eluiert wurden, ist allerdings als ein starker Nachteil dieser Methode anzusehen. So sollte bei der Verwendung einer säulenchromatographischen Auftrennung immer darüber nachgedacht werden, ob die benötigte Bestimmungsgrenze so gering wie möglich sein muss oder Verluste zu Gunsten einer besseren Handhabbarkeit in Kauf genommen werden können. Wenn Proben mit geringer Matrixbelastung und hoher IPL-Konzentration wie beispielsweise Zellextrakte vorliegen, kann auf eine säulenchromatographische Auftrennung verzichtet werden.

Das Ansprechverhalten von intakten polaren Lipiden bei der Elektrosprayionisierung unterscheidet sich sehr stark. Daher ist für eine präzise Quantifizierung die Verfügbarkeit der zu untersuchenden Lipide als Reinsubstanz notwendig. Bei homologen Reihen wie sie häufig bei IPL bakteriellen Ursprungs vorkommen, kann ein Vertreter der entsprechenden Kopfgruppen eine ausreichend genaue Quantifizierung sicherstellen. Viele der häufig vorkommenden intakten polaren Lipide lassen sich kommerziell erwerben. Die Herstellung eigener Standards von weniger häufig auftretenden intakten polaren Lipiden ist zum einen zeitaufwendig und zum anderen oft durch die nicht ausreichende Verfügbarkeit von Probenmaterial oder durch fehlende Geräteausstattung nur begrenzt möglich.

Wie die Untersuchung des Sulfatreduzierers *Desulfovibrio indonensiensis* zeigt, sind auch ohne absolute Quantifizierung wertvolle Aussagen zu Membranveränderungen möglich. Dabei wirkt sich bei der Untersuchung von Reinkulturen, die bei unterschiedlichen Inkubationsbedingungen kultiviert wurden, die Problematik der fehlenden Standards aufgrund einer relativen Vergleichsmöglichkeit nicht aus. Ebenso ist die Messung von Zeitreihen in einem natürlichen Habitat möglich. Dabei kann ein relativer Vergleich der Messdaten zu einer Bezugsprobe erfolgen. Die Grundvoraussetzung für diese beiden Anwendungen ist jedoch, dass sich nur die relativen Verhältnisse von IPL verändern, das sporadische Auftreten von IPL bei bestimmten Inkubationsbedingungen bzw. Zeitpunkten erschwert oder verhindert diese Vorgehensweise. Bei der Analyse von IPL aus Sedimentkernen oder dem Vergleich von unterschiedlichen Habitaten untereinander führt diese relative Methode zum einen aufgrund von möglicherweise unterschiedlichen IPL-Verteilungen zu keinen sinnvollen Ergebnissen. Zum anderen führt eine unterschiedliche Probenmatrix zu einem veränderten Ansprechverhalten bei der LC-ESI-MS-Analyse. Somit kann die Bestimmung von relativen IPL-Verhältnissen in Sedimenten nicht angewendet werden.

Der zweite Bereich der Arbeit befasst sich mit den Anpassungsstrategien von Mikroorganismen an sich verändernde Umweltbedingungen. Die Forschung auf diesem Gebiet ist bisher im Wesentlichen auf der Basis von Fettsäuren (engl. *phospholipid fatty acid*: PLFA) durchgeführt worden, die durch Hydrolyse von intakten polaren Lipiden freigesetzt wurden (z.B. Rajendran et al., 1995; Green & Scow, 2000; Fang et al., 2003; Webster et al., 2006). Dies hat zu einem eingehenden Verständnis der mikrobiellen Anpassungsmechanismen in Bezug auf den apolaren Molekülteil von intakten polaren Lipiden geführt. Studien, welche die Kopfgruppenvariation von intakten polaren Lipiden in Cytoplasmamembranen als Funktion von Kultivierungsbedingungen wie beispielsweise Wachstumstemperatur, Druck, Verfügbarkeit von Sonnenlicht oder Nährstoffen betrachten, sind lückenhaft und decken nur einen Teil der möglichen Aspekte ab (Pluschke & Overath, 1981; Kaneko et al., 2000; Kaye & Baross, 2004). Die im Rahmen dieser Arbeit an *Dv. indonensiensis* durchgeführte Studie liefert einen weiteren Beitrag auf dem Weg zum besseren Verständnis der umweltbedingten Kopfgruppenvariation in Cytoplasmamembranen von Mikroorganismen. Es wurde gezeigt, dass die Veränderung der Molekülstruktur der an das Glycerolgrundgerüst von intakten polaren Lipiden gebundenen Fettsäurereste in mehreren Stufen verläuft. Bei niedrigen Inkubationstemperaturen werden vom untersuchten Sulfatreduzierer *Dv. indonensiensis* intakte polare Lipide mit verzweigten und unverzweigten, einfachungesättigten Fettsäuren synthetisiert. Bei höheren Inkubationstemperaturen werden anstelle dieser einfachungesättigten eher gesättigte und gleichzeitig verzweigte Fettsäuren in die IPL eingebaut. Bei der höchsten untersuchten Inkubationstemperatur liegen höhere Anteile von gesättigten verzweigten Fettsäuren vor. Die

Veränderung des Sättigungsgrads und der Einbau von Kettenverzweigungen zur Cytoplasmamembrananpassung an niedrige Inkubationstemperaturen waren schon vor der durchgeführten Studie bekannt, ein stufenartiger Wechsel der unpolaren IPL-Seitenketten war jedoch bisher unbekannt.

Im dritten Teil der Arbeit wird untersucht, inwieweit intakte polare Lipide und deren habitatbedingte Verteilungsmuster zur Beschreibung der lebenden mikrobiellen Gemeinschaft in natürlichen Habitaten anwendbar sind. Die grundsätzliche Voraussetzung dafür ist ein schneller Abbau von IPL abgestorbener Organismen, da anderenfalls das aktuelle Signal einer mikrobiellen Gemeinschaft durch fossile IPL verfälscht werden würde. Im Rahmen dieser Arbeit wurde der Abbau von IPL mit Ester- und Etherbindungen in anoxischen Wattsedimenten untersucht und so die Aussagen von zwei älteren Publikationen (White et al., 1979; Harvey et al., 1986) überprüft. Die Untersuchung ergab unter den Versuchsbedingungen einen deutlich schnelleren Abbau von IPL mit Esterbindungen, die repräsentativ für bakterielle und eukaryontische Membranlipide sind. Auch IPL mit Etherbindungen, repräsentativ für archaeelle Membranlipide, wurden über die Dauer des Experiments abgebaut, allerdings deutlich langsamer. Die Bedeutung der Ergebnisse für die Untersuchung der tiefen Biosphäre ist nicht eindeutig. So können die für anoxische Sedimente festgestellten unterschiedlich großen Abbauraten potentiell zu Fehlinterpretationen von IPL-Verteilungen führen. Die Ergebnisse einer zuvor veröffentlichten und auf theoretischen Berechnungen basierenden Studie (Lipp & Hinrichs, 2009) legen aber nahe, dass ein Einfluss der unterschiedlichen Abbauraten nur im oberen Bereich des Sedimentkörpers zu erwarten ist.

Die Vielfalt der in unterschiedlichen Organismen synthetisierten Membranbausteine ist beeindruckend und faszinierend. Ihre Untersuchung bietet im Vergleich zu molekularbiologischen Methoden, die DNA- oder RNA-Bausteine nutzen um die Zusammensetzung von mikrobiologischen Gemeinschaften zu bestimmen, eine komplementäre Methode auf der Basis der direkten molekularen Analyse. Wie die Untersuchung von planktonischen Mikroorganismen im Lake Tyrrell gezeigt hat, eignet sich hier die Lipidanalytik zur Bestimmung von Gemeinschaftsstrukturen in Bezug auf die Verhältnisse von Bakterien und Archaeen. Lake Tyrrell stellt dabei aufgrund einer hohen Biomasse-konzentration in der Wassersäule ein Habitat dar, das in Bezug auf die vorliegenden IPL-Konzentrationen vergleichsweise gut zu untersuchen ist. Aufgrund der oxischen Wassersäule und des dadurch bedingten raschen Abbaus der IPL (Harvey et al., 1986) und der hohen Bioproduktion haben die im Abbauversuch festgestellten unterschiedlichen Abbauraten für ester- und ethergebundene IPL hier nur einen sehr geringen Einfluss auf die ermittelten Ergebnisse.

Wie die Lipiduntersuchungen im Lake Tyrrell belegen, eignet sich die Analyse von intakten polaren Lipiden sehr gut, um bakterielle und archaeelle Anteile einer Gemeinschaft zu bestimmen. Allerdings war es nicht möglich, die vergleichsweise einfach zusammengesetzte Mikroorganismengemeinschaft dieses hypersalinen Sees ohne Hilfe von molekularbiologischen Methoden mit ihren einzelnen Vertretern zu beschreiben. Die Ursache hierfür liegt darin begründet, dass der Großteil der dort lebenden Mikroorganismen mehrere Membranlipide synthetisiert, die sich auch in anderen Mitgliedern der Gemeinschaft finden lassen und sich somit das Spektrum an Lipiden der einzelnen Vertreter überschneidet. Dies steht der Verwendung von IPL-Verteilungsmustern als Biomarker zur Beschreibung der Zusammensetzung von mikrobiellen Gemeinschaften entgegen.

Cytoplasmamembranen sind sich selbst organisierende Struktureinheiten. Die sie konstruierenden Lipide werden über van-der-Waals-Kräfte aneinander gebunden und sind aufgrund des flüssigkristallinen Zustands der Membran frei in ihr beweglich (z.B. Engelman, 1970; Esfahani et al., 1971; Cronan & Gelmann, 1975). Aufgrund der Funktion der Cytoplasmamembran sind intakte polare Lipide in ihr nicht chemisch miteinander verknüpft. Bei DNA- oder RNA-Bausteinen liegt eine solche chemische Verknüpfung vor. Bei der zur Analyse notwendigen Extraktion wird zwangsläufig eine weitere Durchmischung aller IPL einer Probe erzeugt, die es im Nachhinein unmöglich macht, auf die Zusammensetzung der Cytoplasmamembran einzelner mikrobieller Mitglieder der untersuchten Gemeinschaft zurückzuschließen oder solche zu identifizieren. Auch die DNA oder RNA von Mikroorganismen wird bei ihrer Extraktion und Analyse in kleinere Bruchstücke zerlegt. Im Gegensatz zu IPL bleibt aber durch die Abfolge der Nukleinbasen eine umfangreichere Information erhalten, die im Anschluss genutzt werden kann, um Organismen zu identifizieren.

In Bezug auf die Identifizierung von Mikroorganismen aus natürlichen Gemeinschaften ist die Analyse von intakten polaren Lipiden im direkten Vergleich zur molekularbiologischen Analyse der Makromoleküle DNA oder RNA unterlegen. Vor dem Hintergrund der Untersuchung der vergleichsweise wenig diversen Struktur von hypersalinen Mikroorganismengemeinschaften (Euzéby, 2013) wird dies umso deutlicher.

Die Analyse von Phospholipidfettsäuren (PLFA) wird unter anderem kommerziell für die Identifizierung von Bakterien aus Reinkulturen und Böden angeboten (Buyer & Sasser, 2012). Allerdings ist auch diese Methode dadurch limitiert, dass eine umfassende Bibliothek mit PLFA-Verteilungsmustern vorliegen muss um aussagekräftige Ergebnisse liefern zu können. Demgegenüber bietet die Analyse von IPL den grundsätzlichen Vorteil, dass hier im Gegensatz zur PLFA-Analyse die apolaren Seitenketten fest an das Lipidgrundgerüst gebunden sind und die damit verknüpfte Information erhalten bleibt.

Es gibt allerdings einige Mikroorganismen, die sehr spezifische intakte polare Lipide synthetisieren, die nur in wenigen Organismen vorkommen. So wurden Sulfonolipide bisher ausschließlich in den Cytoplasmamembranen der beiden Bakterien *Salinibacter ruber* (Corcelli et al., 2004; Corcelli, 2009) und *Salisaeta longa* (Baronio et al., 2010) gefunden. Aufgrund der unterschiedlichen Anforderungen dieser beiden Bakterien an die Salinität ihres aquatischen Lebensraums (Anton et al., 2002; Vaisman & Oren, 2009) können Sulfonolipide als eindeutige Biomarker für diese beiden Mikroorganismen verwendet werden. So existiert eine ganze Reihe von Membranlipiden, bei deren Vorliegen nicht auf einzelne Organismen, wohl aber auf ein bestimmtes Phylum oder die Familie geschlossen werden kann. Beispiele dafür sind sogenannte bakterielle Tetraether oder auch MBTs (engl. *methyl branched tetraethers*), die von in Böden lebenden Acidobakterien (Sinninghe Damsté et al., 2011) synthetisiert werden. Ladderane können als Biomarker für Vertreter der Familie *Planctomycetaceae* (Strous et al., 1999; Sinninghe Damsté et al., 2005; Boumann et al., 2006) verwendet werden. Die Anwesenheit von Phosphoglycerolipiden mit je einer ether- und estergebundenen Seitenkette deutet auf die Präsenz von bestimmten sulfatreduzierenden Bakterien hin (Rütters et al., 2001). Diese Lipide können daher als Biomarker verwendet werden und erlauben zusätzlich zu Informationen über die Gesamtlipidverteilung der extrahierten Proben eine sehr schnelle und einfache Bestimmung der An- oder Abwesenheit ihrer Quellorganismen. Weiterhin können IPL phototropher Organismen in tieferen Sedimenten als Indiz für Transportprozesse von Porenwasser innerhalb des Sedimentköpers verwendet werden (Seidel et al., 2012).

Diese Arbeit hat den oben aufgeführten Beispielen weitere Biomarkerkandidaten hinzugefügt. So wurde in *Dv. indonensiensis* vier Lipide gefunden (Un-1, Un-2, Un-3 und Un-4), die zuvor noch keinen Eingang in die Literatur gefunden haben. Zwar ist ihre chemische Struktur im Rahmen dieser Arbeit nicht vollständig aufgeklärt worden, dennoch stellen diese Lipide mögliche Kandidaten für neue organismus-, genus- oder familienspezifische Biomarker dar. Die Rolle der ebenfalls erstmalig in dieser Arbeit in hypersalinen Habitaten beschriebenen archaeellen IPL mit den Kopfgruppen PI und PIP konnte nicht geklärt werden. Ein großer Teil der in hypersalinen Habitaten vorkommenden Archaeen ist als Reinkultur verfügbar und in Bezug auf IPL-Gehalt und -Zusammensetzung untersucht worden. Leider wird bei der Beschreibung neuer Stämme die bezogen auf die Detektionsgrenzen und die Unterscheidung von IPL mit strukturell ähnlichen Kopfgruppen wenig sensitive Dünnschichtchromatographie als Standarduntersuchungsmethode für Lipiduntersuchungen verwendet (Tindall et al., 2010). Diese Methode ermöglicht es, mit einfachen analytischen Mitteln die wichtigsten Membranlipide durch den Vergleich mit Retentionszeiten bekannter, in Reinsubstanz vorliegender Membranlipide nach Kopfgruppen zu identifizieren. Durch eine Farbreaktion von funktionellen Gruppen wie z.B. Phosphatresten oder zuckerhaltigen Kopfgruppen mit

bestimmten Reagenzien wird eine zusätzliche Absicherung der Ergebnisse erreicht. Diese Technik allein ist jedoch nicht in der Lage, unbekannte Lipide strukturell aufzuklären, sie quantitativ zu erfassen oder im Detail auf molekularer Ebene zu charakterisieren. Erst durch Kombination mit einer Hydrolyse und anschließender gaschromatographischer Analyse der freigesetzten Fettsäuren können weitere Informationen über die Seitenkettenverteilung innerhalb von IPL einer Kopfgruppe gewonnen werden. Die grundsätzliche Verwendung von LC-ESI-MS-Analysetechniken würde zu einer sehr viel breiteren Datenbasis führen. Die zuvor besprochenen Probleme der Probenaufarbeitung und die zum Teil selektiven Wiederfindungen könnten eine Vergleichbarkeit der ermittelten Datensätze erschweren. Es ist jedoch zu erwarten, dass dieses Vorgehen insgesamt einen deutlichen Vorteil für die Beschreibung und Abgrenzung neuer Reinkulturen und damit auch die Nutzung von IPL zur Charakterisierung von mikrobiellen Gemeinschaften bieten würde.

Es ist also sehr wahrscheinlich, dass viele potentiell als Biomarker geeignete IPL übersehen werden und daher das volle Potential dieser Untersuchungsmethode nicht genutzt wird. Damit wird klar, dass sich die Verwendung von IPL zur Beschreibung von Mikroorganismen-gemeinschaften nicht ausschließlich aufgrund von chemisch-biologischen Faktoren als schwierig darstellt, sondern zusätzlich, wie die Untersuchungen an Lake Tyrrell belegen, die derzeit geltenden Standards zur Beschreibung von Reinkulturen die Erstellung einer umfassenden und detaillierten IPL-Datenbank verhindern.

Derzeit eignet sich die Analyse von intakten polaren Lipiden nicht als unabhängige Methode und kann nur komplementär zu molekularbiologischen Untersuchungsmethoden eingesetzt werden. Dies wird sich womöglich auch zukünftig nicht ändern.

Für die Untersuchung und das Verständnis von mikrobiellen Anpassungsstrategien an sich verändernde Umweltbedingungen ist diese Analysetechnik aber unverzichtbar. Sie ermöglicht tiefe Einblicke in die Funktion von biologischen Membranen, die mit anderen Methoden nicht zugänglich wären.

Literaturverzeichnis

Abe, F., Horikoshi, K., 2001. The biotechnological potential of piezophiles. Trends in Biotechnology 19, 102-108.

Aertsen, A., Van Houdt, R., Vanoirbeek, K., Michiels, C.W., 2004. An SOS Response Induced by High Pressure in *Escherichia coli*. Journal of Bacteriology 186, 6133-6141.

Alazard, D., Dukan, S., Urios, A., Verhe, F., Bouabida, N., Morel, F., Thomas, P., Garcia, J.L., Ollivier, B., 2003. *Desulfovibrio hydrothermalis* sp. nov., a novel sulfate-reducing bacterium isolated from hydrothermal vents. International Journal of Systematic and Evolutionary Microbiology 53, 173-178.

Albuquerque, L., Taborda, M., La Cono, V., Yakimov, M., da Costa, M.S., 2012. Natrinema salaciae sp. nov., a halophilic archaeon isolated from the deep, hypersaline anoxic Lake Medee in the Eastern Mediterranean Sea. Systematic and Applied Microbiology 35, 368-373.

Allen, E.E., Facciotti, D., Bartlett, D.H., 1999. Monounsaturated but not polyunsaturated fatty acids are required for growth of the deep-sea bacterium *Photobacterium profundum* SS9 at high pressure and low temperature. Applied and Environmental Microbiology 65, 1710-1720.

Allen, M.A., Goh, F., Leuko, S., Echigo, A., Mizuki, T., Usami, R., Kamekura, M., Neilan, B.A., Burns, B.P., 2008. *Haloferax elongans* sp. nov. and *Haloferax mucosum* sp. nov., isolated from microbial mats from Hamelin Pool, Shark Bay, Australia. International Journal of Systematic and Evolutionary Microbiology 58, 798-802.

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J.H., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research 25, 3389-3402.

Amann, R., Fuchs, B.M., 2008. Single-cell identification in microbial communities by improved fluorescence in situ hybridization techniques. Nature Reviews Microbiology 6, 339-348.

Amann, R.I., Ludwig, W., Schleifer, K.H., 1995. Phylogenetic identification and in-situ detection of individual microbial cells without cultivation. Microbiological Reviews 59, 143-169.

Amann, R.I., Zarda, B., Stahl, D.A., Schleifer, K.H., 1992. Identification of individual prokaryotic cells by using enzyme-labeled, ribosomal-RNA-targeted oligonucleotide probes. Applied and Environmental Microbiology 58, 3007-3011.

Amoozegar, M.A., Makhdoumi-Kakhki, A., Shahzadeh Fazeli, S.A., Azarbaijani, R., Ventosa, A., 2012. *Halopenitus persicus* gen. nov., sp. nov., an archaeon from an inland salt lake. International Journal of Systematic and Evolutionary Microbiology 62, 1932-1936.

Anton, J., Llobet-Brossa, E., Rodriguez-Valera, F., Amann, R., 1999. Fluorescence in situ hybridization analysis of the prokaryotic community inhabiting crystallizer ponds. Environmental Microbiology 1, 517-523.

Anton, J., Oren, A., Benlloch, S., Rodriguez-Valera, F., Amann, R., Rossello-Mora, R., 2002. *Salinibacter ruber* gen. nov., sp nov., a novel, extremely halophilic member of the Bacteria from saltern crystallizer ponds. International Journal of Systematic and Evolutionary Microbiology 52, 485-491.

Anton, J., Rossello-Mora, R., Rodriguez-Valera, F., Amann, R., 2000. Extremely halophilic Bacteria in crystallizer ponds from solar salterns. Applied and Environmental Microbiology 66, 3052-3057.

Antunes, A., Taborda, M., Huber, R., Moissl, C., Nobre, M.F., da Costa, M.S., 2008. *Halorhabdus tiamatea* sp. nov., a non-pigmented, extremely halophilic archaeon from a deep-sea, hypersaline anoxic basin of the Red Sea, and emended description of the genus *Halorhabdus*. International Journal of Systematic and Evolutionary Microbiology 58, 215-220.

Aries, E., Doumenq, P., Artaud, J., Molinet, J., Bertrand, J.C., 2001. Occurrence of fatty acids linked to non-phospholipid compounds in the polar fraction of a marine sedimentary extract from Carteau cove, France. Organic Geochemistry 32, 193-197.

Asker, D., Ohta, Y., 2002. *Haloferax alexandrinus* sp. nov., an extremely halophilic canthaxanthin-producing archaeon from a solar saltern in Alexandria (Egypt). International Journal of Systematic and Evolutionary Microbiology 52, 729-38.

Bale, S.J., Goodman, K., Rochelle, P.A., Marchesi, J.R., Fry, J.C., Weightman, A.J., Parkes, R.J., 1997. *Desulfovibrio profundus* sp. nov, a novel barophilic sulfate-reducing bacterium from deep sediment layers in the Japan Sea. International Journal of Systematic Bacteriology 47, 515-521.

Bardavid, R.E., Khristo, P., Oren, A., 2008. Interrelationships between *Dunaliella*; and halophilic prokaryotes in saltern crystallizer ponds. Extremophiles 12, 5.

Bardavid, R.E., Mana, L., Oren, A., 2007. Haloplanus natans gen. nov., sp. nov., an extremely halophilic, gas-vacuolate archaeon isolated from Dead Sea–Red Sea water mixtures in experimental outdoor ponds. International Journal of Systematic and Evolutionary Microbiology 57, 780-783.

Baronio, M., Lattanzio, V.M.T., Vaisman, N., Oren, A., Corcelli, A., 2010. The acylhalocapnines of halophilic bacteria: structural details of unusual sulfonate sphingoids. Journal of Lipid Research 51, 1878-1885.

Bartlett, D.H., 2002. Pressure effects on in vivo microbial processes. Biochimica et Biophysica Acta 1595, 367-381.

Beck, M., Riedel, T., Graue, J., Köster, J., Kowalski, N., Wu, C.S., Wegener, G., Lipsewers, Y., Freund, H., Böttcher, M.E., Brumsack, H.J., Cypionka, H., Rullkötter, J., Engelen, B., 2011. Imprint of past and present environmental conditions on microbiology and biogeochemistry of coastal Quaternary sediments. Biogeosciences 8, 55-68.

Benning, C., 1998. Biosynthesis and function of the sulfolipid sulfoquinovosyl diacylglycerol. Annual Review of Plant Physiology and Plant Molecular Biology 49, 53-75.

Beyer, H.a.W., W, 1991. Lehrbuch der Organischen Chemie. S. Hirzel Verlag, Stuttgart.

Biddle, J.F., Lipp, J.S., Lever, M.A., Lloyd, K.G., Sorensen, K.B., Anderson, R., Fredricks, H.F., Elvert, M., Kelly, T.J., Schrag, D.P., Sogin, M.L., Brenchley, J.E., Teske, A., House, C.H., Hinrichs, K.U., 2006. Heterotrophic archaea dominate sedimentary subsurface ecosystems off Peru. Proceedings of the National Academy of Sciences of the United States of America 103, 3846-3851.

Bidle, K.A., Bartlett, D.H., 1999. RecD function is required for high-pressure growth of a deep-sea bacterium. Journal of Bacteriology 181, 2330-2337.

Black, G.E., Snyder, A.P., Heroux, K.S., 1997. Chemotaxonomic differentiation between the *Bacillus cereus* group and *Bacillus subtilis* by phospholipid extracts analyzed with electrospray ionization tandem mass spectrometry. Journal of Microbiological Methods 28, 187.

Bodennec, J., Pelled, D., Futerman, A.H., 2003. Aminopropyl solid phase extraction and 2 D TLC of neutral glycosphingolipids and neutral lysoglycosphingolipids. Journal of Lipid Research 44, 218-226.

Boschker, H.T.S., Nold, S.C., Wellsbury, P., Bos, D., de Graaf, W., Pel, R., Parkes, R.J., Cappenberg, T.E., 1998. Direct linking of microbial populations to specific biogeochemical processes by C-13-labelling of biomarkers. Nature 392, 801-805.

Böttcher, C., von Roepenack-Lahaye, E., Willscher, E., Scheel, D., Clemens, S., 2007. Evaluation of matrix effects in metabolite profiling based on capillary liquid chromatography electrospray ionization quadrupole time-of-flight mass spectrometry. Analytical Chemistry 79, 1507-1513.

Boumann, H.A., Hopmans, E.C., van de Leemput, I., Op den Camp, H.J.M., van de Vossenberg, J., Strous, M., Jetten, M.S.M., Damste, J.S.S., Schouten, S., 2006. Ladderane phospholipids in anammox bacteria comprise phosphocholine and phosphoethanolamine headgroups. FEMS Microbiology Letters 258, 297-304.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72, 248-254.

Bright-Gaertner, E., Proulx, P., 1972. Metabolism of phosphoglycerides in *Escherichia coli* during growth at 37 °C and during a cold-induced lag phase. Biochimica et Biophysica Acta - Lipids and Lipid Metabolism 270, 40-49.

Burns, D.G., Camakaris, H.M., Janssen, P.H., Dyall-Smith, A.L., 2004. Combined use of cultivation-dependent and cultivation-independent methods indicates that members of most haloarchaeal groups in an Australian crystallizer pond are cultivable. Applied and Environmental Microbiology 70, 5258-5265.

Burns, D.G., Janssen, P.H., Itoh, T., Kamekura, M., Echigo, A., Dyall-Smith, M.L., 2010a. *Halonotius pteroides* gen. nov., sp. nov., an extremely halophilic archaeon recovered from a saltern crystallizer. International Journal of Systematic and Evolutionary Microbiology 60, 1196-1199.

Burns, D.G., Janssen, P.H., Itoh, T., Kamekura, M., Li, Z., Jensen, G., Rodriguez-Valera, F., Bolhuis, H., Dyall-Smith, M.L., 2007. *Haloquadratum walsbyi* gen. nov., sp nov., the square haloarchaeon of Walsby, isolated from saltern crystallizers in Australia and Spain. International Journal of Systematic and Evolutionary Microbiology 57, 387-392.

Burns, D.G., Janssen, P.H., Itoh, T., Minegishi, H., Usami, R., Kamekura, M., Dyall-Smith, M.L., 2010b. *Natronomonas moolapensis* sp. nov., non-alkaliphilic isolates recovered from a solar saltern crystallizer pond, and emended description of the genus *Natronomonas*. International Journal of Systematic and Evolutionary Microbiology 60, 1173-1176.

Buyer, J.S., Sasser, M., 2012. High throughput phospholipid fatty acid analysis of soils. Applied Soil Ecology 61, 127-130.

Campbell, N.A., Reece, J.B., 2003. Biologie. Spektrum Akademischer Verlag, Heidelberg, Berlin.

Castillo, A.M., Gutiérrez, M.C., Kamekura, M., Ma, Y., Cowan, D.A., Jones, B.E., Grant, W.D., Ventosa, A., 2006a. *Halovivax asiaticus* gen. nov., sp. nov., a novel extremely halophilic archaeon isolated from Inner Mongolia, China. International Journal of Systematic and Evolutionary Microbiology 56, 765-770.

Castillo, A.M., Gutiérrez, M.C., Kamekura, M., Xue, Y., Ma, Y., Cowan, D.A., Jones, B.E., Grant, W.D., Ventosa, A., 2006b. *Halorubrum orientale* sp. nov., a halophilic archaeon isolated from Lake Ejinor, Inner Mongolia, China. International Journal of Systematic and Evolutionary Microbiology 56, 2559-2563.

Castillo, A.M., Gutiérrez, M.C., Kamekura, M., Xue, Y., Ma, Y., Cowan, D.A., Jones, B.E., Grant, W.D., Ventosa, A., 2006c. *Halostagnicola larsenii* gen. nov., sp. nov., an extremely halophilic archaeon from a saline lake in Inner Mongolia, China. International Journal of Systematic and Evolutionary Microbiology 56, 1519-1524.

Castillo, A.M., Gutiérrez, M.C., Kamekura, M., Xue, Y., Ma, Y., Cowan, D.A., Jones, B.E., Grant, W.D., Ventosa, A., 2006d. *Natrinema ejinorense* sp. nov., isolated from a saline lake in Inner Mongolia, China. International Journal of Systematic and Evolutionary Microbiology 56, 2683-2687.

Castillo, A.M., Gutiérrez, M.C., Kamekura, M., Xue, Y., Ma, Y., Cowan, D.A., Jones, B.E., Grant, W.D., Ventosa, A., 2007a. *Halorubrum ejinorense* sp. nov., isolated from Lake Ejinor, Inner Mongolia, China. International Journal of Systematic and Evolutionary Microbiology 57, 2538-2542.

Castillo, A.M., Gutiérrez, M.C., Kamekura, M., Xue, Y., Ma, Y., Cowan, D.A., Jones, B.E., Grant, W.D., Ventosa, A., 2007b. *Halovivax ruber* sp. nov., an extremely halophilic archaeon isolated from Lake Xilinhot, Inner Mongolia, China. International Journal of Systematic and Evolutionary Microbiology 57, 1024-1027.

Cedergren, R.A., Hollingsworth, R.I., 1994. Occurrence of sulfoquinovosyl diacylglycerol in some members of the family *Rhizobiaceae*. Journal of Lipid Research 35, 1452-1461.

Chambers, E., Wagrowski-Diehl, D.M., Lu, Z., Mazzeo, J.R., 2007. Systematic and comprehensive strategy for reducing matrix effects in LC/MS/MS analyses. Journal of Chromatography B 852, 22.

Chang, Y.-Y., Cronan, J.E., 1999. Membrane cyclopropane fatty acid content is a major factor in acid resistance of *Escherichia coli*. Molecular Microbiology 33, 249-259.

Chong, P.L.G., Cossins, A.R., 1983. A differential polarized phase fluorometric study of the effects of high hydrostatic pressure upon the fluidity of cellular membranes. Biochemistry 22, 409-415.

Choquet, C.G., Patel, G.B., Sprott, G.D., Beveridge, T.J., 1994. Stability of pressure-extruded liposomes made from archaeobacterial ether lipids. Applied Microbiology and Biotechnology 42, 375-384.

Chowdhury, T.R., Dick, R.P., 2012. Standardizing methylation method during phospholipid fatty acid analysis to profile soil microbial communities. Journal of Microbiological Methods 88, 285-291.

Cohen, S., Oren, A., Shilo, M., 1983. The divalent cation requirement of Dead Sea halobacteria. Archives of Microbiology 136, 184-190.

Coolen, M.J.L., Overmann, J., 1998. Analysis of subfossil molecular remains of purple sulfur bacteria in a lake sediment. Applied and Environmental Microbiology 64, 4513-4521.

Cooper, R.A., 1978. Influence of increased membrane cholesterol on membrane fluidity and cell function in human red blood cells. Journal of Supramolecular Structure 8, 413-430.

Corcelli, A., 2009. The cardiolipin analogues of *Archaea*. Biochimica et Biophysica Acta - Biomembranes 1788, 2101-2106.

Corcelli, A., Lattanzio, V.M.T., Mascolo, G., Babudri, F., Oren, A., Kates, M., 2004. Novel Sulfonolipid in the Extremely Halophilic Bacterium *Salinibacter ruber*. Applied and Environmental Microbiology 70, 6678-6685.

Cord-Ruwisch, R., 1985. A quick method for the determination of dissolved and precipitated sulfides in cultures of sulfate-reducing bacteria. Journal of Microbiological Methods 4, 33-36.

Cronan, J.E., Gelmann, E.P., 1975. Physical properties of membrane lipids - biological relevance and regulation. Bacteriological Reviews 39, 232-256.

Cui, H.-L., Gao, X., Li, X.-Y., Xu, X.-W., Zhou, Y.-G., Liu, H.-C., Zhou, P.-J., 2010a. *Haloplanus vescus* sp. nov., an extremely halophilic archaeon from a marine solar saltern, and emended description of the genus *Haloplanus*. International Journal of Systematic and Evolutionary Microbiology 60, 1824-1827.

Cui, H.-L., Gao, X., Li, X.-Y., Xu, X.-W., Zhou, Y.-G., Liu, H.-C., Zhou, P.-J., 2010b. *Halosarcina limi* sp. nov., a halophilic archaeon from a marine solar saltern, and emended description of the genus *Halosarcina*. International Journal of Systematic and Evolutionary Microbiology 60, 2462-3466.

Cui, H.-L., Gao, X., Sun, F.-F., Dong, Y., Xu, X.-W., Zhou, Y.-G., Liu, H.-C., Oren, A., Zhou, P.-J., 2010c. *Halogranum rubrum* gen. nov., sp. nov., a halophilic archaeon isolated from a marine solar saltern. International Journal of Systematic and Evolutionary Microbiology 60, 1366-1371.

Cui, H.-L., Gao, X., Yang, X., Xu, X.-W., 2010d. *Halorussus rarus* gen. nov., sp. nov., a new member of the family Halobacteriaceae isolated from a marine solar saltern. Extremophiles 14, 493-499.

Cui, H.-L., Gao, X., Yang, X., Xu, X.-W., 2011a. *Halolamina pelagica* gen. nov., sp. nov., a new member of the family Halobacteriaceae. International Journal of Systematic and Evolutionary Microbiology 61, 1617-1621.

Cui, H.-L., Gao, X., Yang, X., Xu, X.-W., 2011b. *Haloplanus aerogenes* sp. nov., an extremely halophilic archaeon from a marine solar saltern. International Journal of Systematic and Evolutionary Microbiology 61, 965-968.

Cui, H.-L., Li, X.-Y., Gao, X., Xu, X.-W., Zhou, Y.-G., Liu, H.-C., Oren, A., Zhou, P.-J., 2010e. *Halopelagius inordinatus* gen. nov., sp. nov., a new member of the family Halobacteriaceae isolated from a marine solar saltern. International Journal of Systematic and Evolutionary Microbiology 60, 2089-2093.

Cui, H.-L., Lin, Z.-Y., Dong, Y., Zhou, P.-J., Liu, S.-J., 2007a. *Halorubrum litoreum* sp. nov., an extremely halophilic archaeon from a solar saltern. International Journal of Systematic and Evolutionary Microbiology 57, 2204-2206.

Cui, H.-L., Sun, F.-F., Gao, X., Dong, Y., Xu, X.-W., Zhou, Y.-G., Liu, H.-C., Oren, A., Zhou, P.-J., 2010f. *Haladaptatus litoreus* sp. nov., an extremely halophilic archaeon from a marine solar saltern, and emended description of the genus *Haladaptatus*. International Journal of Systematic and Evolutionary Microbiology 60, 1085-1089.

Cui, H.-L., Tohty, D., Feng, J., Zhou, P.-J., Liu, S.-J., 2006a. *Natronorubrum aibiense* sp. nov., an extremely halophilic archaeon isolated from Aibi salt lake in Xin-Jiang, China, and emended description of the genus *Natronorubrum*. International Journal of Systematic and Evolutionary Microbiology 56, 1515-1517.

Cui, H.-L., Tohty, D., Liu, H.-C., Liu, S.-J., Oren, A., Zhou, P.-J., 2007b. *Natronorubrum sulfidifaciens* sp. nov., an extremely haloalkaliphilic archaeon isolated from Aiding salt lake in Xin-Jiang, China. International Journal of Systematic and Evolutionary Microbiology 57, 738-740.

Cui, H.-L., Tohty, D., Zhou, P.-J., Liu, S.-J., 2006b. *Halorubrum lipolyticum* sp. nov. and *Halorubrum aidingense* sp. nov., isolated from two salt lakes in Xin-Jiang, China. International Journal of Systematic and Evolutionary Microbiology 56, 1631-1634.

Cui, H.-L., Tohty, D., Zhou, P.-J., Liu, S.-J., 2006c. *Haloterrigena longa* sp. nov. and *Haloterrigena limicola* sp. nov., extremely halophilic archaea isolated from a salt lake. International Journal of Systematic and Evolutionary Microbiology 56, 1837-1840.

Cui, H.-L., Yang, X., Gao, X., Li, X.-Y., Xu, X.-W., Zhou, Y.-G., Liu, H.-C., Zhou, P.-J., 2010g. *Halogeometricum rufum* sp. nov., a halophilic archaeon from a marine solar saltern, and emended description of the genus *Halogeometricum*. International Journal of Systematic and Evolutionary Microbiology 60, 2613-2617.

Cui, H.-L., Yang, X., Gao, X., Xu, X.-W., 2011c. *Halobellus clavatus* gen. nov., sp. nov. and *Halorientalis regularis* gen. nov., sp. nov., two new members of the family *Halobacteriaceae*. International Journal of Systematic and Evolutionary Microbiology 61, 2682-2689.

Cui, H.-L., Yang, X., Gao, X., Xu, X.-W., 2011d. *Halogranum gelatinilyticum* sp. nov. and *Halogranum amylolyticum* sp. nov., isolated from a marine solar saltern, and emended description of the genus *Halogranum*. International Journal of Systematic and Evolutionary Microbiology 61, 911-915.

Cui, H.-L., Yang, X., Zhou, Y.-G., Liu, H.-C., Zhou, P.-J., Dyall-Smith, M.L., 2012. *Halobellus limi* sp. nov. and *Halobellus salinus* sp. nov., isolated from two marine solar salterns. International Journal of Systematic and Evolutionary Microbiology 62, 1307-1313.

Cypionka, H., 2006. Grundlagen der Mikrobiologie. Springer Verlag, Berlin, Heidelberg, New York.

D'Hondt, S., Jorgensen, B.B., Miller, D.J., Batzke, A., Blake, R., Cragg, B.A., Cypionka, H., Dickens, G.R., Ferdelman, T., Hinrichs, K.U., Holm, N.G., Mitterer, R., Spivack, A., Wang, G.Z., Bekins, B., Engelen, B., Ford, K., Gettemy, G., Rutherford, S.D., Sass, H., Skilbeck, C.G., Aiello, I.W., Guerin, G., House, C.H., Inagaki, F., Meister, P., Naehr, T., Niitsuma, S., Parkes, R.J., Schippers, A., Smith, D.C., Teske, A., Wiegel, J., Padilla, C.N., Acosta, J.L.S., 2004. Distributions of microbial activities in deep subseafloor sediments. Science 306, 2216-2221.

Daiyasu, H., Kuma, K.-I., Yokoi, T., Morii, H., Koga, Y., Toh, H., 2005. A study of archaeal enzymes involved in polar lipid synthesis linking amino acid sequence information, genomic contexts and lipid composition. Archaea 1, 399-410.

De Mendoza, D., Cronan Jr, J.E., 1983. Thermal regulation of membrane lipid fluidity in bacteria. Trends in Biochemical Sciences 8, 49-52.

De Rosa, M., Gambacorta, A., Gliozzi, A., 1986. Structure, biosynthesis, and physicochemical properties of archaebacterial lipids. Microbiology and Molecular Biology Reviews 50, 70-80.

De Rosa, M., Gambacorta, A., Nicolaus, B., Ross, H.N.M., Grant, W.D., Bu'Lock, J.D., 1982. An asymmetric archaebacterial diether lipid from alkaliphilic halophiles. Journal of General Microbiology 128, 343-348.

Dedysh, S.N., Kulichevskaya, I.S., Serkebaeva, Y.M., Mityaeva, M.A., Sorokin, V.V., Suzina, N.E., Rijpstra, W.I.C., Sinninghe Damsté, J.S., 2012. *Bryocella elongata* gen. nov., sp. nov., a member of subdivision 1 of the Acidobacteria isolated from a methanotrophic enrichment culture, and emended description of Edaphobacter aggregans Koch et al. 2008. International Journal 0f Systematic and Evolutionary Microbiology 62, 654-664.

Dees, C., Shively, J.M., 1982. Localization of quantitation of the ornithine lipid of *Thiobacillus thiooxidans*. Journal of Bacteriology 149, 798-799.

Dell'Anno, A., Fabiano, M., Duineveld, G.C.A., Kok, A., Danovaro, R., 1998. Nucleic Acid (DNA, RNA) Quantification and RNA/DNA Ratio Determination in Marine Sediments: Comparison of Spectrophotometric, Fluorometric, and HighPerformance Liquid Chromatography Methods and Estimation of Detrital DNA. Applied and Environmental Microbiology 64, 3238-3245.

DeLong, E., Wickham, G., Pace, N., 1989. Phylogenetic stains: ribosomal RNA-based probes for the identification of single cells. Science 243, 1360-1363.

DeLong, E., Yayanos, A., 1985. Adaptation of the membrane lipids of a deep-sea bacterium to changes in hydrostatic pressure. Science 228, 1101-1103.

Delong, E.F., Yayanos, A.A., 1986. Biochemical function and ecological significance of novel bacterial lipids in deep-sea prokaryotes. Applied and Environmental Microbiology 51, 730-737.

Dembitsky, V.M., 1996. Betaine ether-linked glycerolipids: chemistry and biology. Progress in Lipid Research 35, 1-51.

Denner, E.B.M., McGenity, T.J., Busse, H.-J., Grant, W.D., Wanner, G., Stan-Lotter, H., 1994. *Halococcus salifodinae* sp. nov., an Archaeal Isolate from an Austrian Salt Mine. International Journal of Systematic Bacteriology 44, 774-780.

Dowhan, W., 1997. Molecular basis for membrane phospholipid diversity: Why are there so many lipids? Annual Review of Biochemistry 66, 199-232.

Elshahed, M.S., Savage, K.N., Oren, A., Gutierrez, M.C., Ventosa, A., Krumholz, L.R., 2004. *Haloferax sulfurifontis* sp. nov., a halophilic archaeon isolated from a sulfide- and sulfur-rich spring. International Journal of Systematic and Evolutionary Microbiology 54, 2275-2279.

Enache, M., Itoh, T., Kamekura, M., Teodosiu, G., Dumitru, L., 2007. *Haloferax prahovense* sp. nov., an extremely halophilic archaeon isolated from a Romanian salt lake. International Journal of Systematic and Evolutionary Microbiology 57, 393-397.

Engelen, B., Ziegelmueller, K., Wolf, L., Kopke, B., Gittel, A., Cypionka, H., Treude, T., Nakagawa, S., Inagaki, F., Lever, M.A., Steinsbu, B.O., 2008. Fluids from the oceanic crust support microbial activities within the deep biosphere. Geomicrobiology Journal 25, 56-66.

Engelman, D.M., 1970. X-ray diffraction studies of phase transitions in the membrane of *Mycoplasma laidlawii*. Journal of Molecular Biology 47, 115-117.

Ertefai, T.F., Fisher, M.C., Fredricks, H.F., Lipp, J.S., Pearson, A., Birgel, D., Udert, K.M., Cavanaugh, C.M., Gschwend, P.M., Hinrichs, K.U., 2008. Vertical distribution of microbial lipids and functional genes in chemically distinct layers of a highly polluted meromictic lake. Organic Geochemistry 39, 1572-1588.

Esfahani, M., Limbrick, A.R., Knutton, S., Oka, T., Wakil, S.J., 1971. The Molecular Organization of Lipids in the Membrane of *Escherichia coli*: Phase Transitions. Proceedings of the National Academy of Sciences of the United States of America 68, 3180-3184.

Euzéby, J.P., 2013. List of Prokaryotic names with Standing in Nomenclature. Society for Systematic and Veterinary Bacteriology.

Expedition 301 Scientists, 2005. Site U1301, In: A.T. Fisher, T. Urabe, A. Klaus, Expedition 301 Scientists (Eds.), *Procedings. IODP, 301*. College Station TX (Integrated Ocean Drilling Program Management International, Inc.) doi:10.2204/iodp.proc.301.106.2005pp.

Fan, H., Xue, Y., Ma, Y., Ventosa, A., Grant, W.D., 2004. *Halorubrum tibetense* sp. nov., a novel haloalkaliphilic archaeon from Lake Zabuye in Tibet, China. International Journal of Systematic and Evolutionary Microbiology 54, 1213-1216.

Fang, J., Barcelona, M.J., 1998. Structural determination and quantitative analysis of bacterial phospholipids using liquid chromatography/electrospray ionization/mass spectrometry. Journal of Microbiological Methods 33, 23.

Fang, J.S., Chan, O.V., Kato, C., Sato, T., Peeples, T., Niggemeyer, K., 2003. Phospholipid FA of piezophilic bacteria from the deep sea. Lipids 38, 885-887.

Feio, M.J., Beech, I.B., Carepo, M., Lopes, J.M., Cheung, C.W.S., Franco, R., Guezennec, J., Smith, J.R., Mitchell, J.I., Moura, J.J.G., Lino, A.R., 1998. Isolation and characterisation of a novel sulphate-reducing bacterium of the *Desulfovibrio* genus. Anaerobe 4, 117-130.

Feng, J., Zhou, P.-J., Liu, S.-J., 2004. *Halorubrum xinjiangense* sp. nov., a novel halophile isolated from saline lakes in China. International Journal of Systematic and Evolutionary Microbiology 54, 1789-1791.

Feng, J., Zhou, P., Zhou, Y.-G., Liu, S.-J., Warren-Rhodes, K., 2005. *Halorubrum alkaliphilum* sp. nov., a novel haloalkaliphile isolated from a soda lake in Xinjiang, China. International Journal of Systematic and Evolutionary Microbiology 55, 149-152.

Fichtel, K., Mathes, F., Könneke, M., Cypionka, H., Engelen, B., 2012. Isolation of sulfate-reducing bacteria from sediments above the deep-subseafloor aquifer. Frontiers in Microbiology 3, 1-12.

Geiger, O., Gonzalez-Silva, N., Lopez-Lara, I.M., Sohlenkamp, C., 2010. Amino acid-containing membrane lipids in bacteria. Progress in Lipid Research 49, 46-60.

Geiger, O., Röhrs, V., Weissenmayer, B., Finan, T., Thomas-Oates, J., 1999. The regulator gene phoB mediates phosphate stress-controlled synthesis of the membrane lipid diacylglyceryl-*N*,*N*,*N*-trimethylhomoserine in *Rhizobium* (*Sinorhizobium*) *meliloti*. Molecular Microbiology 32, 63-73.

Giovannoni, S., Stingl, U., 2007. The importance of culturing bacterioplankton in the 'omics' age. Nature Reviews Microbiology 5, 820-826.

Goh, F., Leuko, S., Allen, M.A., Bowman, J.P., Kamekura, M., Neilan, B.A., Burns, B.P., 2006. *Halococcus hamelinensis* sp. nov., a novel halophilic archaeon isolated from stromatolites in Shark Bay, Australia. International Journal of Systematic and Evolutionary Microbiology 56, 1323-1329.

Graue, J., Engelen, B., Cypionka, H., 2011. Degradation of cyanobacterial biomass in anoxic tidal-flat sediments: a microcosm study of metabolic processes and community changes. ISME Journal.

Green, C.T., Scow, K.M., 2000. Analysis of phospholipid fatty acids (PLFA) to characterize microbial communities in aquifers. Hydrogeology Journal 8, 126-141.

Griffiths, R.I., Whiteley, A.S., O'Donnell, A.G., Bailey, M.J., 2000. Rapid method for coextraction of DNA and RNA from natural environments for analysis of ribosomal DNA- and rRNA-based microbial community composition. Applied and Environmental Microbiology 66, 5488-5491.

Grogan, D.W., Cronan, J.E., 1997. Cyclopropane ring formation in membrane lipids of bacteria. Microbiology and Molecular Biology Reviews 61, 429-&.

Gruber, C., Legat, A., Pfaffenhuemer, M., Radax, C., Weidler, G., Busse, H.J., Stan-Lotter, H., 2004. *Halobacterium noricense* sp nov., an archaeal isolate from a bore core of an alpine Permian salt deposit, classification of *Halobacterium* sp NRC-1 as a strain of *H. salinarum* and emended description of *H. salinarum*. Extremophiles 8, 431-439.

Guckert, J.B., Antworth, C.P., Nichols, P.D., White, D.C., 1985. Phospholipid, ester-linked fatty-acid profiles as reproducible assays for changes in prokaryotic community structure of estuarine sediments. FEMS Microbiology Ecology 31, 147-158.

Gutiérrez, C.M., Kamekura, M., Holmes, M.L., Dyall-Smith, M.L., Ventosa, A., 2002. Taxonomic characterization of *Haloferax* sp. ("*H. alicantei*") strain Aa 2.2: description of *Haloferax lucentensis* sp. nov. Extremophiles 6, 479-483.

Gutiérrez, M.C., Castillo, A.M., Corral, P., Kamekura, M., Ventosa, A., 2011. *Halorubrum aquaticum* sp. nov., an archaeon isolated from hypersaline lakes. International Journal of Systematic and Evolutionary Microbiology 61, 1144-1148.

Gutiérrez, M.C., Castillo, A.M., Corral, P., Minegishi, H., Ventosa, A., 2010. *Natronorubrum sediminis* sp. nov., an archaeon isolated from a saline lake. International Journal of Systematic and Evolutionary Microbiology 60, 1802-1806.

Gutiérrez, M.C., Castillo, A.M., Kamekura, M., Ventosa, A., 2008a. *Haloterrigena salina* sp. nov., an extremely halophilic archaeon isolated from a salt lake. International Journal of Systematic and Evolutionary Microbiology 58, 2880-2884.

Gutiérrez, M.C., Castillo, A.M., Kamekura, M., Xue, Y., Ma, Y., Cowan, D.A., Jones, B.E., Grant, W.D., Ventosa, A., 2007. *Halopiger xanaduensis* gen. nov., sp. nov., an extremely halophilic archaeon isolated from saline Lake Shangmatala in Inner Mongolia, China. International Journal of Systematic and Evolutionary Microbiology 57, 1402-1407.

Gutiérrez, M.C., Castillo, A.M., Pagaling, E., Heaphy, S., Kamekura, M., Xue, Y., Ma, Y., Cowan, D.A., Jones, B.E., Grant, W.D., Ventosa, A., 2008b. *Halorubrum kocurii* sp. nov., an archaeon isolated from a saline lake. International Journal of Systematic and Evolutionary Microbiology 58, 2031-2035.

Hamamoto, T., Takata, N., Kudo, T., Horikoshi, K., 1994. Effect of temperature and growth phase on fatty acid composition of the psychrophilic *Vibrio* sp. strain no. 5710. FEMS Microbiology Letters 119, 77-81.

Harvey, H.R., Fallon, R.D., Patton, J.S., 1986. The effect of organic-matter and oxygen on the degradation of bacterial-membrane lipids in marine-sediments. Geochimica et Cosmochimica Acta 50, 795-804.

Heberling, C., Lowell, R.P., Liu, L., Fisk, M.R., 2010. Extent of the microbial biosphere in the oceanic crust. Geochemistry Geophysics Geosystems 11, 1-15.

Heidelberg, K.B., Nelson, W.C., Holm, J.B., Eisenkolb, N., Andrade, K., Emerson, J.B., 2013. Characterization of eukaryotic microbial diversity in hypersaline Lake Tyrrell, Australia. Frontiers in Microbiology 4.

Herczeg, A.L., Barnes, C.J., Macumber, P.G., Olley, J.M., 1992. A stable isotope investigation of groundwater-surface water interactions at Lake Tyrrell, Victoria, Australia. Chemical Geology 96, 19-32.

Herrmann, A.G., Knake, D., Schneider, J., Peters, H., 1973. Geochemistry of modern seawater and brines from salt pans: Main components and bromine distribution. Contributions to Mineralogy and Petrology 40, 1-24.

Hezayen, F.F., Gutiérrez, M.C., Steinbüchel, A., Tindall, B.J., Rehm, B.H.A., 2010. *Halopiger aswanensis* sp. nov., a polymer-producing and extremely halophilic archaeon isolated from hypersaline soil. International Journal of Systematic and Evolutionary Microbiology 60, 633-637.

Hezayen, F.F., Rehm, B.H., Tindall, B.J., Steinbüchel, A., 2001. Transfer of *Natrialba asiatica* B1T to *Natrialba taiwanensis* sp. nov. and description of *Natrialba aegyptiaca* sp. nov., a novel extremely halophilic, aerobic, non-pigmented member of the Archaea from Egypt that produces extracellular poly(glutamic acid). International Journal of Systematic and Evolutionary Microbiology 51, 1133-42.

Hezayen, F.F., Tindall, B.J., Steinbüchel, A., Rehm, B.H.A., 2002. Characterization of a novel halophilic archaeon, *Halobiforma haloterrestris* gen. nov., sp. nov., and transfer of *Natronobacterium nitratireducens* to *Halobiforma nitratireducens* comb. nov. International Journal of Systematic and Evolutionary Microbiology 52, 2271-80.

Hinrichs, K.U., Hayes, J.M., Sylva, S.P., Brewer, P.G., DeLong, E.F., 1999. Methane-consuming archaebacteria in marine sediments. Nature 398, 802-805.

Hölzl, G., Dörmann, P., 2007. Structure and function of glycoglycerolipids in plants and bacteria. Progress in Lipid Research 46, 225.

Hopmans, E.C., Weijers, J.W.H., Schefuss, E., Herfort, L., Sinninghe Damsté, J.S., Schouten, S., 2004. A novel proxy for terrestrial organic matter in sediments based on branched and isoprenoid tetraether lipids. Earth and Planetary Science Letters 224, 107-116.

Horsfield, B., Schenk, H.J., Zink, K., Ondrak, R., Dieckmann, V., Kallmeyer, J., Mangelsdorf, K., di Primlo, R., Wilkes, H., Parkes, R.J., Fry, J., Cragg, B., 2006. Living microbial ecosystems

within the active zone of catagenesis: Implications for feeding the deep biosphere. Earth and Planetary Science Letters 246, 55-69.

Hu, L., Pan, H., Xue, Y., Ventosa, A., Cowan, D.A., Jones, B.E., Grant, W.D., Ma, Y., 2008. *Halorubrum luteum* sp. nov., isolated from Lake Chagannor, Inner Mongolia, China. International Journal of Systematic and Evolutionary Microbiology 58, 1705-1708.

Ihara, K., Watanabe, S., Tamura, T., 1997. *Haloarcula argentinensis* sp. nov. and *Haloarcula mukohataei* sp. nov., Two new extremely halophilic archaea collected in argentina. International Journal of Systematic Bacteriology 47, 73-77.

Inagaki, F., Nunoura, T., Nakagawa, S., Teske, A., Lever, M., Lauer, A., Suzuki, M., Takai, K., Delwiche, M., Colwell, F.S., Nealson, K.H., Horikoshi, K., D'Hondt, S., Jørgensen, B.B., 2006. Biogeographical distribution and diversity of microbes in methane hydrate-bearing deep marine sediments on the Pacific Ocean Margin. Proceedings of the National Academy of Sciences of the United States of America 103, 2815-2820.

Inoue, K., Itoh, T., Ohkuma, M., Kogure, K., 2011. Halomarina oriensis gen. nov., sp. nov., a halophilic archaeon isolated from a seawater aquarium. International Journal of Systematic and Evolutionary Microbiology 61, 942-946.

Ishii, A., Sato, T., Wachi, M., Nagai, K., Kato, C., 2004. Effects of high hydrostatic pressure on bacterial cytoskeleton FtsZ polymers in vivo and in vitro. Microbiology 150, 1965-1972.

Itoh, T., Yamaguchi, T., Zhou, P., Takashina, T., 2005. *Natronolimnobius baerhuensis* gen. nov., sp. nov. and *Natronolimnobius innermongolicus* sp. nov., novel haloalkaliphilic archaea isolated from soda lakes in Inner Mongolia, China. Extremophiles 9, 111-116.

Jaeschke, A., Rooks, C., Trimmer, M., Nicholls, J.C., Hopmans, E.C., Schouten, S., Damste, J.S.S., 2009. Comparison of ladderane phospholipid and core lipids as indicators for anaerobic ammonium oxidation (anammox) in marine sediments. Geochimica et Cosmochimica Acta 73, 2077-2088.

Jones, B.F., Hanor, J.S., Evans, W.R., 1994. Sources of Dissolved Salts in the Central Murray Basin, Australia. Chemical Geology 111, 135-154.

Josephson, K.L., Gerba, C.P., Pepper, I.L., 1993. Polymerase chain-reaction detection of nonviable bacterial pathogens. Applied and Environmental Microbiology 59, 3513-3515.

Kallmeyer, J., Pockalny, R., Adhikari, R.R., Smith, D.C., D'Hondt, S., 2012. Global distribution of microbial abundance and biomass in subseafloor sediment. Proceedings of the National Academy of Sciences of the United States of America, (doi:10.1073/pnas.1203849109).

Kamekura, M., Dyall-Smith, M.L., Upasani, V., Ventosa, A., Kates, M., 1997. Diversity of Alkaliphilic Halobacteria: Proposals for Transfer of *Natronobacterium vacuolatum, Natronobacterium magadii,* and *Natronobacterium pharaonis* to *Halorubrum*, *Natrialba*, and *Natronomonas* gen. nov., Respectively, as *Halorubrum vacuolatum* comb. nov., *Natrialba magadii* comb. nov., and *Natronomonas pharaonis* comb. nov., Respectively. International Journal of Systematic Bacteriology 47, 853-857.

Kamekura, M., Kates, M., 1999. Structural diversity of membrane lipids in members of halobacteriaceae. Bioscience, Biotechnology, and Biochemistry 63, 969-972.

Kamimura, K., Fuse, H., Takimura, O., Yamaoka, Y., 1993. Effects of growth pressure and temperature on fatty-acid composition of a barotolerant deep-sea bacterium. Applied and Environmental Microbiology 59, 924-926.

Kanai, H., Kobayashi, T., Aono, R., Kudo, T., 1995. *Natronococcus amylolyticus* sp. nov., a Haloalkaliphilic Archaeon. International Journal of Systematic Bacteriology 45, 762-766.

Kaneko, H., Takami, H., Inoue, A., Horikoshi, K., 2000. Effects of hydrostatic pressure and temperature on growth and lipid composition of the inner membrane of barotolerant Pseudomonas sp BT1 isolated from the deep-sea. Bioscience, Biotechnology, and Biochemistry 64, 72-79.

Kannenberg, E.L., Poralla, K., 1999. Hopanoid biosynthesis and function in bacteria. Naturwissenschaften 86, 168-176.

Kates, M., 1977. The phytanyl ether-linked polar lipids and isoprenoid neutral lipids of extremely halophilic bacteria. Progress in the Chemistry of Fats and other Lipids 15, 301-342.

Kates, M., 1993. Biology of halophilic bacteria, Part II. Cellular and Molecular Life Sciences 49, 1027-1036.

Kates, M., 1996. Structural analysis of phospholipids and glycolipids in extremely halophilic archaebacteria. Journal of Microbiological Methods 25, 113-128.

Kates, M., Deroo, P.W., 1973. Structure determination of the glycolipid sulfate from the extreme halophile *Halobacterium cutirubrum*. Journal of Lipid Research 14, 438-445.

Kato, C., Bartlett, D.H., 1997. The molecular biology of barophilic bacteria. Extremophiles 1, 111-116.

Kato, C., Inoue, A., Horikoshi, K., 1996. Isolating and characterizing deep-sea marine microorganisms. Trends in Biotechnology 14, 6-12.

Kato, C., Sato, T., Horikoshi, K., 1995. Isolation and properties of barophilic and barotolerant bacteria from deep-sea mud samples. Biodiversity and Conservation 4, 1-9.

Kaye, J.Z., Baross, J.A., 2004. Synchronous effects of temperature, hydrostatic pressure, and salinity on growth, phospholipid profiles, and protein patterns of four Halomonas species isolated from deep-sea hydrothermal-vent and sea surface environments. Applied and Environmental Microbiology 70, 6220-6229.

Kharroub, K., Lizama, C., Aguilera, M., Boulahrouf, A., Campos, V., Ramos-Cormenzana, A., Monteoliva-Sánchez, M., 2008. *Halomicrobium katesii* sp. nov., an extremely halophilic archaeon. International Journal of Systematic and Evolutionary Microbiology 58, 2354-2358.

Kharroub, K., Quesada, T., Ferrer, R., Fuentes, S., Aguilera, M., Boulahrouf, A., Ramos-Cormenzana, A., Monteoliva-Sánchez, M., 2006. *Halorubrum ezzemoulense* sp. nov., a halophilic archaeon isolated from Ezzemoul sabkha, Algeria. International Journal of Systematic and Evolutionary Microbiology 56, 1583-1588.

Khelaifia, S., Fardeau, M.-L., Pradel, N., Aussignargues, C., Garel, M., Tamburini, C., Cayol, J.-L., Gaudron, S., Gaill, F., Ollivier, B., 2011. *Desulfovibrio piezophilus* sp. nov., a piezophilic, sulfate-reducing bacterium isolated from wood falls in the Mediterranean Sea. International Journal of Systematic and Evolutionary Microbiology 61, 2706-2711.

Kim, K.K., Lee, K.C., Lee, J.-S., 2011. *Halogranum salarium* sp. nov., a halophilic archaeon isolated from sea salt. Systematic and Applied Microbiology 34, 576-580.

Koga, Y., Morii, H., 2006. Special methods for the analysis of ether lipid structure and metabolism in archaea. Analytical Biochemistry 348, 1-14.

Kroth, P., Strotmann, H., 1999. Diatom plastids: Secondary endocytobiosis, plastid genome and protein import. Physiologia Plantarum 107, 136-141.

Kuhlmann, F.E., Apffel, A., Fischer, S.M., Goldberg, G., Goodley, P.C., 1995. Signal enhancement for gradient reverse-phase high-performance liquid chromatography electrospray ionization mass spectrometry analysis with trifluoroacetic and other strong acid modifiers by postcolumn addition of propionic acid and isopropanol. Journal of the American Society for Mass Spectrometry 6, 1221-1225.

Kushwaha, S.C., Kates, M., Juez, G., Rodriguez-Valera, F., Kushner, D.J., 1982. Polar lipids of an extremely halophilic bacterial strain (R-4) isolated from salt ponds in Spain. Biochimica et Biophysica Acta - Lipids and Lipid metabolism 711, 19.

Lai, D., Springstead, J., Monbouquette, H., 2008. Effect of growth temperature on ether lipid biochemistry in *Archaeoglobus fulgidus*. Extremophiles 12, 271-278.

Langworthy, T.A., Mayberry, W.R., Smith, P.F., 1974. Long-chain glycerol diether and polyol dialkyl glycerol triether lipids of *Sulfolobus acidocaldarius*. Journal of Bacteriology 119, 106-116.

Lattanzio, V.M.T., Corcelli, A., Mascolo, G., Oren, A., 2002. Presence of two novel cardiolipins in the halophilic archaeal community in the crystallizer brines from the salterns of Margherita di Savoia (Italy) and Eilat (Israel). Extremophiles 6, 437-444.

Lauro, F., Bartlett, D., 2008. Prokaryotic lifestyles in deep sea habitats. Extremophiles 12, 15-25.

Lee, S.H., Kemp, P.F., 1994. Single-cell RNA-content of natural marine planktonik bachteria measured by hybridization with multiple 16S ribosomal-RNA-targeted fluorescent-probes. Limnology and Oceanography 39, 869-879.

Lever, M.A., Alperin, M., Engelen, B., Inagaki, F., Nakagawa, S., Steinsbu, B.O., Teske, A., Sci, I.E., 2006. Trends in basalt and sediment core contamination during IODP Expedition 301. Geomicrobiology Journal 23, 517-530.

Levin, D.E., 2005. Cell Wall Integrity Signaling in *Saccharomyces cerevisiae*. Microbiology and Molecular Biology Reviews 69, 262-291.

Lin, Y.S., Lipp, J.S., Yoshinaga, M.Y., Lin, S.H., Elvert, M., Hinrichs, K.U., 2010. Intramolecular stable carbon isotopic analysis of archaeal glycosyl tetraether lipids. Rapid Communications in Mass Spectrometry 24, 2817-2826.

Lipp, J.S., Hinrichs, K.U., 2009. Structural diversity and fate of intact polar lipids in marine sediments. Geochimica et Cosmochimica Acta 73, 6816-6833.

Lipp, J.S., Morono, Y., Inagaki, F., Hinrichs, K.-U., 2008. Significant contribution of Archaea to extant biomass in marine subsurface sediments. Nature.

Litchfield, C.D., Irby, A., Kis-Papo, T., Oren, A., 2000. Comparisons of the polar lipid and pigment profiles of two solar salterns located in Newark, California, U.S.A., and Eilat, Israel. Extremophiles 4, 259.

Lizama, C., Monteoliva-Sánchez, M., Suárez-García, A., Roselló-Mora, R., Aguilera, M., Campos, V., Ramos-Cormenzana, A., 2002. *Halorubrum tebenquichense* sp. nov., a novel halophilic archaeon isolated from the Atacama Saltern, Chile. International Journal of Systematic and Evolutionary Microbiology 52, 149-55.

Logemann, J., 2007. Diplomarbeit. Vergleich und Bewertung unterschiedlicher Aufarbeitungsmethoden für die Analyse polarer Lipide unter besonderer Berücksichtigung intakter Phospholipide. Institut for Chemie und Biologie des Meeres (ICBM). Carl von Osszietzky Universität. Oldenburg.

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

Long, D.T., Fegan, N.E., McKee, J.D., Lyons, W.B., Hines, M.E., Macumber, P.G., 1992. Formation of alunite, jarosite and hydrous iron oxides in a hypersaline system: Lake Tyrrell, Victoria, Australia. Chemical Geology 96, 183-202.

Lorenz, M.G., Wackernagel, W., 1987. Adsorption of DNA to sand and variable degradation rates of adsorbed DNA. Applied and Environmental Microbiology 53, 2948-2952.

Lunau, M., Lemke, A., Walther, K., Martens-Habbena, W., Simon, M., 2005. An improved method for counting bacteria from sediments and turbid environments by epifluorescence microscopy. Environmental Microbiology 7, 961-968.

Lyons, W.B., Tyler, S.W., Gaudette, H.E., Long, D.T., 1995. The use of strontium isotopes in determining groundwater mixing and brine fingering in a playa spring zone, Lake Tyrrell, Australia. Journal of Hydrology 167, 225-239.

Macdonald, A.G., 1988. Application of the theory of homeoviscous adaptation to excitable membranes: pre-synaptic processes. Biochemical Journal 256, 313-327.

Macumber, P.G., 1992. Hydrological processes in the Tyrrell Basin, southeastern Australia. Chemical Geology 96, 1-18.

Madigan, M.T., Martinko, J.M., Brock, T.D., Thomm, M., 2006. Brock Mikrobiologie. Pearson Education, München.

Makhdoumi-Kakhki, A., Amoozegar, M.A., Ventosa, A., 2012. *Halovenus aranensis* gen. nov., sp. nov., an extremely halophilic archaeon from Aran-Bidgol salt lake. International Journal of Systematic and Evolutionary Microbiology 62, 1331-1336.

Makula, R.A., Finnerty, W.R., 1975. Isolation and characterization of an ornithine-containing lipid from *Desulfovibrio gigas*. Journal of Bacteriology 123, 523-529.

Mancinelli, R.L., Landheim, R., Sánchez-Porro, C., Dornmayr-Pfaffenhuemer, M., Gruber, C., Legat, A., Ventosa, A., Radax, C., Ihara, K., White, M.R., Stan-Lotter, H., 2009. *Halorubrum chaoviator* sp. nov., a haloarchaeon isolated from sea salt in Baja California, Mexico, Western Australia and Naxos, Greece. International Journal of Systematic and Evolutionary Microbiology 59, 1908-1913.

Manecki, M., 2008. Optimierung einer etablierten Auftrennungsgsmethode für intakte Phospholipide. Carl von Ossetzki Universität. Oldenburg.

Mangelsdorf, K., Zink, K.G., Birrien, J.L., Toffin, L., 2005. A quantitative assessment of pressure dependent adaptive changes in the membrane lipids of piezosensitive deep sub-seafloor bacterium. Organic Geochemistry 36, 1459-1479.

Marr, A.G., Ingraham, J.L., 1962. Effect of temperature on the composition of fatty acids in *Escherichia coli*. Journal of Bacteriology 84, 1260-1267.

Matos, A.R., Pham-Thi, A.-T., 2009. Lipid deacylating enzymes in plants: Old activities, new genes. Plant Physiology and Biochemistry 47, 491-503.

Matsubara, T., Iida-Tanaka, N., Kamekura, M., Moldoveanu, N., Ishizuka, I., Onishi, H., Hayashi, A., Kates, M., 1994. Polar lipids of a non-alkaliphilic extremely halophilic archaebacterium strain 172: a novel bis-sulfated glycolipid. Biochimica et Biophysica Acta - Lipids and Lipid metabolism 1214, 97.

Maturrano, L., Santos, F., Rossello-Mora, R., Anton, J., 2006. Microbial diversity in Maras salterns, a hypersaline environment in the Peruvian Andes. Applied and Environmental Microbiology 72, 3887-3895.

McGenity, T.J., Gemmell, R.T., Grant, W.D., 1998. Proposal of a new halobacterial genus *Natrinema* gen. nov., with two species *Natrinema pellirubrum* nom. nov. and *Natrinema pallidum* nom. nov. International Journal of Systematic Bacteriology 48, 1187-1196.

McGenity, T.J., Grant, W.D., 1995. Transfer of *Halobacterium saccharovorum*, *Halobacterium sodomense*, *Halobacterium trapanicum* NRC 34021 and *Halobacterium lacusprofundi* to the Genus *Halorubrum* gen. nov., as *Halorubrum saccharovorum* comb. nov., *Halorubrum sodomense* comb. nov., *Halorubrum trapanicum* comb. nov., and *Halorubrum lacusprofundi* comb. nov. Systematic and Applied Microbiology 18, 237-243.

Mei, H., Hsieh, Y., Nardo, C., Xu, X., Wang, S., Ng, K., Korfmacher, W.A., 2003. Investigation of matrix effects in bioanalytical high-performance liquid chromatography/tandem mass spectrometric assays: application to drug discovery. Rapid Communications in Mass Spectrometry 17, 97-103.

Michiels, C., Bartlett, D.H., Aertsen, A., 2008. High-pressure microbiology. ASM Press, Washington, DC, USA.

Miller, C.S., Baker, B.J., Thomas, B.C., Singer, S.W., Banfield, J.F., 2011. EMIRGE: reconstruction of full-length ribosomal genes from microbial community short read sequencing data. Genome Biology 12.

Molina-Höppner, A., Sato, T., Kato, C., Gänzle, M.G., Vogel, R.F., 2003. Effects of pressure on cell morphology and cell division of lactic acid bacteria. Extremophiles 7, 511–516.

Montalvo-Rodriguez, R., Lopez-Garriga, J., Vreeland, R.H., Oren, A., Ventosa, A., Kamekura, M., 2000. *Haloterrigena thermotolerans* sp. nov., a halophilic archaeon from Puerto Rico. International Journal of Systematic and Evolutionary Microbiology 50, 1065-71.

Montalvo-Rodriguez, R., Vreeland, R.H., Oren, A., Kessel, M., Betancourt, C., Lopez-Garriga, J., 1998. *Halogeometricum borinquense* gen. nov., sp. nov., a novel halophilic archaeon from Puerto Rico. International Journal of Systematic Bacteriology 48, 1305-1312.

Morii, H., Yagi, H., Akutsu, H., Nomura, N., Sako, Y., Koga, Y., 1999. A novel phosphoglycolipid archaetidyl(glucosyl)inositol with two sesterterpanyl chains from the aerobic hyperthermophilic archaeon *Aeropyrum pernix K1*. Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids 1436, 426.

Müller, K.D., Husmann, H., Nalik, H.P., Schomburg, G., 1990. Trans-esterification of fatty acids from microorganisms and human blood serum by trimethylsulfonium hydroxide (TMSH) for GC analysis. Chromatographia 30, 245-248.

Muyzer, G., Dewaal, E.C., Uitterlinden, A.G., 1993. Profiling of complex microbial-populations by denaturing gradient gel-electrophoresis analysis of polymerase chain reaction-amplified genes-coding for 16S ribosomal RNA. Applied and Environmental Microbiology 59, 695-700.

Muyzer, G., Teske, A., Wirsen, C.O., Jannasch, H.W., 1995. Phylogenetic-relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturating gradient gel-electrophoresis of 16S rDNA fragments. Archives of Microbiology 164, 165-172.

Nagaoka, S., Minegishi, H., Echigo, A., Shimane, Y., Kamekura, M., Usami, R., 2011. *Halostagnicola alkaliphila* sp. nov., an alkaliphilic haloarchaeon from commercial rock salt. International Journal of Systematic and Evolutionary Microbiology 61, 1149-1152.

Nagaoka, S., Minegishi, H., Echigo, A., Usami, R., 2010. *Halostagnicola kamekurae* sp. nov., an extremely halophilic archaeon from solar salt. International Journal of Systematic and Evolutionary Microbiology 60, 2828-2831.

Namwong, S., Tanasupawat, S., Kudo, T., Itoh, T., 2011. *Haloarcula salaria* sp. nov. and *Haloarcula tradensis* sp. nov., isolated from salt in Thai fish sauce. International Journal of Systematic and Evolutionary Microbiology 61, 231-236.

Namwong, S., Tanasupawat, S., Visessanguan, W., Kudo, T., Itoh, T., 2007. *Halococcus thailandensis* sp. nov., from fish sauce in Thailand. International Journal of Systematic and Evolutionary Microbiology 57, 2199-2203.

Narasingarao, P., Podell, S., Ugalde, J.A., Brochier-Armanet, C., Emerson, J.B., Brocks, J.J., Heidelberg, K.B., Banfield, J.F., Allen, E.E., 2012. De novo metagenomic assembly reveals abundant novel major lineage of Archaea in hypersaline microbial communities. ISME Journal 6, 81-93.

Neira, C., Rackemann, M., 1996. Black spots produced by buried macroalgae in intertidal sandy sediments of the Wadden Sea: Effects on the meiobenthos. Journal of Sea Research 36, 153-170.

Nichols, D.S., Miller, M.R., Davies, N.W., Goodchild, A., Raftery, M., Cavicchioli, R., 2004. Cold adaptation in the antarctic archaeon *Methanococcoides burtonii* Involves membrane lipid unsaturation. Journal of Bacteriology 186, 8508-8515.

Ochsenreiter, T., Pfeifer, F., Schleper, C., 2002. Diversity of *Archaea* in hypersaline environments characterized by molecular-phylogenetic and cultivation studies. Extremophiles 6, 267-274.

Oh, D., Porter, K., Russ, B., Burns, D., Dyall-Smith, M., 2010. Diversity of *Haloquadratum* and other haloarchaea in three, geographically distant, Australian saltern crystallizer ponds. Extremophiles 14, 161-169.

Orcutt, B.N., Sylvan, J.B., Knab, N.J., Edwards, K.J., 2011. Microbial ecology of the dark ocean above, at, and below the seafloor. Microbiology and Molecular Biology Reviews 75, 361-422.

Oren, A., 1994. Characterization of the halophilic archaeal community in saltern crystallizer ponds by means of polar lipid analysis. International Journal of Salt Lake Research 3, 15-29.

Oren, A., 2005. A hundred years of Dunaliella research: 1905-2005. Saline Systems 1, 2.

Oren, A., 2011. Diversity of Halophiles, In: K. Horikoshi (Ed.), *Extremophiles Handbook*. Springer Japanpp. 309-325.

Oren, A., Arahal, D.R., Ventosa, A., 2009. Emended descriptions of genera of the family *Halobacteriaceae*. International Journal of Systematic and Evolutionary Microbiology 59, 637-642.

Oren, A., Duker, S., Ritter, S., 1996. The polar lipid composition of walsby's square bacterium. FEMS Microbiology Letters 138, 135.

Oren, A., Elevi, R., Watanabe, S., Ihara, K., Corcelli, A., 2002. *Halomicrobium mukohataei* gen. nov., comb. nov., and emended description of *Halomicrobium mukohataei*. International Journal of Systematic and Evolutionary Microbiology 52, 1831-5.

Oren, A., Gurevich, P., Gemmell, R.T., Teske, A., 1995. *Halobaculum gomorrense* gen. nov., sp. nov., a Novel Extremely Halophilic Archaeon from the Dead Sea. International Journal of Systematic Bacteriology 45, 747-754.

Oren, A., Rodriguez-Valera, F., 2001. The contribution of halophilic bacteria to the red coloration of saltern crystallizer ponds. FEMS Microbiology Ecology 36, 123-130.

Oren, A., Ventosa, A., 1996. A Proposal for the Transfer of *Halorubrobacterium distributum* and *Halorubrobacterium coriense* to the Genus *Halorubrum* as *Halorubrum distributum* comb. nov. and *Halorubrum coriense* comb. nov., Respectively. International Journal of Systematic Bacteriology 46, 1180.

Oren, A., Ventosa, A., Gutiérrez, M.C., Kamekura, M., 1999. *Haloarcula quadrata* sp. nov., a square, motile archaeon isolated from a brine pool in Sinai (Egypt). International Journal of Systematic Bacteriology 49, 1149-1155.

Pernthaler, A., Pernthaler, J., Amann, R., 2002. Fluorescence in situ hybridization and catalyzed reporter deposition for the identification of marine bacteria. Applied and Environmental Microbiology 68, 3094-3101.

Pesenti, P.T., Sikaroodi, M., Gillevet, P.M., Sánchez-Porro, C., Ventosa, A., Litchfield, C.D., 2008. *Halorubrum californiense* sp. nov., an extreme archaeal halophile isolated from a crystallizer pond at a solar salt plant in California, USA. International Journal of Systematic and Evolutionary Microbiology 58, 2710-2715.

Pluschke, G., Overath, P., 1981. Function of phospholipids in *Escherichia coli.* Influence of changes in polar head group composition on the lipid phase transition and characterization of a mutant containing only saturated phospholipid acyl chains. Journal of Biological Chemistry 256, 3207-3212.

Pulfer, M., Murphy, R.C., 2003. Electrospray mass spectrometry of phospholipids. Mass Spectrometry Reviews 22, 332-364.

Qiu, D.-F., Games, M.P.L., Xiao, X.-Y., Games, D.E., Walton, T.J., 1998. Application of high-performance liquid chromatography/electrospray mass spectrometry for the characterization of membrane lipids in the haloalkaliphilic archaebacterium Natronobacterium magadii. Rapid Communications in Mass Spectrometry 12, 939-946.

Rajendran, N., Matsuda, O., Imamura, N., Urushigawa, Y., 1995. Microbial community structure analysis of euxinic sediments using phospholipid fatty acid biomarkers. Journal of Oceanography 51, 21-38.

Ravenschlag, K., Sahm, K., Knoblauch, C., Jorgensen, B.B., Amann, R., 2000. Community structure, cellular rRNA content, and activity of sulfate-reducing bacteria in marine Arctic sediments. Applied and Environmental Microbiology 66, 3592-3602.

Roh, S., Bae, J.-W., 2009. *Halorubrum cibi* sp. nov., an extremely halophilic archaeon from salt-fermented seafood. Journal of Microbiology 47, 162-166.

Roh, S.W., Lee, M.-L., Bae, J.-W., 2010. *Haladaptatus cibarius* sp. nov., an extremely halophilic archaeon from seafood, and emended description of the genus *Haladaptatus*. International Journal of Systematic and Evolutionary Microbiology 60, 1187-1190.

Roh, S.W., Nam, Y.-D., Chang, H.-W., Kim, K.-H., Sung, Y., Kim, M.-S., Oh, H.-M., Bae, J.-W., 2009. *Haloterrigena jeotgali* sp. nov., an extremely halophilic archaeon from salt-fermented food. International Journal of Systematic and Evolutionary Microbiology 59, 2359-2363.

Roh, S.W., Nam, Y.-D., Chang, H.-W., Sung, Y., Kim, K.-H., Lee, H.-J., Oh, H.-M., Bae, J.-W., 2007. *Natronococcus jeotgali* sp. nov., a halophilic archaeon isolated from shrimp jeotgal, a traditional fermented seafood from Korea. International Journal of Systematic and Evolutionary Microbiology 57, 2129-2131.

Romano, I., Poli, A., Finore, I., Huertas, F.J., Gambacorta, A., Pelliccione, S., Nicolaus, G., Lama, L., Nicolaus, B., 2007. *Haloterrigena hispanica* sp. nov., an extremely halophilic

archaeon from Fuente de Piedra, southern Spain. International Journal of Systematic and Evolutionary Microbiology 57, 1499-1503.

Rossel, P.E., Lipp, J.S., Fredricks, H.F., Arnds, J., Boetius, A., Elvert, M., Hinrichs, K.U., 2008. Intact polar lipids of anaerobic methanotrophic archaea and associated bacteria. Organic Geochemistry 39, 992-999.

Russell, N.J., 1984. Mechanisms of thermal adaptation in bacteria: blueprints for survival. Trends in Biochemical Sciences 9, 108-112.

Rütters, H., Sass, H., Cypionka, H., Rullkötter, J., 2001. Monoalkylether phosphoslipids in the sulfate-reducing bacteria *Desulfosarcina variablis* and *Desulforhabus amnigenus*. Archives of Microbiology 176, 435-442.

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

Sanyal, S., Menon, A.K., 2009. Flipping Lipids: Why an' What's the Reason for? ACS Chemical Biology 4.

Savage, K.N., Krumholz, L.R., Oren, A., Elshahed, M.S., 2007. *Haladaptatus paucihalophilus* gen. nov., sp. nov., a halophilic archaeon isolated from a low-salt, sulfide-rich spring. International Journal of Systematic and Evolutionary Microbiology 57, 19-24.

Savage, K.N., Krumholz, L.R., Oren, A., Elshahed, M.S., 2008. *Halosarcina pallida* gen. nov., sp. nov., a halophilic archaeon from a low-salt, sulfide-rich spring. International Journal of Systematic and Evolutionary Microbiology 58, 856-860.

Schippers, A., Neretin, L.N., Kallmeyer, J., Ferdelman, T.G., Cragg, B.A., Parkes, R.J., Jørgensen, B.B., 2005. Prokaryotic cells of the deep sub-seafloor biosphere identified as living bacteria. Nature 433, 861-864.

Schouten, S., Hopmans, E.C., Schefuss, E., Sinninghe Damsté, J.S.S., 2002. Distributional variations in marine crenarchaeotal membrane lipids: a new tool for reconstructing ancient sea water temperatures? Earth and Planetary Science Letters 204, 265-274.

Schouten, S., Middelburg, J.J., Hopmans, E.C., Sinninghe Damsté, J.S.S., 2010. Fossilization and degradation of intact polar lipids in deep subsurface sediments: A theoretical approach. Geochimica et Cosmochimica Acta 74, 3806-3814.

Schubotz, F., Stuart, G.W., Julius, S.L., Helen, F.F., Kai-Uwe, H., 2009. Detection of microbial biomass by intact polar membrane lipid analysis in the water column and surface sediments of the Black Sea. Environmental Microbiology.

Seidel, M., 2009. Dissertation. Intact polar membrane lipids as biomarkers for characterization of microbial communitiesin Wadden Sea sediments. . Institut für Chemie und Biologie des Meeres (ICBM), Fakultät V - Mathematik und Naturwissenschaften Carl von Ossietzky Universität Oldenburg. Oldenburg.

Seidel, M., Graue, J., Engelen, B., Köster, J., Sass, H., Rullkötter, J., 2012. Advection and diffusion determine vertical distribution of microbial communities in intertidal sediments as revealed by combined biogeochemical and molecular biological analysis. Organic Geochemistry 52, 114-129.

Shimane, Y., Hatada, Y., Minegishi, H., Echigo, A., Nagaoka, S., Miyazaki, M., Ohta, Y., Maruyama, T., Usami, R., Grant, W.D., Horikoshi, K., 2011. *Salarchaeum japonicum* gen. nov., sp. nov., an aerobic, extremely halophilic member of the Archaea isolated from commercial salt. International Journal of Systematic and Evolutionary Microbiology 61, 2266-2270.

Shimane, Y., Hatada, Y., Minegishi, H., Mizuki, T., Echigo, A., Miyazaki, M., Ohta, Y., Usami, R., Grant, W.D., Horikoshi, K., 2010. *Natronoarchaeum mannanilyticum* gen. nov., sp. nov., an aerobic, extremely halophilic archaeon isolated from commercial salt. International Journal of Systematic and Evolutionary Microbiology 60, 2529-2534.

Simon, M., Azam, F., 1989. Protein-Content And Protein-Synthesis Rates Of Planktonic Marine-Bacteria. Marine Ecology-Progress Series 51, 201-213.

Sinensky, M., 1974. Homeoviscous adaptation - A homeostatic process that regulates the viscosity of membrane lipids in *Escherichia coli*. Proceedings of the National Academy of Sciences of the United States of America 71, 522-525.

Sinninghe Damsté, J.S., Rijpstra, W.I.C., Geenevasen, J.A.J., Strous, M., Jetten, M.S.M., 2005. Structural identification of ladderane and other membrane lipids of planctomycetes capable of anaerobic ammonium oxidation (anammox). FEBS Journal 272.

Sinninghe Damsté, J.S., Rijpstra, W.I.C., Hopmans, E.C., Weijers, J.W.H., Foesel, B.U., Overmann, J., Dedysh, S.N., 2011. 13,16-Dimethyl octacosanedioic acid (iso-diabolic acid), a common membrane-spanning lipid of *Acidobacteria* subdivisions 1 and 3. Applied and Environmental Microbiology 77, 4147-4154.

Smith, P.B.W., Snyder, A.P., Harden, C.S., 1995. Characterization of bacterial phospholipids by electrospray-ionization tandem mass-spectrometry. Analytical Chemistry 67, 1824-1830.

Somero, G.N., 1992. Adaptations to high hydrostatic pressure. Annual Review of Physiology 54, 557-577.

Souverain, S., Rudaz, S., Veuthey, J.-L., 2004. Matrix effect in LC-ESI-MS and LC-APCI-MS with off-line and on-line extraction procedures. Journal of Chromatography A 1058, 61-66.

Souza, J.V.B., Junior, R., Koshikene, D., Silva, E.S., 2007. Applications of fluorescent in situ hybridization (FISH) in environmental microbiology. Journal of Food Agriculture & Environment 5, 408-411.

Sprott, G.D., 1992. Structures of archaebacterial membrane-lipids. Journal of Bioenergetics and Biomembranes 24, 555-566.

Sprott, G.D., Larocque, S., Cadotte, N., Dicaire, C.J., McGee, M., Brisson, J.R., 2003. Novel polar lipids of halophilic eubacterium *Planococcus* H8 and archaeon *Haloferax volcanii*. Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids 1633, 179.

Stan-Lotter, H., Pfaffenhuemer, M., Legat, A., Busse, H.-J.r., Radax, C., Gruber, C., 2002. *Halococcus dombrowskii* sp. nov., an archaeal isolate from a Permian alpine salt deposit. International Journal of Systematic and Evolutionary Microbiology 52, 1807-14.

Stiehl, T., Rullkötter, J., Nissenbaum, A., 2005. Molecular and isotopic characterization of lipids in cultured halophilic microorganisms from the Dead Sea and comparison with the sediment record of this hypersaline lake. Organic Geochemistry 36, 1242-1251.

Stouthamer, A.H., 1979. The search for correlation between theoretical and experimental growth yields. International Reviews in Biochemistry 21, 1-15.

Strous, M., Fuerst, J.A., Kramer, E.H.M., Logemann, S., Muyzer, G., van de Pas-Schoonen, K.T., Webb, R., Kuenen, J.G., Jetten, M.S.M., 1999. Missing lithotroph identified as new planctomycete. Nature 400, 446-449.

Stüber, M., Reemtsma, T., 2004. Evaluation of three calibration methods to compensate matrix effects in environmental analysis with LC-ESI-MS. Analytical and Bioanalytical Chemistry 378, 910-916.

Sturt, H.F., Summons, R.E., Smith, K., Elvert, M., Hinrichs, K.U., 2004. Intact polar membrane lipids in prokaryotes and sediments deciphered by high-performance liquid chromatography/electrospray ionization multistage mass spectrometry - new biomarkers for biogeochemistry and microbial ecology. Rapid Communications in Mass Spectrometry 18, 617-628.

Sumper, M., Berg, E., Mengele, R., Strobel, I., 1990. Primary structure and glycosylation of the S-layer protein of *Haloferax volcanii*. Journal of Bacteriology 172, 7111-7118.

Süß, J., Engelen, B., Cypionka, H., Sass, H., 2004. Quantitative analysis of bacterial communities from Mediterranean sapropels based on cultivation-dependent methods. FEMS Microbiology Ecology 51, 109-121.

Takano, Y., Chikaraishi, Y., Ogawa, N.O., Nomaki, H., Morono, Y., Inagaki, F., Kitazato, H., Hinrichs, K.U., Ohkouchi, N., 2010. Sedimentary membrane lipids recycled by deep-sea benthic archaea. Nature Geosciences 3, 858-861.

Tang, L., Kebarle, P., 1993. Dependence of ion intensity in electrospray mass spectrometry on the concentration of the analytes in the electrosprayed solution. Analytical Chemistry 65, 3654-3668.

Tapingkae, W., Tanasupawat, S., Itoh, T., Parkin, K.L., Benjakul, S., Visessanguan, W., Valyasevi, R., 2008. *Natrinema gari* sp. nov., a halophilic archaeon isolated from fish sauce in Thailand. International Journal of Systematic and Evolutionary Microbiology 58, 2378-2383.

Taylor, P.J., 2005. Matrix effects: the Achilles heel of quantitative high-performance liquid chromatography–electrospray–tandem mass spectrometry. Clinical Biochemistry 38, 328-334.

Teixidor, P., Grimalt, J.O., Pueyo, J.J., Rodriguezvalera, F., 1993. Isopranylglycerol diethers in nonalkaline evaporitic environments. Geochimica et Cosmochimica Acta 57, 4479-4489.

Tenchov, B., Vescio, E.M., Sprott, G.D., Zeidel, M.L., Mathai, J.C., 2006. Salt tolerance of archaeal extremely halophilic lipid membranes. Journal of Biological Chemistry 281, 10016-10023.

Teske, A., Sorensen, K.B., 2008. Uncultured archaea in deep marine subsurface sediments: have we caught them all? ISME Journal 2, 3-18.

Tindall, B.J., 1990. Lipid composition of *Halobacterium lacusprofundi*. FEMS Microbiology Letters 66, 199-202.

Tindall, B.J., Rosselló-Móra, R., Busse, H.-J., Ludwig, W., Kämpfer, P., 2010. Notes on the characterization of prokaryote strains for taxonomic purposes. International Journal of Systematic and Evolutionary Microbiology 60, 249-266.

Tindall, B.J., Tomlinson, G.A., Hochstein, L.I., 1989. Notes: Transfer of *Halobacterium denitrificans* (Tomlinson, Jahnke, and Hochstein) to the Genus *Haloferax* as *Haloferax denitrificans* comb. nov. International Journal of Systematic Bacteriology 39, 359-360.

Trincone, A., Nicolaus, B., Lama, L., De Rosa, M., Gambacorta, A., Gran, W.D., 1990. The glycolipid of *Halobacterium sodomense*. Journal of General Microbiology 136, 2327-2331.

Trincone, A., Trivellone, E., Nicolaus, B., Lama, L., Pagnotta, E., Grant, W.D., Gambacorta, A., 1993. The glycolipid of *Halobacterium trapanicum* Biochimica et Biophysica Acta 1210, 35-40.

Ulrih, N.P., Gmajner, D., Raspor, P., 2009. Structural and physicochemical properties of polar lipids from thermophilic archaea. Applied Microbiology and Biotechnology 84, 249-260.

Upasani, V.N., Desai, S.G., Moldoveanu, N., Kates, M., 1994. Lipids of extremely halophilic archaeobacteria from saline environments in india - a novel glycolipid in Natronobacterium strains. Microbiology 140, 1959-1966.

v. Wintzingerode, F., Gobel, U.B., Stackebrandt, E., 1997. Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. FEMS Microbiology Reviews 21, 213-229.

Vaisman, N., Oren, A., 2009. *Salisaeta longa* gen. nov., sp. nov., a red, halophilic member of the Bacteroidetes. International Journal of Systematic and Evolutionary Microbiology 59, 2571-2574.

Van Mooy, B.A.S., Fredricks, H.F., Pedler, B.E., Dyhrman, S.T., Karl, D.M., Koblizek, M., Lomas, M.W., Mincer, T.J., Moore, L.R., Moutin, T., Rappe, M.S., Webb, E.A., 2009. Phytoplankton in the ocean use non-phosphorus lipids in response to phosphorus scarcity. Nature 458, 69.

Vancanneyt, M., Witt, S., Abraham, W.R., Kersters, K., Fredrickson, H.L., 1996. Fatty acid content in whole-cell hydrolysates and phospholipid fractions of pseudomonads: A taxonomic evaluation. Systematic and Applied Microbiology 19, 528-540.

Ventosa, A., Gutiérrez, M.C., Kamekura, M., Dyall-Smith, M.L., 1999. Proposal to transfer *Halococcus turkmenicus*, *Halobacterium trapanicum* JCM 9743 and strain GSL-11 to

Haloterrigena turkmenica gen. nov., comb. nov. International Journal of Systematic Bacteriology 49, 131-136.

Ventosa, A., Gutiérrez, M.C., Kamekura, M., Zvyagintseva, I.S., Oren, A., 2004. Taxonomic study of *Halorubrum distributum* and proposal of *Halorubrum terrestre* sp. nov. International Journal of Systematic and Evolutionary Microbiology 54, 389-392.

Vreeland, R., Straight, S., Krammes, J., Dougherty, K., Rosenzweig, W., Kamekura, M., 2002. *Halosimplex carlsbadense* gen. nov., sp. nov., a unique halophilic archaeon, with three 16S rRNA genes, that grows only in defined medium with glycerol and acetate or pyruvate. Extremophiles 6, 445-452.

Wagner, R., 1994. The regulation of ribosomal-RNA synthesis and bacterial cell growth. Archives of Microbiology 161, 100-109.

Wainø, M., Tindall, B.J., Ingvorsen, K., 2000. *Halorhabdus utahensis* gen. nov., sp. nov., an aerobic, extremely halophilic member of the Archaea from Great Salt Lake, Utah. International Journal of Systematic and Evolutionary Microbiology 50, 183-90.

Wang, A.-Y., Cronan, J.E., 1994. The growth phase-dependent synthesis of cyclopropane fatty acids in *Escherichia coli* is the result of an RpoS(KatF)-dependent promoter plus enzyme instability. Molecular Microbiology 11, 1009-1017.

Wang, F., Xiao, X., Ou, H.-Y., Gai, Y., Wang, F., 2009. Role and regulation of fatty acid biosynthesis in the response of *Shewanella piezotolerans* WP3 to different temperatures and pressures. Journal of Bacteriology 191, 2574–2584.

Wang, Q.-f., Li, W., Yang, H., Liu, Y.-l., Cao, H.-h., Dornmayr-Pfaffenhuemer, M., Stan-Lotter, H., Guo, G.-q., 2007. *Halococcus qingdaonensis* sp. nov., a halophilic archaeon isolated from a crude sea-salt sample. International Journal of Systematic and Evolutionary Microbiology 57, 600-604.

Wang, S., Yang, Q., Liu, Z.-H., Sun, L., Wei, D., Zhang, J.-Z., Song, J.-Z., Yuan, H.-F., 2010. *Haloterrigena daqingensis* sp. nov., an extremely haloalkaliphilic archaeon isolated from a saline-alkaline soil. International Journal of Systematic and Evolutionary Microbiology 60, 2267-2271.

Webster, G., Watt, L.C., Rinna, J., Fry, J.C., Evershed, R.P., Parkes, R.J., Weightman, A.J., 2006. A comparison of stable-isotope probing of DNA and phospholipid fatty acids to study prokaryotic functional diversity in sulfate-reducing marine sediment enrichment slurries. Environmental Microbiology 8, 1575-1589.

Weijers, J.W.H., Schouten, S., Hopmans, E.C., Geenevasen, J.A.J., David, O.R.P., Coleman, J.M., Pancost, R.D., Sinninghe Damste, J.S., 2006. Membrane lipids of mesophilic anaerobic bacteria thriving in peats have typical archaeal traits. Environmental Microbiology 8, 648-657.

Weissenmayer, B., Gao, J.-L., López-Lara, I.M., Geiger, O., 2002. Identification of a gene required for the biosynthesis of ornithine-derived lipids. Molecular Microbiology 45, 721–733.

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

White, D.C., Frerman, F.E., 1967. Extraction, Characterization, and Cellular Localization of the Lipids of *Staphylococcus aureus*. Journal of Bacteriology 94, 1854-1867.

Whitman, W.B., Coleman, D.C., Wiebe, W.J., 1998. Prokaryotes: The unseen majority. Proceedings of the National Academy of Sciences of the United States of America 95, 6578-6583.

Wilansky, B., 1936. Nature 138, 467.

Wilms, R., Köpke, B., Sass, H., Chang, T.S., Cypionka, H., Engelen, B., 2006. Deep biosphere-related bacteria within the subsurface of tidal flat sediments. Environmental Microbiology 8, 709-719.

Wilms, R., Sass, H., Köpke, B., Cypionka, H., Engelen, B., 2007. Methane and sulfate profiles within the subsurface of a tidal flat are reflected by the distribution of sulfate-reducing bacteria and methanogenic archaea. FEMS Microbiology Ecology 59, 611-621.

Wirsen, C.O., Jannasch, H.W., Wakeham, S.G., Canuel, E.A., 1986. Membrane lipids of a psychrophilic and barophilic deep-sea bacterium. Current Microbiology 14, 319-322.

Xin, H., Itoh, T., Zhou, P., Suzuki, K., Kamekura, M., Nakase, T., 2000. *Natrinema versiforme* sp. nov., an extremely halophilic archaeon from Aibi salt lake, Xinjiang, China. International Journal of Systematic and Evolutionary Microbiology 50, 1297-303.

Xin, H., Itoh, T., Zhou, P., Suzuki, K., Nakase, T., 2001. *Natronobacterium nitratireducens* sp. nov., a aloalkaliphilic archaeon isolated from a soda lake in China. International Journal of Systematic and Evolutionary Microbiology 51, 1825-9.

Xu, X.-W., Liu, S.-J., Tohty, D., Oren, A., Wu, M., Zhou, P.-J., 2005a. *Haloterrigena saccharevitans* sp. nov., an extremely halophilic archaeon from Xin-Jiang, China. International Journal of Systematic and Evolutionary Microbiology 55, 2539-2542.

Xu, X.-W., Ren, P.-G., Liu, S.-J., Wu, M., Zhou, P.-J., 2005b. *Natrinema altunense* sp. nov., an extremely halophilic archaeon isolated from a salt lake in Altun Mountain in Xinjiang, China. International Journal of Systematic and Evolutionary Microbiology 55, 1311-1314.

Xu, X.-W., Wu, M., Zhou, P.-J., Liu, S.-J., 2005c. *Halobiforma lacisalsi* sp. nov., isolated from a salt lake in China. International Journal of Systematic and Evolutionary Microbiology 55, 1949-1952.

Xu, X.-W., Wu, Y.-H., Wang, C.-S., Oren, A., Zhou, P.-J., Wu, M., 2007a. *Haloferax larsenii* sp. nov., an extremely halophilic archaeon from a solar saltern. International Journal of Systematic and Evolutionary Microbiology 57, 717-720.

Xu, X.-W., Wu, Y.-H., Zhang, H.-b., Wu, M., 2007b. *Halorubrum arcis* sp. nov., an extremely halophilic archaeon isolated from a saline lake on the Qinghai–Tibet Plateau, China. International Journal of Systematic and Evolutionary Microbiology 57, 1069-1072.

Xu, Y., Wang, Z., Xue, Y., Zhou, P., Ma, Y., Ventosa, A., Grant, W.D., 2001. *Natrialba hulunbeirensis* sp. nov. and *Natrialba chahannaoensis* sp. nov., novel haloalkaliphilic archaea from soda lakes in Inner Mongolia Autonomous Region, China. International Journal of Systematic and Evolutionary Microbiology 51, 1693-8.

Xu, Y., Zhou, P., Tian, X., 1999. Characterization of two novel haloalkaliphilic archaea *Natronorubrum bangense* gen. nov., sp. nov. and *Natronorubrum tibetense* gen. nov., sp. nov. International Journal of Systematic Bacteriology 49, 261-266.

Xue, Y., Fan, H., Ventosa, A., Grant, W.D., Jones, B.E., Cowan, D.A., Ma, Y., 2005. *Halalkalicoccus tibetensis* gen. nov., sp. nov., representing a novel genus of haloalkaliphilic archaea. International Journal of Systematic and Evolutionary Microbiology 55, 2501-2505.

Yachai, M., Tanasupawat, S., Itoh, T., Benjakul, S., Visessanguan, W., Valyasevi, R., 2008. *Halobacterium piscisalsi* sp. nov., from fermented fish (pla-ra) in Thailand. International Journal of Systematic and Evolutionary Microbiology 58, 2136-2140.

Yang, X., Cui, H.-L., 2012. *Halomicrobium zhouii* sp. nov., a halophilic archaeon from a marine solar saltern. International Journal of Systematic and Evolutionary Microbiology 62, 1235-1240.

Yang, Y., Cui, H.-L., Zhou, P.-J., Liu, S.-J., 2006. *Halobacterium jilantaiense* sp. nov., a halophilic archaeon isolated from a saline lake in Inner Mongolia, China. International Journal of Systematic and Evolutionary Microbiology 56, 2353-2355.

Yang, Y., Cui, H.-L., Zhou, P.-J., Liu, S.-J., 2007. *Haloarcula amylolytica* sp. nov., an extremely halophilic archaeon isolated from Aibi salt lake in Xin-Jiang, China. International Journal of Systematic and Evolutionary Microbiology 57, 103-106.

Yano, Y., Nakayama, A., Ishihara, K., Saito, H., 1998. Adaptive changes in membrane lipids of barophilic bacteria in response to changes in growth pressure. Applied and Environmental Microbiology 64, 479-485.

Yayanos, A.A., 1995. Microbiology To 10,500 Meters In The Deep-Sea. Annual Review of Microbiology 49, 777-805.

Yayanos, A.A., van Boxtel, R., Dietz, A.S., 1984. High-pressure-temperature gradient instrument: use for determining the temperature and pressure limits of bacterial growth. Applied and Environmental Microbiology 48, 771-776.

Yilmaz, L.S., Noguera, D.R., 2004. Mechanistic Approach to the Problem of Hybridization Efficiency in Fluorescent In Situ Hybridization. Applied and Environmental Microbiology 70, 7126-7139.

Yoshinaga, M.Y., Kellermann, M.Y., Rossel, P.E., Schubotz, F., Lipp, J.S., Hinrichs, K.U., 2011. Systematic fragmentation patterns of archaeal intact polar lipids by high-performance liquid chromatography/electrospray ionization ion-trap mass spectrometry. Rapid Communications in Mass Spectrometry 25, 3563-3574.

Zak, J.C., Willig, M.R., Moorhead, D.L., Wildman, H.G., 1994. Functional diversity of microbial communities: A quantitative approach. Soil Biology and Biochemistry 26, 1101-1108.

Zelles, L., 1997. Phospholipid fatty acid profiles in selected members of soil microbial communities. Chemosphere 35, 275-294.

Zelles, L., 1999. Fatty acid patterns of phospholipids and lipopolysaccharides in the characterisation of microbial communities in soil: a review. Biology and Fertility of Soils 29, 111-129.

Zhang, Y.-M., Rock, C.O., 2008. Membrane lipid homeostasis in bacteria. Nature Reviews Microbiology 6, 222.

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

Zobell, C.E., Cobet, A.B., 1964. Filament formation by *Escherichia coli* at increased hydrostatic pressures. Journal of Bacteriology 87, 710-719.

Zobell, C.E., Johnson, F.H., 1949. The influence of hydrostatic pressure on the growth and viability of terrestrial and marine bacteria. Journal of Bacteriology 57, 179–189.

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Eidesstattliche Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit selbständig angefertigt habe und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe. Zusätzlich erkläre ich, dass diese Dissertation weder in ihrer Gesamtheit noch in Teilen einer anderen wissenschaftlichen Hochschule zur Begutachtung in einem Promotionsverfahren vorliegt oder vorgelegen hat.

Oldenburg, den 4. Juli 2013

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