Deep-Sea and Sub-Seafloor Frontiers (DS^3F)

Workshop
May 3-4, 2011,
Aarhus, Denmark

Report on
Workpackage 3

The Deep Biosphere

Workshop held at the Center for Geomicrobiology, Aarhus University, Denmark

Organizers: Bo Barker Jørgensen and R. John Parkes
Local hosts: Bo Barker Jørgensen and Camilla Nissen Toftdal

Table of contents

1. Workshop program and objectives

Program

Monday, May 2
Arrival in the afternoon or evening. Joint dinner at 20:00.

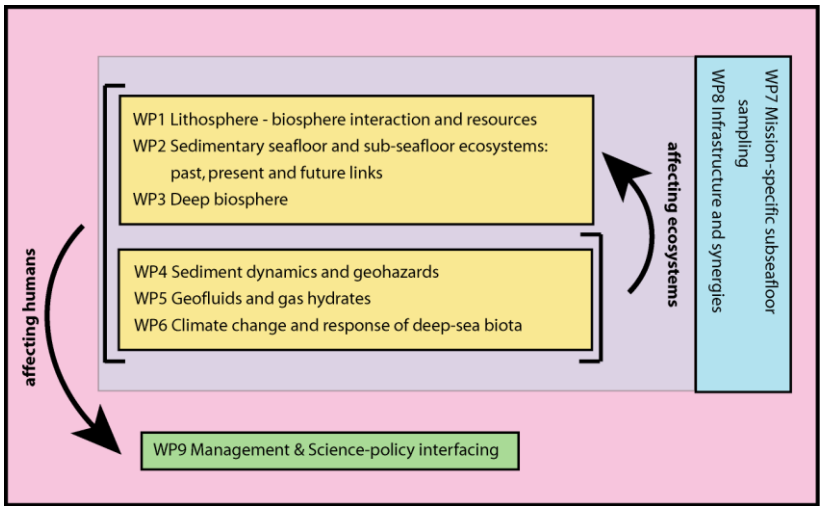
Tuesday, May 3
09:00-17:00
Scientific presentations and discussions at the Center for Geomicrobiology, Aarhus University.
A light lunch is served. Return to hotel after 17:00. Workshop dinner at 19:00.

Wednesday, May 4
09:00-13:00
Open discussions of scientific goals, research opportunities, and funding strategies.
A report summarizing discussions and proposals will be written during and shortly after the workshop. The meeting ends with lunch at 1 pm.

Objectives

(the folloowing information was sent to participants before the workshop)

The objective of this workshop is to discuss and propose future directions of marine deep biosphere research and how this may be more strongly integrated and funded in Europe. We invite a group of active researchers in this field to share their ideas of the major scientific challenges and new opportunities that will move the science forward in the coming years. We wish to strengthen the European engagement in future drilling proposals and expeditions. We invite participants to contribute their opinions and expertise to a report that, together with a series of other reports from workshops during 2010-11, will contribute to a white paper that will provide background information for the planning of the EU Framework Program 8.



During winter-spring 2010-2011, each of the work packages 1 to 8 of DS^{3}F (see graphics) will arrange separate workshops and write a report on their conclusions and

recommendations. These reports are due end of June 2011 and will be attached to the final white paper.

Workshop tasks

The primary task of the workshop participants will be to contribute with information, ideas and suggestions for deep biosphere research in the new IODP and the new EU framework program, both starting in 2013. The product of the workshop will be a written report that summarizes the outcome and the conclusions and provides sufficient background information to support these. The proposed format for the workshop protocol and report is shown below.

It is the responsibility of R. John Parkes and Bo Barker Jørgensen to edit and submit the report. It is the responsibility of all workshop participants to contribute to the writing of the report.

We ask all participants, before the workshop, to consider the main objectives and themes to discuss. In the report, we need to define:

- What is the current state of knowledge?
- What are the key open questions?
- What are the recommendations for future research?
- What are the instrumental and logistic needs for this research?
- What are the infrastructure and funding needs to reach the goals?
- What are the priorities for future drilling sites?

We propose to spend the first morning of the workshop introducing these questions and structuring our discussion. We will work in breakout groups during parts of the workshop in order to discuss specific themes. We do not ask for formal talks, and the workshop is not a time for us to give specific presentations of our data, but we invite you to consider how you may best contribute to the discussion and the report. If you have useful background information and a few graphs that could be useful for discussion or for the report, please bring them along.

For further information, please see the DS^{3}F webpage (**www.deep-sea-frontier.eu**).

2. Participants

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DS^{3}F Workpackage 3 Group photo

From left to right:
Beth Orcutt, Karen Lloyd, Ian Head, Bert Engelen, R. John Parkes, Achim Kopf, Britta Gribsholt, Laura Wehrmann, Kai Mangelsdorf, Pierre Regnier, Kai-Uwe Hinrichs, Mathias Middelboe, Laurent Toffin, Ingunn Throseth, Konstantinos Kormas, Bente Aa. Lomstein, Mark Lever, Hans Røy, Kasper U. Kjeldsen, Nils Risgaard-Petersen, and Bo Barker Jørgensen. (Photo by Luc Riolon).

3. Abstract

During the DS^{3}F Workpackage 3 workshop in Aarhus, 3-4 May 2011, 22 scientists from seven European nations met for two days to discuss marine deep biosphere research and how this may be more strongly integrated and funded in Europe during the coming decade. The group comprised expertise within the fields of microbiology, molecular ecology, geology, organic geochemistry and mathematical modeling. There were no lectures presented but the participants discussed the relevant themes in plenum and in breakout groups. The outcome of these discussions was summarized on the second day of the workshop and writing tasks were distributed among all participants while some participants were appointed rapporteurs with the task of coordinating each chapter.

This report is structured in five chapters corresponding to the five breakout groups. The "Microbial diversity, biomass, physiology, evolution & biogeography" chapter discusses background knowledge about life of the deep biosphere and the open questions to be addressed. The great progress in DNA/RNA and biomarker based techniques has opened up new research on the deep communities that are comprised of basically non-growing cells. Future research shall address to which extent the cells are active or dormant and what is the relationship between the phylogenetic and the functional diversity, on the community level and the single-cell level. New approaches to cultivation and experimentation are needed in order to understand how the predominant archaea and bacteria are adapted to the energy-deprived subsurface world.

The "Microbial activity and link to global element cycles" chapter reports how the pathways of organic carbon degradation and the rates of predominant reactions can be explored by new analytical techniques, by more sensitive experimentation, and by mathematical modeling. The controls on the dynamics and efficiency of organic matter mineralization are a particular challenge as these are critical for the carbon and other element balances on a geological time scale and thereby exert a feed-back on global climate. The "Energy, environment and physiological adaptations" chapter discusses the potential energy sources for the deep biosphere and how these set the energetic limits for subsurface life. Other limits must also be studied in the future in order to understand the full range of adaptations of subsurface microorganisms. Such studies must also address the questions of how to determine the absence of life. The role of viruses needs to be better understood as does the role of the spatial structure of communities and environment.

The "Instrumental, methodological, and logistic needs" chapter argues for improved analytical methods and for high-throughput and automated techniques. Contamination tests remain a critical issue for all deep biosphere research and must be carried out on all relevant drilling cruises. In situ instrumentation is now being developed and will become even more important in the future in order to study and monitor microbial populations and processes over time. Also on-board technology is needed to maintain pressure and temperature in retrieved samples from the deep sub-seafloor.

The participants finally proposed and discussed a number of target sites by which basic deep biosphere questions can be optimally addressed. The "Priorities for future drilling sites" chapter lists five types of such sites that will be particularly important to study during the next phase of the IODP and that will be particular relevant and unique for European deep biosphere research.

4. Microbial diversity, biomass, physiology, evolution & biogeography
Rapporteur: Mark Lever

Our knowledge of the diversity of organisms (bacteria, archaea, eukaryotes, viruses) that inhabit the deep biosphere is still fragmentary. To significantly advance our understanding, we need to improve methods for comprehensive and quantitative studies of microbial diversity, biomass, physiological state, metabolism, distribution, dispersal and biogeography. These efforts will require the design of novel methods as well as the improvement and adaptation of already existing methods. In addition, the inter-calibration and comparability of results obtained by different laboratories from a range of samples needs to be improved. This can only be achieved by standardizing methods. In the following sections we go over examples from the study of microbial diversity, biomass, physiology, evolution and biogeography that we think require particular focus to advance our understanding of life in the deep biosphere.

Microbial diversity

Microbial life is ubiquitous in the marine deep biosphere, pervading into the deepest sediments drilled so far as well as into the oceanic crust. Studies over the past two decades have revealed a high diversity of deep biosphere life with complex prokaryotic communities consisting of hundreds to thousands of different species. This discovery has resulted in new important research questions regarding the extent and origin of diversity, how diversity is linked to space and time, and which organisms carry out key ecosystem functions in the deep biosphere. To reliably address these questions it is necessary to improve existing methods for studying microbial diversity, to design/implement new ones, and to expand sampling efforts.

Retrieval and interpretation of taxonomic marker molecules

Knowledge on deep biosphere prokaryotic diversity mostly stems from cultivation-independent studies of taxonomic marker genes and membrane lipids. The extraction and purification of nucleic acids and membrane lipids from deep biosphere samples is not a trivial task; yet it is pivotal for the exploration of the microbial diversity of this ecosystem. Extraction and purification methods already exist but need to be further improved in terms of their detection limit, and accuracy of quantification. Our current knowledge is biased to an unknown extent by the choice of method and marker molecule. Perhaps for this reason, results obtained by different methods often show poor agreement. Therefore a key challenge for future diversity studies will be to improve methods for robust and quantitative retrieval of taxonomic marker molecules, even with deeply buried and/or ultra-oligotrophic samples.

The performance of extraction and purification methods is likely to vary with the type of sample matrix and thus methods should be modified accordingly. Identifying the biases inherent to individual methods will be particularly challenging but invaluable for cross-sample comparisons. Furthermore, it is important to acknowledge the need for more replication (Prosser 2010; Caporaso et al. 2011). New methodological developments should therefore be compatible with high throughput analysis strategies. Improved replication will lead to more robust analyses of microbial biogeography, spatio-temporal occurrences, and provide clues to the identity of syntrophic partnerships (Chaffron et al. 2010).

Deep biosphere microbial communities live at a very low energy flux. Hence, distinguishing live from dead cells and active from inactive or dormant microbial taxa is very important when analyzing and interpreting microbial diversity data. Special focus should be devoted to resolving the performance of commonly used taxonomic markers (DNA, RNA

and membrane lipids) as proxies for microbial identity, cellular integrity and activity. How do results obtained with these biomarkers differ and why? Do discrepancies result from methodological biases? Are they due to differential preservation of the biomarker molecules in dead and inactive cells? Or are discrepancies indeed informative about the physiological states of cells in the environment?

The implementation and development of methods allowing the analysis of alternative marker molecules, for example proteins and cell wall constituents, as well as validating methods for pure cultures and environmental whole community or single cell extracts represent further important future tasks.

Eukaryotes and viruses

Most studies of microbial diversity in the deep biosphere have focused on the prokaryotic component while eukaryotic and virus diversity has been mostly overlooked. Consequently, the abundance and ecological role of eukaryotes and virus in the deep biosphere is not known.

A recent study applying 18S rRNA as a marker gene for studying eukaryotic diversity in deep marine sediments is promising (Edgcomb et al. 2011). However, PCR primers targeting this marker gene have not yet been thoroughly evaluated and likely need further refinement along with nucleic acid extraction procedures for eukaryotic cells given their potential large size, different cell wall composition and cellular organization as compared to prokaryotes. In addition, the 18S rRNA gene may have insufficient taxonomic resolution for resolving eukaryotic diversity below the genus level. Efforts should therefore be made to assay other eukaryotic taxonomic marker genes that are less evolutionary conserved.

Fluorescence and electron microscopy-based approaches have pointed to the presence of viruses in marine sediments (Filippini & Middelboe 2007). This was recently confirmed by first quantifications of viruses within deep subsurface sediments (Engelhardt et al. 2011; Middelboe et al. 2011). Assessing virus diversity is hampered by several methodological challenges, however. Firstly, of all their small size and the diverse nature of their genome (dsDNA, ssDNA or RNA) may bias their detection by existing nucleic acid extraction methods developed for studying prokaryotic diversity. Secondly, their high genetic diversity has so far prevented the identification of conserved marker genes universally distributed among the virus, which prevents their broad identification by PCR-based approaches. Knowledge on the diversity of virus in the deep biosphere may be advanced through: (i) refined isolation procedures for virus particles and nucleic acids combined with metagenomic approaches for retrieving suitable taxonomic marker gene sequences, (ii) development of proteomic methods for analyzing virus-derived proteins (e.g. capsid constituents), and (iii) analyzing CRISPR regions in prokaryotic genomes or prokaryotic metagenomic fragments (e.g. Anderson et al. 2011).

PCR-based retrieval of marker genes

Most knowledge on environmental prokaryotic diversity is derived from PCR-based generation of 16S rRNA gene sequence inventories. Primer bias is an inherent limitation of PCR-based approaches even for highly conserved 16S rRNA genes (Teske & Sørensen 2008). Continuous re-evaluation and refinement of 16S rRNA gene-targeted primers and the comparative use of multiple primer sets are important to obtain the most accurate PCR-based assessment of prokaryotic diversity and abundance. The micro-diversity of deep biosphere microbial communities remains incompletely resolved and should be studied by implementing and advancing the use of PCR-assays of less conserved taxonomic marker genes (e. g. 16S rRNA-23S rRNA transcribed spacer regions, *rpoB* or *recA*).

Typically, functional traits cannot be reliably linked to taxonomic marker genes. For this reason very little is known about the diversity and abundance of individual metabolic guilds in the deep biosphere. Existing PCR assays targeting functional marker genes (*dsrA* and *B* for sulfate reducers, *mcrA* for methanogens, *acsA* for acetogens, etc.) should be more widely implemented and novel assays targeting key ecosystem functions, e.g. autotrophy, extracellular hydrolysis, or fermentation, should be developed. Finally, functional and taxonomic marker gene-based results should be evaluated in concert.

Ab initio detection of diversity

Metagenomic, metatranscriptomic and metaproteomic approaches hold great promise for future exploration of the deep biosphere microbial diversity. These methodologies allow *ab initio* (primer/probe-independent) determination of taxonomic and functional diversity thus offering a systems biology approach (Ideker et al. 2001) for studying the deep biosphere; and facilitating the discovery of diversity e.g. eukaryotes and virus as well as completely novel diversity (Wu et al. 2011). The implementation of these methods in deep biosphere research is currently suffering from their high demands on the concentration and purity of the nucleic acid/protein samples to be analyzed and the high sampling (sequencing) effort needed for reliably analyzing diverse microbial communities. Methodological advances are being made, however, and first results are promising (Biddle et al. 2011).

Sharing of methods and know-how

In order for efficient sharing of basic methodologies, or of novel methodological breakthroughs, and for communicating across the deep biosphere research communities in Europe and beyond, it would be beneficial to establish an internet-based platform. This platform could follow a similar format as the IODP databases in which ship-based methods for IODP expeditions are shared.

Microbial biomass and physiological state

Among the fundamental objectives in deep sub-seafloor biosphere research are to detect and count prokaryotes, eukaryotes and viruses, as well as estimate their physiological state. A variety of approaches is necessary to assess these objectives. Cell abundance in marine subsurface sediments has conventionally been evaluated by routine microscopic observations of sediments and rocks using Acridine Orange Direct Counts (AODC). These counts have been made on a wide range of ODP sediment cores (Parkes et al. 2000) and have demonstrated that the community sizes of prokaryotic cells decrease with depth and age according to a power law function.

The AODC counting technique requires special training for recognizing and counting cells. Over the past decade, another nucleic acid dye, SYBR Green I, has been found to be more effective due to higher fluorescence intensity and greater specificity to nucleic acids. Using SYBR Green I a new method has been developed that allows discrimination of fluorescence stained cells from background fluorescent signals based on differences in fluorescent spectra and the use automated cell counting (Morono et al. 2008). While this method has greatly improved the through-put, problems have persisted with sediments harboring very low cell numbers, e.g. within ocean gyres. Due to the extremely low cell densities in oligotrophic or ultra-oligotrophic deep sub-seafloor sediments, whole cells need to be extracted and filtered from the sediment matrix prior to counting (D'Hondt et al. 2009). This method may be combined with flow cytometry, which has the advantage over automated microscopic enumerations of being even higher in sample throughput and enabling better counting statistics by large numbers of individual samples and by more consistent criteria for

cell identification. Flow cytometry will therefore result in higher data resolution and more consistent cell counts than obtained previously. This technique has the added advantage of being able to discriminate and also count FISH-labeled cells.

Further information on the distribution of sub-seafloor populations can be obtained by quantitative real-time PCR (qPCR). This method has high throughput once nucleic acids have been extracted. However, due to differential biases of nucleic acid extraction methods and PCR primers (Teske & Sørensen 2008), estimates of relative abundances of bacteria and archaea in sub-seafloor sediments can vary greatly (Schippers et al. 2005; Lipp et al. 2008). Considerable effort will therefore be necessary to develop nucleic acid extraction methods that are exhaustive and PCR probes that have good phylogenetic coverage.

Cell quantifications based on direct counts or qPCR do not reliably discriminate between living, dormant, or even dead cells. The physiological state of microorganisms living in deeply buried sediments has therefore remained questionable. Quantification of viable cells can be done more reliably by fluorescent *in situ* hybridization (FISH) or CARD-FISH techniques, by which living or active microbial cells can be detected using taxon-specific, fluorescently labeled oligonucleotide probes. Spores can be identified independently of cell-counting or other methods of quantification by their dipicolinic acid content. The reliable identification of non-spore-forming dormant cells is currently not possible and represents an important scientific goal for the future.

In spite of the differences in results obtained even with the same general methods of cell quantification, e.g. qPCR, standardization of protocols and techniques are a long-term goal necessary to compare microbial biomass and basic oceanographic, geological and geochemical features. Standardization using pre-specified protocols for biomass quantification will help identify important environmental parameters that control the distribution and abundance of microbial populations.

Cultivation and Microbial Physiology

The gold standard in microbial ecology is still the isolation of indigenous microorganisms as pure cultures for studying their metabolic versatility and for linking this information to ecological questions (Batzke et al. 2007). Unfortunately, the majority of species in a given habitat that were detected by cultivation-independent methods have not been cultivated so far. Due to the extreme conditions in the deep biosphere, the number of isolated microorganisms is very small. The combination of low energy and nutrient availability leads to extremely low growth rates that can not easily be mimicked in laboratory experiments. Thus, the study of microbial energy production and biomass synthesis in the deep biosphere requires new, innovative cultivation-based techniques in combination with molecular methods.

While long-term cultivation over years might be necessary for slow-growing microorganisms, high-throughput cultivation may help to isolate subsurface microbes with a potential to grow fast. Innovative high-throughput techniques may involve culture and isolation procedures based on the combination principle, including the community culture of microbial cells incorporated in gel micro-droplets and followed by sorting and microplate cultivation (Zengler et al., 2002). Then the cell-cell communications may be maintained by using a flow-through culture in parallel micro-bioreactors nourished by community culture medium and metabolite products. The range of substrates used in the enrichment of deep biosphere representatives should be expanded by, e.g. including insoluble compounds or macromolecules, since virtually nothing is known about the microorganisms that are involved in breaking down complex organic matter. These novel cultivation approaches should also include co-cultivation experiments to study syntrophic growth, high pressure, varying redox

conditions (aerobic/anaerobic), or enrichments with or without substrates such as sediment particles or basalt.

By establishing state-of-the-art cultivation-independent methods, the potential metabolism of unknown microbes can be inferred from their genomes or isotopic compositions. Thus, isolations are not always necessary, since enrichments, e.g. when coupled with isotope probing and radiotracer approaches, can be highly informative and help to link microbial identity to metabolic properties. For instance, radiotracer incubations can be analyzed by autoradiography or nano-SIMS in combination with other identification methods such as micro-MAR-FISH (Nielsen et al. 2004), or Gene-FISH (Moraru et al. 2010) or they might be subjected to stable isotope probing (SIP; Radajewski et al. 1999). The isotopic composition of cells and cell components isolated directly from environmental samples can serve as an indicator of microbial physiology.

Single-cell genomics is a potential way to link identity to activity on a cellular level to geochemistry and net ecosystem processes. The identification of specific metabolic pathways might guide novel isolation strategies. However, it remains unknown how representative the analyses of single-cell genomes are to understand larger-scale processes occurring in the environment. While the most abundant cells are most likely to be targeted, keystone species are often rare, and large numbers of genomes might need to be sequenced and annotated to understand the ecosystem. Finally, the inferred genomic potential does not always reliably indicate the reactions a microbe actually performs in the environment. This might be overcome by *in-situ* experiments, e.g. under natural pressure, colonization experiments on natural substrates (e.g. via CORKs), manipulations of substrate concentrations, or the transfer of samples to different environments to observe growth there.

Biogeography and Evolution

In our efforts to understand sub-seafloor ecosystems, we still fall short of answering basic questions regarding the distribution and evolution of sub-seafloor life. What is the maximum depth to which life exists? What is the global distribution and zonation of life across oceanic sediments and crustal habitats, and what does this tell us about their degree of interconnectivity? Are sub-seafloor microbes active colonizers that are adapted to the prevailing conditions, or are they passively distributed, dying remnants of microbial communities adapted to other environments? Could life have originated in the sub-seafloor? Are sub-seafloor ecosystems, like surface environments, characterized by temporal succession and spatial patterns? To address these questions, we will need deeper, and yet contamination-free drilling technologies. Moreover, we will need to investigate oceanic regions that have never been drilled for microbiology-focused purposes.

To obtain an accurate mapping of the deep biosphere, we need to ensure that the description of the species composition is based on DNA from intact cells rather than extracellular pools. Special effort should be directed towards eukaryotes, since most of the current knowledge deals only with prokaryotes. Determining the distribution of eukaryotic life may reveal whether and to what depth there is a grazing pressure on prokaryotes, as well as providing insights to paleoceanography and paleoclimate (e.g. distribution of pollen).

The currently available data stress the need to distinguish between microorganisms that are indigenous to the subsurface (i.e. adapted to the subsurface conditions and therefore metabolically active) and ones that were introduced from other habitats. Detailed geochemical and geophysical description of the prevailing conditions in drilling sites will assist our efforts to identify distinct microbial distribution patterns and provinces (sensu Schrenk et al. 2010). Moreover, we need focused approaches to determine whether the microorganisms are deeply buried survivors of communities deposited on or living at the

seafloor thousands of years ago or whether they were transported from other sediment layers and actively colonized the deep biosphere. Identifying the origin and degree of endemicity of sub-seafloor microbes will help us answer these fundamental questions, in addition to providing clues concerning key organisms across or within different environments.

Adaptations to sub-seafloor conditions could be investigated by in situ approaches (e.g. observatories, see Orcutt et al. 2011). Such approaches should be emphasized and strengthened since they go beyond inferring metabolism and processes and directly test hypotheses in the field. Some of the quests that could be tackled with in situ approaches include temporal succession, competition and competitive outcomes between different microbial species and/or communities under similar environmental conditions, as well as allelopathy.

New ways to study rates of evolution should be examined. Existing molecular clocks may fail or require recalibration with sub-seafloor microorganisms, since time scales and viability tend to be different from those in the surface world. The evolution of genes may provide important clues about the evolution of the organism hosting them, eg. if progressive/linear changes in vertically inherited genes can be detected, or there is evidence for horizontal gene transfer. Certain genes are transferred frequently between organisms whereas others form part of the "core genome" and do not seem to transfer. What, if anything, does this tell us about an organism's physiology, and how does it influence our view of what defines a "microbial species"? Sediment layers that are isolated by geological barriers or form "islands" between layers with discrepant physicochemical conditions, e.g. sapropels, may make suitable model environments for the development of new methods and testing of hypotheses related to the evolution of microorganisms in the deep biosphere.

Ample data exist already on sub-seafloor microbial occurrence in the world's oceans. Hence, considerable insights regarding the distribution of microbes can be gained from meta-analyses of existing, published data. In analyzing these data, caution needs to be taken to account for methodological biases (i.e. sampling and lab analysis), e.g. by applying network analysis, as is already done in surface environments by the Hotspot Ecosystem Research and Man's Impact on European Seas project (HERMIONE, http://www.eu-hermione.net), where the distribution of deep-sea nematodes is examined, a group of animals with no planktonic life stage but yet showing biogeographic patterns. Similar analyses could depict the first overarching/global patterns in microbial distributions in deep sea habitats, if they exist, as well as provide improved interpretation of archived samples and help select sites for further drilling.

5. Microbial activity and link to global element cycles

Rapporteur: Kai Mangelsdorf

With the finding of a widespread and diverse deep microbial biosphere, major questions arise regarding its metabolic activity, the involved physiology, and the role of deep microbial life for the global carbon and other elemental cycles. Compared to surface sediments the rates of microbial metabolic activity in the deep biosphere are orders of magnitude lower impeding the detection of microbial activity in the deep biosphere. Thus, microbial processes in the deep biosphere are operating on a significantly lower level and on different time scales that require the development of more sensitive analytical methods. Little is known about microbial physiologies of the deep subsurface. What metabolic strategies are applied to

obtain sufficient energy for life? How do microorganisms scavenge appropriate substrates from an environment of recalcitrant organic matter? Although the community size decreases strongly with depth, the deep biosphere colonizes a huge space in the subsurface and constitutes a major fraction of living biomass on Earth (Whitman et al., 1998). Due to its huge size, wide dissemination, and diversity of metabolic processes, the deep biosphere plays an important role in global elemental cycles and impacts Earth's climate on geological time scales.

Microbial activity

The influence of the sub-seafloor microbial communities and the relative importance of abiotic versus biotic processes on elemental cycling are poorly understood. This is to a large extent due to a lack of appropriate techniques to quantify microbial activity (respiration, fermentation and assimilation) under realistic deep biosphere conditions. In many cases, conventional techniques are not sensitive enough to detect the microbial activity in the deep biosphere. Two complementary strategies may advance the field: a) inference of microbial activity from inverse modeling of terminal electron acceptors and electron donors, and b) direct measurements of microbial activity by experimental incubations using radioactive or stable isotope labeled tracers. Major constrains are low abundance and low activity of the microbial community (Jørgensen and D'Hondt 2006), incomplete knowledge of the relevant microbial pathways, or insufficient knowledge about the microbial habitat. The determination of microbial activity requires careful sampling of geochemical and geophysical parameters, development of new systems for long term incubations (either in the laboratory or in situ based in natural laboratories) that exert minimal alternations of the environment to be studied, and development and application of new sensitive analytical techniques (e.g., nanoSIMS together with labeled compounds).

Because microbial doubling times are long and reaction rates are low in the deep biosphere (D'Hondt et al., 2002; Parkes et al., 2005), biogeochemical models can be used at spatial and temporal scales that are not accessible through laboratory experiments. Such models can provide quantitative information on rates and kinetics of substrate utilization and of microbial activity (Arndt et al. 2006). They are also well suited to assess the environmental controls on reaction rates and the response of the microbial community to changes in substrate supply (Dale et al., 2008a) and organic matter accumulation rates over geological time scales (Arndt et al., 2009, Marquardt et al., 2010). Biogeochemical models are complementary to the growing ODP/IODP database because they allow reconstruction, based on present-day observations of deep biosphere environments, of the system's evolution over many thousands or millions of years (e.g., Arndt et al., 2006; 2009; Meister et al., 2007). Mathematical modeling of microbial activities and identification of the involved metabolic processes under deep biosphere conditions should therefore be an integrated part of deep biosphere research.

Microbial carbon and energy sources

The analysis of microbial activity in the deep biosphere is often complicated because the relevant microbial pathways are poorly known. The analysis has so far focused on a limited number of well known metabolic pathways (e.g., sulfate reduction, methane oxidation, methanogenesis, iron reduction etc.) and the turnover of a limited number of substrates. These pathways may, however, represent only a narrow subset of the total metabolic diversity of processes that are important in the deep biosphere. Insights provided from microbial molecular ecology (e.g., metagenomics and proteomics) may provide hints to other microbial processes (Chivian et al., 2008) and can help design tools to quantify the microbial activity.

The presence of a widely disseminated deep biosphere raises questions as to the carbon and energy sources of the microorganisms in the subsurface and the mechanisms by which these substrates become available. To most extent, the sedimentary sub-seafloor biosphere extracts metabolic energy from fossil organic material that originates from the photic zone of the oceans. During burial, the original biomolecules are altered and turned into poorly characterized geo-macromolecules by biotic and abiotic processes (Hedges et al., 2000). Below the most reactive layer of the seabed, this so-called kerogen – Earth's largest pool of organic matter – is the central fuel for the deep biosphere. The mechanisms involved in microbially mediated breakdown of kerogen may be key to understand the energy flux available to the deep biosphere. The enzymatic and abiotic reactions that break chemical bonds and thereby release molecules into the pool of dissolved organic matter (DOM) are critical for both the fate of organic matter and for the deep biosphere. Previous studies of carbon flow in the deep biosphere have typically focused on low-molecular-weight compounds known as substrates for terminal metabolism, e.g. acetate, methane, hydrogen, and amino acids (Hinrichs et al., 2006; Heuer et al., 2009). However, their production is presumably intimately linked to the slow degradation of geo-macromolecules (Glombitza et al., 2009a; 2009b), a process that is not yet understood.

This poor mechanistic understanding is also reflected in the quantitative modeling of the complex reaction pathways of organic matter degradation and substrate utilization, which remain limited by the lack of appropriate reaction stoichiometries, rate expressions, and kinetic parameters (Regnier et al., 2011). Theoretical approaches such as the power law or reactive continuum models provide quantitative descriptions of organic matter decomposition during burial, from the shallow subsurface to the deep biosphere. Yet, they do not relate the substrate reactivity to the nature and accessibility of individual compounds or to the microbial and environmental factors that may affect the fate of organic matter. In this respect, a number of recent developments in the field of biogeochemical modeling could become key to an improved quantitative understanding of the sub-seafloor processes and energy fluxes that sustain life in the deep biosphere. Those include expressions that account for the dependence of reaction rates on the energy yield of useable substrates (Jin and Bethke, 2005; La Rowe and Van Cappellen, 2011), explicit representations of the fate of metabolic intermediates such as hydrogen, formate or acetate (Dale et al., 2008b; Orcutt and Meile, 2008; Alperin and Hoehler, 2009), or linking the rate of substrate production/consumption to the growth and decay of different resident microbial populations (e.g. Rittmann and VanBriesen, 1996).

Recalcitrant organic matter

After intense microbial degradation of the sedimentary organic matter in the top layers of the seabed, the residual organic matter becomes increasingly recalcitrant with depth. Less altered kerogen, however, is still rich in small ester-linked fatty acids such as formate and acetate and other potential substrates for the deep biosphere (Glombitza et al., 2009b). These substrates might be available to exo-enzymatic attack, but it is also conceivable that these ester-linked compounds are in equilibrium with the corresponding compounds in the surrounding pore water, thus replenishing consumed substrates by an abiotic process. Molecular characterization of DOM in pore waters of deep sub-seafloor sediments and of the buried kerogen matrix could be key to disentangling relationships between structural properties of organic matter, kinetics of degradation, production of low-molecular-weight substrates, and ultimately the energy flux to the deep biosphere. Ideally, field observations should be combined with laboratory microcosm experiments with sub-seafloor sediments

during which compositional changes of DOM and kerogen are monitored. Promising techniques to tap the information encoded in molecular DOM composition and kerogen include chemical degradation, FT-ICR-MS, and NMR (cf. Hedges et al., 2000; Kujawinski, 2002). First studies of DOM in sedimentary pore water suggest that biogeochemical processes may results in recognizable molecular-level signatures in this pool (Schmidt et al., 2009; 2011). First studies on structural kerogen characterization reveal the potential of kerogen to fuel deep microbial communities with substrates over geologic time (Glombitza et al., 2009b).

Future studies exploring the link between organic matter and the deep biosphere should be guided by the following working hypotheses: The energy flux to the sedimentary sub-seafloor biosphere is closely linked to the kinetics of degrading organic macromolecules down to molecules small enough to be incorporated by a microbe through its cell wall. The kinetics are probably controlled by structural properties of the poorly characterized macromolecular organic matter, the frequency of bond scission by exo-enzymes and abiotic mechanisms, and the abilities of the microbial communities to utilize this pool. In low-temperature sediments, structural modification and degradation of organic matter are closely linked to the activity of microbial communities. Thereby, abundance and quality in terms of bioavailability of the organic matter are essential for the level of microbial activity. Important insights into the mechanisms and compound types affected by microbially mediated modification can be obtained through molecular analysis of the pool of dissolved organic matter and of kerogen. The processes directly influence the partitioning of carbon in several major pools of global relevance, i.e., dissolved inorganic and organic carbon in the ocean and dispersed particulate organic carbon in sediments and rocks.

In deeper sedimentary successions inert chemical bonds within the kerogen matrix are re-activated by rising geothermal temperature (Parkes et al., 2007; Wellsbury et al., 1997) which increases the bioavailability of organic compounds from kerogen. With depth, the first geothermally driven break-down processes (early catagenesis) start at temperatures that are still compatible with microbial life (Glombitza et al., 2009; Vu et al., 2009). Thus, there are indications that in deeper zones geothermal processes may sustain deep microbial life (Horsfield et al., 2007; Zink et al., 2003). In addition to high quality deep sediment cores in specific areas with appropriate geothermal heat flow, long-term laboratory experiments with heating of sediment material from the deep biosphere and kinetic modeling of the generation potential of the buried organic matter are required to elucidate substrate delivery processes and mechanisms in the deeper and warm parts of the inhabited subsurface.

Organic-fueled metabolic activity in the most oligotrophic regions of the deep biosphere is extremely low. It is possible, however, that energy sources other than those derived from organic matter may be important, e.g., CO_2 and H_2 (Pedersen, 2000). One potential source is the radiolysis of water, whereby the electron donor H_2 can be supplied by in situ radiolysis or by transport of radiolytic H_2 from a much deeper, biologically dead environment. Water radiolysis has been described as a potential source of energy for ecosystems in hard rock far beneath continental surfaces (Pedersen 1996, Lin et al. 2005). Also, earthquakes can stimulate the production and release of H_2 from quartz rich granites (Kameda et al., 2003; Bräuer et al., 2005). Dedicated laboratory experiments and in situ measurements are required to elucidate the quantitative importance of radiolysis in order to constrain the role of biological vs. geological substrate delivery in the deep biosphere.

It is also of importance that studies on the deep biosphere include the characterization of the sedimentary environment as the habitat of deep microbial communities. The composition of sedimentary sequences in terms of organic carbon, inorganic ions, fluids, gases and minerals

(electron donors, electron acceptors and nutrients) as well as physical parameters such as porosity, permeability and mineral surface areas have a strong impact on the abundance, distribution and activity of deep microbial communities. Also the history of the sedimentary body is fundamental as it provides information on basin subsidence and uplift and the associated heating history. These have strong impact on the delivery of geothermally generated substrates for the deep biosphere (Horsfield et al., 2007) and on the distribution of the deep biosphere in the subsurface considering the concept of paleo-pasteurisation (Wilhelms et al., 2001). Thus, measurements of geochemical and physical sediment parameters as well as basin modeling are important tools to gain a holistic picture of the deep biosphere.

Impact on the global carbon cycle and other elemental cycles

The influence of the deep biosphere on global elemental cycles is not well understood. However, its widespread distribution in the subsurface suggests that the deep biosphere has a strong impact on global cycles, for instance on the carbon, nitrogen, iron and sulfur cycles. Although operating at significantly lower rates than surface microorganisms, deep subsurface microbial ecosystems control the remineralization of organic matter in the deep subsurface. With the production of gaseous compounds, the migration of these compounds up through the sediments, and finally their emission at the surface, the subsurface "geological carbon cycle" is linked to the surface "biological cycle". Moreover, gaseous compounds such as biogenic methane and CO_2 are important greenhouse gases. Thus, the deep biosphere may have a strong effect on the temperature regime on Earth by interfering with the emission of greenhouse gases or the decay of gas hydrates which are natural collectors of these gases in continental margin and permafrost areas (Kvenvolden, 1999).

6. Energy, environment and physiological adaptations

Rapporteur: Beth Orcutt

Energetics

Currently, only a few types of metabolism have been proposed to support life in the deep marine subsurface. The metabolic pathways that have been shown, through direct measurement of reactants and products, to be energetically favorable include the reduction of oxygen, nitrate, manganese, iron and sulfate, as well as production of methane. These processes are often energetically favorable with small, reduced organic and inorganic molecules such as hydrogen, acetate, or formate as the electron donors. However, the range of substrates available to deep subsurface microorganisms is much broader than this and includes: various fermentation products from the gradual breakdown of organic matter (Lever et al. 2010), reduced seawater ions, transition metals, and a variety of intermediate oxidation states of sulfur, as well as reduced mineral compounds in the oceanic crust. The importance of abiotic organic compounds and molecular hydrogen formed by subsurface water-rock reactions (e.g. serpentinization) compared to that of biogenic material from the surface world is not known (Delacour et al. 2008; Proskurowski et al. 2008; Mason et al. 2010). The potential range of metabolisms present in the subsurface appears to be as diverse as the microorganisms that have thus far only been identified with 16S rRNA gene markers. Therefore, implementation of new measurement procedures to quantify these potential

substrates in deep subsurface environments will be key to understanding their importance to natural microbial populations.

Current microbial detection methods (cell staining, analysis of lipid or nucleic acid biomarkers) do not provide information about the activity level of each individual cell. Given that energy-yielding processes appear to be very slow in the deep oceanic subsurface, it is likely that some of the microbes we can detect are either temporarily or permanently dormant. This makes it difficult to determine the energy available to each living cell. Future modeling studies could address the energetic constraints of cells moving in and out of dormancy vs. maintaining a low but steady metabolic level. Research could also focus on what are the ecological stress factors on microbes that lie dormant for thousands of years. Further research could search for a biomarker that is indicative of the quantity of live cells in the environment.

Alternative energy sources

One of the most important questions in deep biosphere research is what powers and maintains the significant populations of microorganisms that inhabit the deep subsurface? Photosynthetically derived organic carbon that has survived early diagenesis is clearly an important component of the energy budget of the deep biosphere, and there is evidence that burial and moderate heating can result in the biogenic release of labile energy sources such as short chain fatty acids and hydrogen (Parkes et al., 2007). Nevertheless, photosynthetically derived organic carbon is unlikely to be the only source of energy for the deep biosphere, especially under conditions where there is a paucity of organic carbon. One of the prime candidates for an alternative energy source is abiotically generated hydrogen. Several sources of hydrogen to fuel the deep biosphere have been proposed. In addition to biogenic hydrogen from fermentation of organic carbon, organic carbon may give rise to hydrogen from aromatization of saturated ring structures (Parkes et al., 2007; Jones et al., 2008). Potential inorganic sources of hydrogen to drive the deep biosphere include serpentinization of ultrabasic rocks at high temperatures, oxidation of reduced iron minerals iron sulfide formation from pyrite, and fracture-induced reduction of water and radiolysis of water (Lin et al., 2005a). The last of these is considered the most plausible source of hydrogen in many deep subsurface environments (Lin et al., 2005a,b). However there is a need to better constrain the role of different potential sources of reductant as energy sources for deep subsurface organisms in different settings, and also to investigate novel mechanisms that have hitherto received little attention, such as “biomechanical” generation of hydrogen which may provide a novel link between tectonic processes and the deep biosphere (Parkes et al., 2011).

Although hydrogen from radiolytic splitting of water is likely an important energy source in the deep biosphere, an important, but overlooked, aspect of radiolysis of water is that it not only generates hydrogen but also oxidizing species such as hydrogen peroxide and even oxygen (Bjergbakke et al., 1989). The quantities of oxygen produced may be comparable to the amounts of hydrogen generated (Draganic, 1991). Since most subsurface environments are anoxic, oxidized species generated from radiolysis of water are assumed to be rapidly consumed by oxidation of reduced sulfur and iron minerals, thereby maintaining anoxic conditions (Lin et al., 2005b). In addition to abiotic production of oxidized sulfur and iron-based electron acceptors, the oxidizing species generated from radiolysis of water may also support the growth of aerobic organisms in nominally anoxic subsurface environments. The role of such oxidized species in the deep subsurface is currently unknown and both theoretical and experimental analyses will be required to establish the potential for the deep subsurface to harbor a “cryptic” community of aerobic microorganisms (Parkes et al., 2011).

What are the limits on life, how deep does life go, and can we detect the absence of life?

The foremost questions in deep biosphere research are highlighted in the IODP draft science plan: What are the limits of life in the sub-seafloor biosphere? How deep and how extreme does life persist? Are there places in the sub-seafloor where life does not exist? If so, what explains the absence of life – high pressure, high temperature, absence of essential nutrients, carbon and/or energy, extremes in pH or salinity, or other factors? Scientific ocean drilling is required to evaluate the influence of some of these factors, such as high pressure (which requires deep drilling). Other types of seafloor and subsurface sampling may assist the evaluation of others – such as seabed rock drilling in high temperature environments, or deep piston coring in organic-poor sediments.

To assist in the evaluation of these factors, new or improved tools and techniques are needed, as well as dedicated microbiological sampling. Improved coring devices designed to recover high-pressure and high-temperature samples under *in situ* conditions for laboratory manipulations, similar to the DeepIsoBUG (Parkes et al. 2009) and new high-temperature/pressure incubation vessels (Takai et al. 2008), would be invaluable for evaluating the influence of high temperature and high pressure on deep life. As any evaluation of the absence of life will require strict contamination controls during sample collection and handling, microbiologists need to have leading roles in designing coring campaigns and in evaluating sampling devices for improvements to avoid contamination. Limits of detection in molecular methods such as cell counting and DNA extraction need further improvement to lower the limits of detection.

On the subject of improved detection limits, the scientific community needs to critically evaluate whether we can really know if life is absent or if it is just below a detection limit. Significant advances in cell separation techniques for cell counting have improved the limits of detection of this method to roughly 1000 cells per cubic centimeter of sediment (D'Hondt et al. 2009). It is not clear whether this method can be improved further to get a statistically reliable count on the order of 100, 10, 1 or no cells per cubic centimeter. Likewise, improvements are needed in DNA and lipid extraction methods to improve yields from low biomass samples.

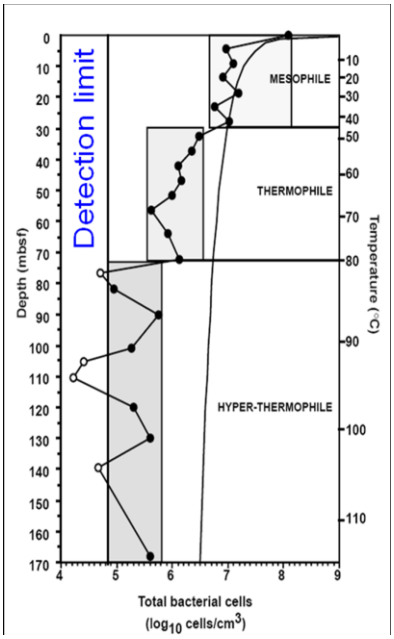

Figure 6.1. Cell counts from Juan de Fuca sediments showing ca. 10^5 to 10^6 cells/cm^3 in deep hot sediments

The currently established thermal limit for life is 121-122^{o}C (Kashefi et al., 2007; Takai et al., 2008). However, it is not clear whether microbial life in deep subsurface sediments can survive the extremes that support life in near surface environments or hydrothermal vents. For example, empirical data on the occurrence of biodegraded petroleum reservoirs suggest that reservoirs hosted in sediments that have been heated to only 80-90^{o}C are not biodegraded (Wilhelms et al., 2001) suggesting that the indigenous microbial communities are inactivated at this temperature. This is the basis of the paleo-pasteurization hypothesis. Furthermore, there are few reports of deep subsurface hyperthermophiles that have been isolated at temperatures greater than 90^{o}C (Grassia et al., 1996) and, interestingly, methanogenesis and sulfate reduction could be measured only at temperatures between 70 to 83^{o}C in produced waters from Californian petroleum reservoirs and not at higher temperatures, even when the temperature of the reservoir from which the samples came was up to 120^{o}C (Orphan et al., 2003). Nevertheless, in deeper sediments which experience temperatures in the range for hyperthermophiles, substantial cell numbers have been observed (Parkes et al., 2000; Figure 6.1.).

One reason for modulated maximum thermal limit for life in some subsurface sediments may be that the rates of metabolism that can be sustained in a metabolically constrained system do not allow for the regeneration of labile molecules involved in conservation of energy (e.g. ATP and NADH) sufficiently quickly to support the maintenance energy requirements of cells (Wilhelms et al., 2001). It has been shown that cells with low metabolic activity have reduced tolerance to environmental extremes (Lloyd et al., 2005). This may be exacerbated by additional energy requirements to maintain cell integrity in a chemically demanding environment such as crude oil. There are many other extremes found in deep subsurface sediments that may also conspire to limit the tolerances of microbial life and it is known that interactions between different environmental factors can act to increase or decrease an organism's sensitivity to other environmental stressors (Edgcomb et al., 2004; Figure 6.2.).

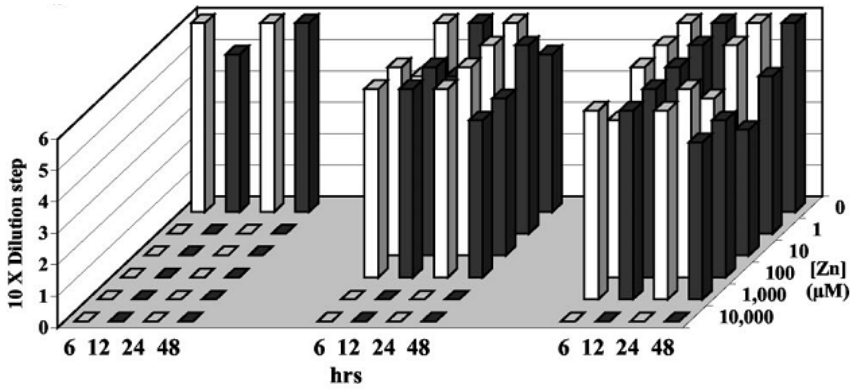

Figure 6.2. Effect of sulfide on survival of a *Pyrococcus* sp. after exposure to increasing concentrations of Zn for 6, 12, 24, and 48 h. The portion of surviving cells equals the number of 10-fold dilution steps from 1 to 6 (6 equals 100% survival) of the culture that regrew after exposure. Data from three parallel experiments with different levels of sulfide exposure are shown: Left; no sulfide added. Centre; 0.4 mM sulfide added. Right; 2 mM sulfide added. High sulfide results in precipitation of metal sulfides reducing the effective metal concentration and increasing the metal tolerance of the culture. From Edgcomb et al. 2004.

The effects of such interactions are important to define the range of deep subsurface habitats that are likely to be inhabited by active microorganisms. They are also important to determine the balance between the energy available from the thermodynamic disequilibrium of an environment relative to the energy requirement to combat the detrimental effects of environmental stressors (Hoehler et al., 2007). Thus, it is important to understand the interactions between factors such as temperature, pressure, salinity, metal concentration, and hydrocarbon concentration in limiting the habitability of different regions in the deep biosphere.

Viruses

Viruses have recently been found to occur in high abundance in the deep biosphere, decreasing with sediment depth from 1 x 10^8 viruses cm^{-3} at 4 mbsf to 5 x 10^6 viruses cm^{-3} at 96 mbsf, corresponding to an increase in sediment age from 0.5 to 2 Ma (Middelboe et al. 2011). In surface sediments, viruses are known to cause significant mortality to bacterial communities with implications for bacterial activity and benthic nutrient cycling (e.g. Middelboe & Glud 2006, Danovaro et al. 2008), and the production and abundance of viruses are closely correlated with benthic mineralization rates (Middelboe et al 2006, Middelboe & Glud 2006). The conditions for virus-host interactions and for virus production and dispersal are, however, fundamentally different in deep sediments with extremely low metabolic activity and limited microbial mobility. The presence of viruses in the deep biosphere thus raises questions regarding their origin, activity, fate and impact on the microbial communities in these environments. Is there sufficient energy available to sustain a virus production in such environments or are the viruses remnants from active surface sediments that have been permanently buried? What are the mechanisms and strategies for the production and persistence of deep biosphere viruses? How do viruses and prokaryotes interact in these environments (lytic infections, lysogenic induction)?

If we assume that the observed decline in viral abundance with depth in the deep biosphere (Middelboe et al. 2011) reflects a gradual decay of the viral assemblage over time, then the estimated decay rate of the viral community is 1.2 (± 0.3) × 10^{-6} y^{-1}, corresponding to a half-life of the viral community of 5.8 × 10^5 y. It is likely that part of the viral community in the deep biosphere represents extremely old viruses that were produced several hundred thousand years ago in the upper 20-30 m of the sediment and that subsequently became protected from decay and essentially permanently buried in the deep biosphere. However, this does not exclude a production and decay of viruses that occur at a time scale of months or less, sustained by a small and active prokaryotic community in the deep biosphere. Consequently, it is not known to what extent viruses in the deep biosphere are active controllers of microbial community dynamics and carbon cycling, as they are in surface sediments, or whether they merely represent a dead end for benthic viruses.

Deep biosphere viruses possibly represent a giant reservoir of relict viral DNA, and the exploration of this pool may contribute significantly to our understanding of microbial evolution over geological time scales. We propose, therefore, to perform metagenomic analyses of deep viral populations in combination with characterization of specific viral isolates to reveal the genetic composition, origin and diversity of deep biosphere viral communities.

Only very few studies have investigated deep biosphere viruses, and our current knowledge of the production and fate of viruses in the deep biosphere does not provide answers to even fundamental questions about their role as regulators of prokaryotic activity and population dynamics, or their genetic diversity and origin. Exploration of deep biosphere viruses therefore represents an important challenge for future research.

Spatial structure is important

Cells in surface sediments are on average only a few cell diameters apart, many are motile, and cell-to-cell communication is of vital importance. Cells in deeply buried sediments are two to six orders of magnitude more sparse, and the mean energy flux per cell does not allow motility. Thus, most of the deeply buried sediment is not in direct contact with organisms on a micro-scale and presumably the organisms are not in direct contact with each other. Current research on microbial diversity and ecophysiology treat the sediment as a homogeneous matrix which is clearly not the case. Research should be focused on understanding the interactions between microorganisms, between microorganisms and their viruses, and the interactions between microorganisms and their substrates on the spatial scale of the individual cells. We need to consider whether cells are clumped around micro-scale 'hot spots' of activity or evenly distributed, which substrate compounds are adsorbed versus available, and what the spatial separation between producers and consumers of intermediate substrates mean for kinetic or thermodynamic controlled interactions. The distribution of cells could be visualized in intact sediments, for example with techniques like SEM, SIMS, or optical microscopy, and the resulting data could be coupled to cell metabolism via measurements and numerical simulation. Knowledge about the mobility of organisms is of vital importance for such studies since this will constrain the access to immobile substrates and constrain the exchange of organisms, and thus genes, across sediment depths.

7. Instrumental, methodological, and logistic needs

Rapporteur: Beth Orcutt

Recommendations for a European drilling network

Cross-site evaluation of data from deep biosphere samples would be strengthened by the development of standardized methods that are routinely applied. Standardized sampling parameters (using specific protocols) would allow comparison of microbial communities in relation to basic oceanographic, geological, and geochemical features. Standardization of methods and data would also help to determine which environmental factors are more important in structuring microbial communities. Parameters such as total cell counts should be integrated into the set of routinely measured parameters during each ocean drilling expedition to broaden the database of such basic and important parameters. This would require training shipboard technicians in the methods for analysis, and education of the scientific community to request and evaluate the data. A workshop to develop and define the standard parameters and protocols to use would greatly benefit the scientific community.

The study of sub-seafloor life depends on the support of government agencies in Europe to acquire ship time and access to large scientific instrumentation such as ROVs and submersibles. For example, the EU project EUROFLEETS provides access to European research vessels and associated equipment for marine scientists in Europe. Implementation of standardized sampling protocols within this fleet should be recommended to enable a broader suite of cross-comparisons.

Improvements in analytical methods, automation, and high-throughput techniques

Standardized contamination tests on board

The prevention of contamination during sampling is paramount to accurate down-stream geochemical and microbiological analyses. Drilling fluid contamination can be

quantified using perfluorocarbon tracers (PFT), which are supplied to the drilling fluid at constant concentration and subsequently analyzed by headspace gas analyses of sediment samples. Alternatively, fluorescent microspheres can be used. A bag containing these microspheres is placed above the core catcher inside the core liner and breaks immediately once the core penetrates the liner during coring. Microsphere presence/absence within samples is determined microscopically (Smith et al. 2000a).

The PFT method is far more sensitive and time-efficient than the microsphere method, and has the advantage of being quantitative. A protocol designed by Smith et al. (2000b) and modified by Lever et al. (2006) has shown to be effective. However, this method is not being implemented routinely during drilling operations. As a result, IODP technicians are not being trained to carry out PFT analyses, and shipboard scientists spend precious time familiarizing themselves with the gas chromatograph on board. By training IODP technicians to perform PFT quantifications, analyses could be carried out from the very beginning of cruises, enabling shipboard geochemists and microbiologists to save valuable time by focusing on samples that have been show to be clean.

So far, contamination tests using PFT can only be performed during IODP drilling operations. For samples obtained by gravity coring or drilling not involving the pumping of drilling fluid (e.g. the MeBo (Meeresboden-Bohrgerät) of MARUM Bremen), the use of microspheres is more feasible. This method could be improved by using microspheres that can be more clearly distinguished from cells and background than those used in the past (e.g. Smith et al. 2000a) and by use of automated microscopic (e.g. Morono et al. 2008) or flow cytometric methods for faster analyses of samples. Furthermore, novel analytical targets serving as reliable proxies for seawater contamination may be identified.

Wider range of analytes & analytical tools

Currently the range of geochemical compounds analyzed routinely is narrow. Many key organic compounds that may play important roles in carbon turnover, microbial energy production and/or biomass synthesis, e.g. amino acids, carbohydrates, alcohols, methyl sulfides and amines, are not quantified. For some of these compounds, useful methods for analyses exist but are not or are only rarely implemented, for example for amino acids (e.g. Mitterer 2006). For other potential key compounds, such as complex polymeric compounds, carbohydrates and alcohols, there are no methods with sufficient sensitivity for quantification at natural concentrations in the deep biosphere.

A focus of geochemical analyses in the near future should therefore be, a) the implementation of existing methods to quantify a wider range of chemical species, b) the lowering of the detection limits of existing methods to allow quantifications at lower concentrations and smaller sample volumes, and c) the development of methods for analytes that are commonly ignored, such as alcohols, monosaccharides, or methylated compounds. The relative importance of these compounds will remain unknown until we can quantify them.

An analytical tool that holds great promise for the future is the development of non-destructive microsensor-based methods for quantifying analytes. This approach facilitates direct measurements on retrieved cores, thereby reducing biases associated with pore water extraction, such as chemical oxidation and loss of volatile and gaseous compounds. To save resources, it would be useful to share instruments within a consortium of laboratories studying deep biosphere processes.

High-throughput

Currently, analytical time imposes a constraint on the number of samples that can be analyzed. As a result, studies with high degrees of replication are often not feasible or can

only be achieved by compromising the number of sites or depths analyzed. Therefore, a greater effort in deep biosphere research needs to be made to (re)design or improve geochemical and microbiological methods, so they can be carried out at much higher throughput by scientists or via automated methods. The use of auto-analyzers needs to be expanded to a wider range of chemical analyses, for whole cell extraction and total cell counts, as well as nucleic acid extraction and amplification. Much can be learned from other fields, e.g., medical research, where automated methods are already used in high-throughput nucleic acids-based approaches.

Instrumental needs for the future – *in situ* technology

Ultimately, it will be necessary to confirm laboratory-based geochemical and microbiological analyses using instruments that can accurately quantify and characterize the same analytes under *in situ* conditions. Over the past decade, significant technological developments have been made in the fields of *in situ* analyses and experimentation relevant to deep biosphere research, including the use of long-term subsurface observatories such as CORKs for studying microbial life in deep oceanic crust (Cowen et al. 2003; Fisher et al. 2005; Orcutt et al. 2011), and the development of special sampling devices for retrieving deep sediment samples (Parkes et al. 2009). To further advance our understanding of microbial activity and community dynamics in the deep biosphere, our scientific community would benefit from improved *in situ* technologies for measuring a greater range of physical, chemical and biological properties and conducting short- and long-term experiments and monitoring. Development of new or improved *in situ* tools for deep biosphere research - such as *in situ* mass spectrometers (for measuring gas concentrations; Wankel et al. 2010), *in situ* voltammetric sensors (for measuring a suite of redox species; Glazer and Rouxel, 2009); and *in situ* sensors (for quantifying cell densities by deep UV fluorescence; Bhartia et al. 2010) – will expand the range of parameters that could be measured *in situ* with minimal sample disturbance. Further refinement of such technologies for inclusion on wireline logging tools (for use during scientific ocean drilling) and for long-term autonomous monitoring would greatly enhance our understanding of processes in the deep biosphere. Building on the initial successes of long-term observatories in the oceanic crust, future development of long-term sediment observatories is also essential for studying processes *in situ*. By partnering with scientists in other fields, such as paleoceanographers that are developing sophisticated long (~50 m length) piston coring devices (as summarized in the DS3F WP7 report), we may be able to achieve these objectives.

To achieve our long-term goal of studying and monitoring microbial populations within intact sub-seafloor sediment and oceanic crust, further technological advances will be necessary. For example, we would benefit from a suite of submersible robots that can extract and preserve cells or nucleic acids, or methods allowing online monitoring of cell identity and possibly gene expression, such as microfluidics-based DNA chip technologies or *in situ* DNA sequencers. Similar technologies are in development for conducting *in situ* molecular biological analysis on water samples with the ESP instrument (Scholin et al. 2009). Such innovations need to be brought into the deep biosphere. To study rates of biogeochemical processes, devices by which radioisotope labeled substrates can be injected into deep sediment or crust and turnover measured *in situ* will be crucial, as will be equipment with which stable-isotope labeled substrates can be released and stable isotope incorporation into metabolites and biomass can be measured *in situ* using mass spectrometers and/or nanoSIMS.

Recommendations on sampling and storage methods

To analyze *in situ* characteristics of sub-seafloor environments the development of advanced sampling technologies and of storage and incubation systems are required. Development of a pressure- and temperature- maintaining coring system is required to access, for example, depths greater than 2500 m below the sea surface. To this end, construction of an onboard high-pressure core transfer system equipped with multiple (micro-) sensors, gas and fluid extraction ports, and tracer injection systems is needed. As ship-board based analyses of retrieved material is not always possible, new technology that enables shipping and storage of live biological material under conditions that maintain *in situ* properties is essential.

8. Priorities for future drilling sites

Rapporteur: Karen Lloyd

The combined efforts of the Integrated Ocean Drilling Program (IODP), the Ocean Drilling Program (ODP), and the Deep Sea Drilling Program (DSDP) have covered much of Earth's oceans (Figure 5.1), however, only a handful of these expeditions have included a significant deep biosphere component. Many of these previously-drilled sites could be revisited for studies with a microbiological emphasis. A few sites are particularly interesting to the European deep biosphere community, and will be discussed below. This it not intended as an exhaustive list of sites that are important for deep biosphere research, but rather some examples of areas of potentially high scientific impact.

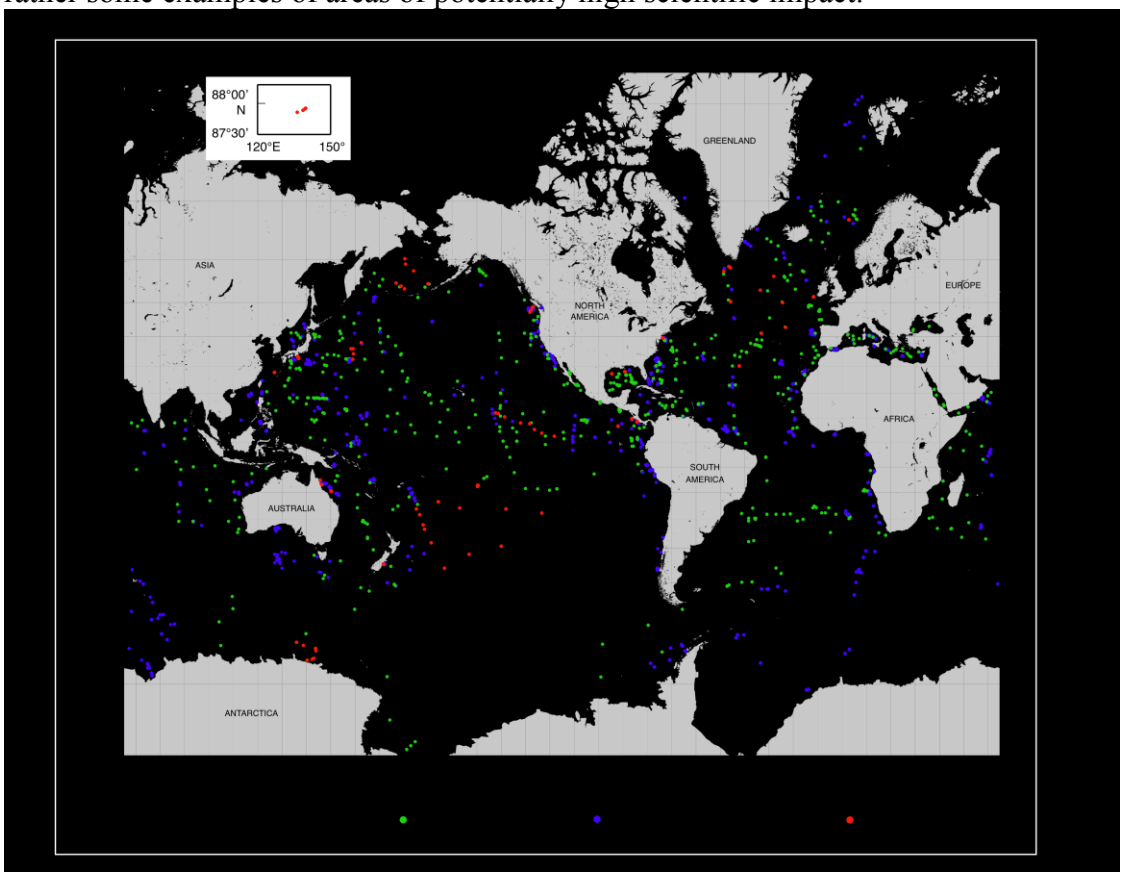

Figure 8.1. Map of all previous IODP, ODP, and DSDP drilling sites.

Mediterranean and Black Sea

The Mediterranean and the Black Sea should be hot spots for European drilling activities due to their proximity and the wide variety of contrasting environmental features that can be discovered within a fairly close spatial range. The sediments at different sites include active mud volcanoes, brines that derive from evaporates, or periodically occurring sapropels. The Black Sea can be seen as a modern analogue of the Mediterranean in times of sapropel formation during deep-water anoxia. In an expedition similar to ODP leg 201, which was the first leg dedicated to microbiology, microbial abundance, diversity and the response of indigenous microorganisms to the conditions in these extreme habitats should be explored.

Mud volcanoes are generally out of chemical equilibrium exhibiting very low microbial activities but seem to stimulate growth of unique microbial communities near the seafloor (Niemann et al. 2006). Their analysis offers a direct look into the deep biosphere as mud, fluids, gases and probably microorganisms are rising up from deeper reservoirs. However, it has never been shown whether the detected species have survived the sediment maturation processes and have been pushed up from below.

Very ancient microbial communities might be found within or below the Mediterranean brine pools (Sass et al. 2001). So far, it is unknown how the constant high salt concentration in the evaporites affects life in the deep subsurface. Deep drilling would be necessary to discover whether microorganisms could exist under these hypersaline conditions.

Mediterranean sapropels represent stepping stones back in Earth's history, a history that can be accurately dated. They are organic-rich sediment layers with organic carbon contents of 20-30% that are formed after regional climate changes that appear due to astronomical cycles approximately every 21.000 years. They are embedded in organic poor intervals that are deposited at times similar to today's climate. Elevated cell counts, determined on ODP legs 160 and 161, indicated elevated microbial activities in sapropels, even in the deepest sapropel layers sampled (Parkes et al. 2000). In some cases, deep sulfate intrusions might additionally drive life within the different zones. Unfortunately, cell counts are the only microbiological data available from the Pliocene sapropels since the previous ODP expeditions were primarily paleoceanographic cruises. High microbial activity was demonstrated by using advanced methods to analyze younger sapropels that are accessible by gravity coring (Coolen et al.2004; Süß et al. 2008). Diversity studies in this material have identified a specific microbial community (Süß et al. 2004; Süß et al. 2008). However, it is unknown if the detected microorganisms have adapted after burial or if allochthonous organisms took advantage of the new environment as conditions have changed. Thus, a revisit to previously drilled sites would offer the opportunity to look with improved methodology at subsurface communities in organic-poor and organic-rich layers that have developed over millions of years.

Deep drilling into the Black Sea will provide a unique access to sediments that were buried in times of dramatic environmental changes over the past two million years. Similar to the Baltic Sea, the water column has varied between limnic and marine and even hypersaline settings. These changes have influenced microbial communities within the sediments due to large differences in organic matter supply. While a sapropel is currently formed, an Eemian sapropel that has a counterpart in the Mediterranean S5 strata was found only one time by the former DSDP drilling. Recently, this app. 120,000 years old sapropel was recovered by gravity coring at a steep slope off the coast of Turkey. The first microbial analyses indicate similarities in community composition between the Mediterranean S5 and the Black Sea

Eemian sapropels. Whether this holds for older sapropels can only be determined by deep drilling. Furthermore, from the large number of hydrocarbon seeps at the seafloor it can be inferred that highly active communities are degrading deeply buried organic matter within the deep biosphere of the Black Sea.

Baltic Sea Basin

The Baltic Sea Basin is one of the world's largest intra-continental basins. It has served as depositional sink throughout at least the last several hundred thousand years, and its sediments comprise a unique high-resolution archive of the paleoenvironmental history of the huge drainage area, the basin itself and neighbouring sea areas. The recurrently waning and waxing of the Scandinavian Ice Sheet has resulted in a complex development, characteristic for many glaciated regions of the Northern Hemisphere: repeated glaciations, sensitive responses to sea level and gateway threshold changes, large shifts in sedimentation patterns and high sedimentation rates. Its position makes it a unique link between the Eurasian and the NW European terrestrial records and as such also serves as a link to North Atlantic marine records and Greenland ice cores. Some of the sediments can be resolved on interannual timescales which makes the Baltic Sea unique for sampling sediments from the last glacial cycle. Decades of marine geological and geophysical research in BSB have given a good understanding of the thickness and distribution of the Quaternary deposits, but no deep drillings for scientific purposes have been performed.

Scientific drilling in the Baltic Sea will provide unique possibilities to study several of the basic deep biosphere questions:

- How has the alternation between a) limnic, brackish and marine conditions, b) oxic and suboxic/anoxic conditions, c) low and temperate temperature, or d) low and high organic sedimentation, controlled the prokaryotic communities and the biogeochemical processes in the seabed?
- Are microorganisms that presently live in the deep sediments remnants of these limnic and marine populations or are they selected by the modern sedimentary environment?
- Do chemical and genetic fossils (i.e. biomarkers and DNA) of the original prokaryotic organisms persist today and are they useful as paleoceanographic indicators?
- Which biogeochemical processes predominate today in the glacial and interglacial deposits, what are their rates, and which are the microorganisms carrying them out?
- How does the phylogenetic diversity of the deep biosphere in this intra-continental sea differ from that of deep open-ocean communities?

Specific Baltic Sea goals will be to understand how the environmental and depositional history of the Baltic Sea system through the Saalian, Eemian, Weichselian and Holocene has affected the phylogenetic diversity of the microbial communities. This will be addressed by analyzing the microbiological and biogeochemical responses to major shifts: a) between limnic, brackish and marine phases, b) between high and low deposition of terrestrial vs. marine organic and clastic material. A special challenge will be to understand how the post-glacial diffusive penetration of conservative seawater ions has altered the chemistry and the microbial physiology in the sub-seafloor biosphere.

Scientific drilling in the Baltic Sea calls for a mission specific platform and is an ideal drilling mission for the ECORD. A Baltic Sea proposal, "Paleoenvironmental evolution of the Baltic Sea basin through the last glacial cycle" (lead proponent Thomas Andrén) is pending by the IODP and has been forwarded to the Operations Task Force for potential implementation.

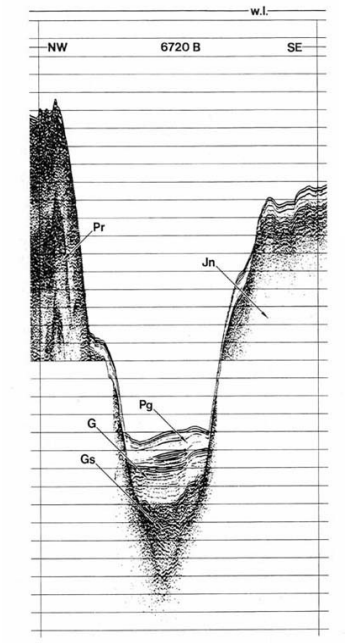

Figure 8.2. Seismic reflection profile (airgun, frequency interval 250-500 Hz) crossing the northern part of the Landsort Trench (NW to SE).

Pg=Postglacial sediments
G=Late Glacial sediments
Gs=Glaciofluvial sediments and til

Total thickness of the Gs unit is ca 80 m.

Potential Arctic deep drill sites

Multiple objectives relevant to WP3 and other WPs could be accomplished with deep drilling in Arctic regions, e.g., off Greenland, Siberia, and Scandinavia. For example, the relationship of sea-ice cover on sub-seafloor microbial communities and their activity and physiology could be constrained in a transect from permanently ice-covered to temporally ice-covered to ice free sites with similar water depth (cf. Boetius and Damm, 1998). Quantitatively and qualitatively distinct fluxes of organic matter are expected to influence sites in such a transect and make it possible to identify relationships between these variables and sub-seafloor life.

Another target in Arctic regions are submarine permafrost deposits with gas hydrates that are subject to increasing destabilization due to global warming. Synergies with the International Continental Drilling Program (ICDP) could be exploited by combining drilling in nearby marine and terrestrial sites. Scientific problems to be tackled range from the impact of destabilizing hydrate on subsurface geochemical processes and microbial communities to the quantification of methane release (cf. Koch et al., 2009). Another highly interesting topic is the link between the deep biosphere and climate and oceanography. The Arctic has experienced dramatic changes in temperature and salinity that are recorded in sub-seafloor sediments (Brinkhuis et al., 2006; Sluijs et al., 2006). Do deep biosphere communities in the respective sediment horizons reflect such dramatic changes in climate and oceanography of the Arctic region?

Arctic mid-ocean spreading ridges

Whereas sulfur-based metabolisms of black smokers systems are well known, it has more recently been suggested that oxidation of Fe(II) released by water-rock reactions may be a principal energy source for an extensive low-temperature, basalt-hosted deep biosphere. Furthermore, the finding of hydrogen and methane production by ultramafic rock-water reaction implies that H_2 or CH_4-based chemosynthetic ecosystems may be widespread in the deep oceanic subsurface where water interacts with ultramafic rock. There is also new

compelling evidence for abiotic synthesis of organic compounds in subsurface hydrothermal environments where ultramafic rocks are involved.

The ultraslow Arctic Mid-Ocean Spreading Ridges (AMOR) provide exiting future drilling sites in both basaltic and ultramafic rocks where these geochemical and microbial subsurface processes can be studied. Due to the slow spreading rate these ridges consist of both volcanic active basalt segments as well as segments with no volcanic activity where ultramafic mantle rocks (peridotite) have been brought to the seafloor by tectonic processes. The variability in thermal gradients and rock types in these regions is the basis for a variety of hydrothermal systems and subsurface environments.

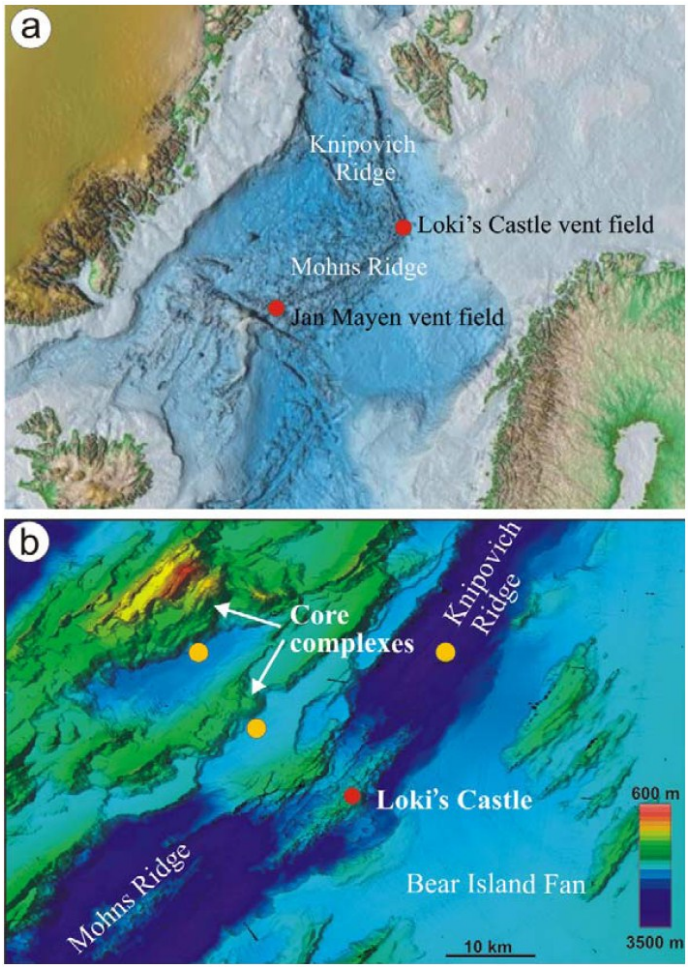

Figure 8.3. a) Location of the recently discovered hydrothermal vent fields at the arctic Knipovich Ridge and Mohns Ridge in the Norwegian-Greenland Sea. b) Proposed drilling sites (yellow spheres) at the southernmost Knipovich Ridge for studies of lower crust and mantle rocks (core complexes) at the NW flank of the ridge, and sedimented zero-age crust in the rift valley to the north of Loki's Castle. The drilling site are based on results from the EUROMARC project "H2DEEP - Ultra-slow spreading and hydrogen-based deep biosphere: A site survey proposal for zero-age drilling of the Kniopvich Ridge" (2007-2012). The objective of the project is to lay the ground for a European lead IOPD proposal aimed to test hypotheses on the geodynamics and the extent and nature of hydrothermal activity, water-rock interactions and the deep biosphere at the ultraslow end of the spreading rate spectrum.

The Knipovich Ridge is one of the AMOR in the Norwegian-Greenland Sea (Fig. 4a) and represents one of the slowest spreading ridge segments on Earth. Because of the proximity to the continental margin it is partially covered by sediments. In 2008 the

northernmost black smoker hydrothermal vent field (Loki's Castle) was discovered here at a water depth of 2400 m (Pedersen et al. 2010a). The field is located on an active volcanic segment that is bordered by a tectonic terrain dominated by lower crust and mantle rocks to the NW, by a ridge flank that is buried by continental sediments from the Bear Island Fan to the SE, and by a sediment-filled rift valley basin to the north (Fig. 4b). Fluid compositions are anomalous to other basalt-hosted vent fields and indicate interactions with sediments buried below the volcanic rocks. Evidences for an increased geothermal gradient in the sediment basin to the north suggest underlying magma or hot rocks. The sediment cover in the Knipovich Ridge area provides a unique opportunity for drilling zero-age ocean crust, which potentially may provide groundbreaking new insight as to the existence of a hydrogen-based deep biosphere sustained by the formation and alteration of oceanic crust and mantle by ultraslow spreading.

An additionally interesting drilling site is located at the Jan Mayen vent fields at the southern part of the Mohns Ridge, another of the AMOR in the Norwegian-Greenland Sea (Fig. 4a). A shallow basalt-hosted white smoker field is here surrounded by diffuse low-temperature venting areas where hydrothermal fluids escape through basalt talus and hyaloclastites (basaltic glass sediment) in the rift valley (Pedersen et al. 2010b). This area represents a unique opportunity for shallow drilling to study geochemical and microbial sub-seafloor processes in a basaltic habitat at a range of temperatures.

Spent North Sea oil reservoir

Petroleum reservoirs are potential hotspots for life in the deep biosphere and the widespread occurrence of biodegraded heavy oil reservoirs is the most significant manifestation of the activity of the deep biosphere on a global scale over geological time (Head et al., 2003). Despite this, our understanding of the interactions between the subsurface biosphere and geosphere in petroleum reservoirs is rudimentary. This is a significant omission in deep biosphere research given the enormous economic and societal importance of petroleum as a source of energy and chemical feedstocks.

There have been relatively few studies of heavily biodegraded petroleum reservoirs (Grabowski et al., 2005; Hubert et al., 2011) yet most of the world's petroleum is biodegraded (Head et al., 2003) and consequently we have no systematic understanding of how microbial ecology and biogeochemistry differ between biodegraded and non-degraded petroleum reservoirs (Head et al., 2010). It has been demonstrated empirically, on the basis of geochemical signatures characteristic of biodegraded oil, that oil reservoirs that have been heated to greater than 80^{o}C during their geological history are not biodegraded (Willhelms et al., 2001) and petroleum provinces where reservoirs are buried to different depths and temperatures showing gradients of biodegradation associated with burial history would be ideal for determining how temperature shapes microbial communities and affects crude oil biodegradation. Peace River in Western Canada is one such province (Adams et al., 2006) and in Europe, the North Sea Gullfaks field shows a strong gradient of oil biodegradation from North-East to South-West in reservoirs with contrasting thermal histories (Horstad et al., 1992; Willhelms et al., 2001; Jones et al., 2008; Figure 8.4).

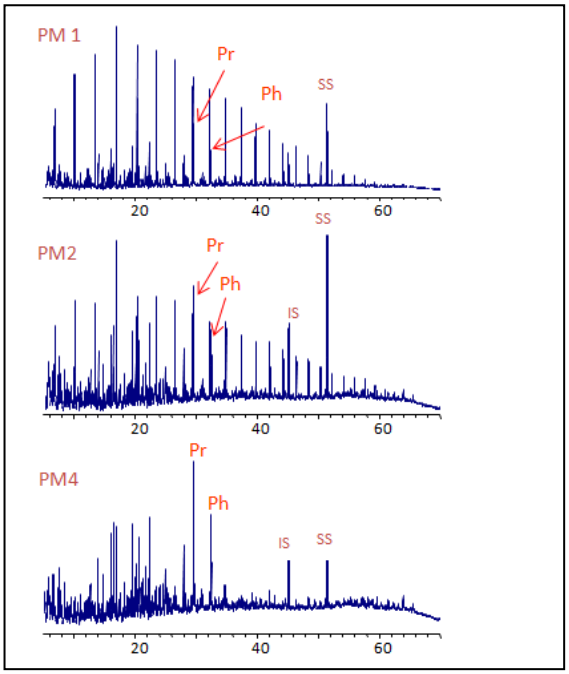

Figure 8.4. Gas chromatograms of total hydrocarbon fractions from oils of different reservoirs across the Gullfaks field showing different degrees of biodegradation. PM 1 to PM4 represent the degree of biodegradation on the Peters and Moldowan biodegradation scale (the higher the PM the greater the biodegradation). Loss of alkanes is evident and the ratio of Pristane (Pr) and Phytane (Ph) relative to the linear alkanes can be seen to increase with increasing PM level. IS and SS are internal and surrogate extraction standards respectively

Understanding the deep petroleum biosphere may also offer more than fundamental understanding of how the subsurface microorganisms shape important Earth processes. There is evidence that *in-reservoir* petroleum biodegradation proceeds through methanogenesis (Jones et al., 2008) and methane in the giant Troll gas field in the North Sea has been formed by biodegradation of residual oil (Horstad & Larter, 1997). It has therefore been proposed that stimulation of methanogenic oil biodegradation *in situ* may offer a route for the recovery of stranded residual oil as methane since the volumetrics of gas recovery (ca. 70% recovery) are much more favourable than the volumetrics of oil recovery (ca. 35% recovery) (Jones et al., 2008).

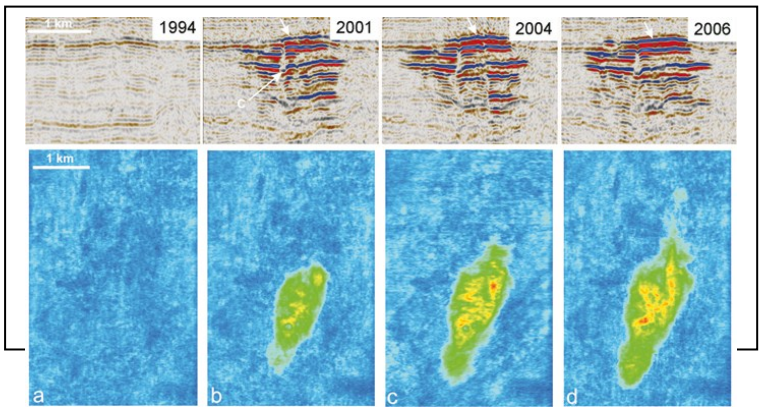

Figure 8.5 : Time-lapse seismic images from Sleipner showing vertical slices through the expanding plume in 1994, 2001, 2004 and 2006. The total height of the plume is about 250 metres, with a total width in 2001 of around 2 km. (Images courtesy of BGS involvement in the SACS, SACS2 and CO2STORE projects; http://www.bgs.ac.uk/science/CO2/home.html).

Carbon capture and storage (CCS) in petroleum reservoirs is one potential route for disposal of fossil carbon dioxide to control atmospheric CO_2 levels. This is already practiced

by Statoil in the Sleipner field in the North Sea, where almost 10 million tons of CO_2 has been disposed of since CCS operation began in 1996 (Figure 8.5). Carbon dioxide sequestration in this way is likely to have many geochemical effects which may influence the activity of deep biosphere microorganisms. For example, pH is likely to decrease substantially affecting mineral dissolution and microbial activity both of which may have effects on rock permeability and capacity to retain sequestered carbon.

Storegga Slide

The Storegga Slide is about 3500 km^3 of material which slid of Norway's continental shelf ca 8150 years BP, producing a very large tsunami in the North Sea and North Atlantic Ocean. Maximum thickness is about 700 m and sub-surface studies reveal a large number of palaeoslides with movements that have occurred on a number of different failure surfaces. In some areas sediments have moved as flows, whereas in others they remain as more-or-less coherent blocks and there are compression zones and these are associated with seabed seeps. In addition, the Ormen Lange gas field lies near the headwall of the landslide, and which is the second largest gas field in Norway.

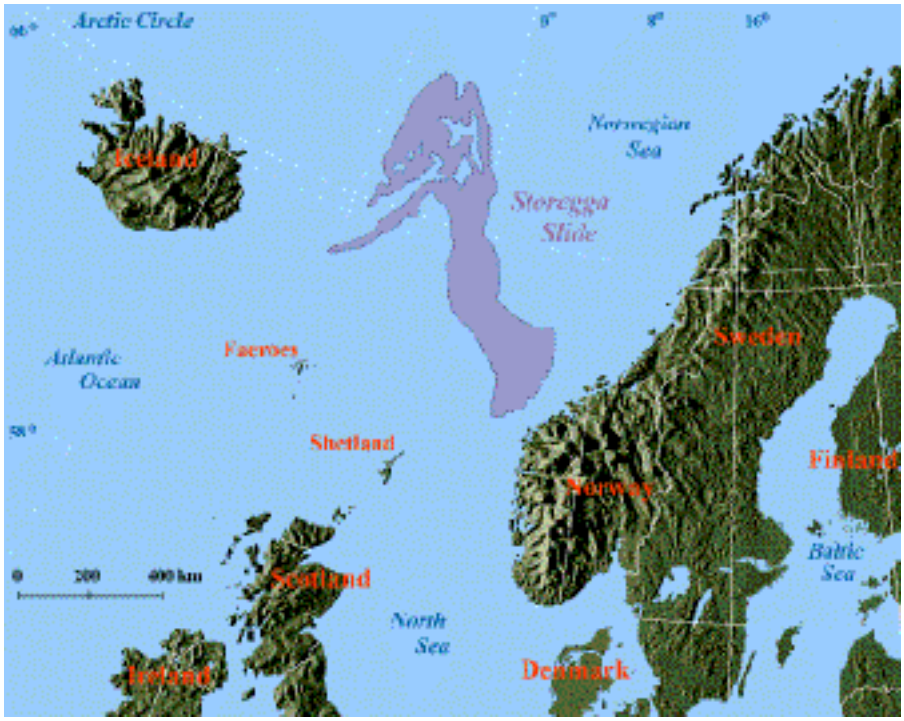

Fig 8.6. Map of the Storegga Slide

There are also pockmarks, in a few places, diapirs cropping out forming dome-like elevations, and subsurface gas hydrates. The presence of a BSR (Bottom Simulating Reflector, marking the end of hydrate and presence of free methane gas) in the sedimentary section within the slide scar area implies the repositioning of hydrates to newly established equilibrium conditions after the slide event. Hence, there are a number of important and contrasting potential subsurface prokaryotic habitats and energy sources within Storegga Slide sediments, including hydrogen from mechanochemistry (Parkes *et al*., 2011) due mineral fracturing and compression. Already, a range of *Archaea* have been documented in association with tube worms around cold seeps in the area (Lazar *et al*., 2010) and subsurface gas hydrates have been shown to be biogeochemically active and distinct

prokaryotic habitats (Wellsbury *et al*., 2000). In contrast, in the top 20 m of the re-deposited sediments there are low rates of microbial reactions, which may be due to its refractory organic matter content (Paul *et al*., 2010). However, it would be very interesting to know how the now buried previously near surface prokaryotic communities have adapted to almost instantaneous burial.

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