

THE DEBITS PROJECT: INVESTIGATION OF DEEP MICROBIAL ECOSYSTEMS IN A TERRESTRIAL ENVIRONMENT

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The investigation of the extent and dynamics of deep microbial ecosystems in sedimentary basins is a relatively young and intriguing topic in today's geoscience research. With the finding of ubiquitous deep microbial life on Earth, inevitably the question arises as to how microorganisms can survive in such ancient and, from a surface perspective, hostile habitats. In addition to elevated temperature and pressure conditions, nutrient limitation and limited porosity and permeability, deep microbial communities have to cope with a decrease in the available carbon and energy sources, because of the sedimentary organic matter becoming more recalcitrant with depth (Parkes *et al.*, 2000). The activation and usability of such food or substrate sources with increasing depth is, therefore, of specific interest when investigating deep microbial populations. There are relatively few investigations of the microbiology of sub-surface coal-bearing formations.

The international DEBITS (Deep Biosphere In Terrestrial Systems) project was started in February 2004 in the Waikato coal area on the North Island of New Zealand and is especially dedicated to terrestrial deep microbial ecosystems. The aim of the project is the investigation of the indigenous microbial populations and the characterisation of their habitats using biogeochemical, organic-geochemical and microbiological approaches.

The Waikato coal area represents a perfect natural laboratory for terrestrial deep biosphere research, because in this area organic carbon-rich lithologies are intercalated with coarser grained sediments. While the coaly seams are potential substrate providers (feeder lithologies), the coarser grained lithologies might act as habitats for microorganisms (carrier lithologies), having enough permeability to enable sufficient supply of respiratory compounds and hence for effective metabolism. Within the DEBITS project a 148 m deep well was drilled at Ohinewai in the Waikare Coalfield, taking strict precautions to prevent or at least to control any contamination of the core material by surface microorganisms. The DEBITS-1 well penetrates a complex succession of interbedded organic carbon-rich layers and coarser grained mudstones, siltstones and sandstones. At a depth of about 76 m the core intersected an

unconformity. Sediments below the unconformity were previously buried to more than 2000 m and therefore have experienced significantly higher temperatures, resulting in sub-bituminous coal rank, compared to the organic carbon-rich lithologies above the unconformity, of lignite rank.

Microbial *life markers (cell membrane phospholipids)* and intact and viable prokaryotic cells were detected above and below the unconformity, indicating either that sediments below this boundary have not been sterilized by increased burial and heating or that these sediments have been re-colonized after uplift. The phospholipid *life marker* profile decreases from the top to the base of the DEBITS-1 core. In contrast, the prokaryotic cell counts show no overall decrease with depth. An explanation for this discrepancy might be that microorganisms in deeper parts of the DEBITS-1 core are not necessarily smaller in number but in size due to the more extreme environmental conditions. There is a high molecular prokaryotic diversity, with some groups of *Bacteria* and *Archaea* similar to the marine deep biosphere. Methanogens (methane-producing microorganisms) were also detected and methane production occurred in long term slurry incubations.

Comparing the phospholipid (PL) abundances with the organo- and lithofacies in selected transects from organic carbon-rich to coarser grained organic carbon-poor lithologies, distinct trends can be recognized. While the organic carbon rich lithologies contain almost no PLs, the highest PL signals are generally detected at or near the transition zones, decreasing into the adjacent clay/silt/sand layers. The PL *life marker* distribution points to better conditions for the deep microbial populations in the coarser grained sediments close to the organic carbon-rich lithologies. Water extraction experiments show highest amounts of small fatty acid anions (e.g. acetate) near the transition zones (Vieth et al., this volume). A release of substrate from the organic carbon-rich seams into the adjacent sediments is suggested. Thus, the distribution of the microbial populations appears to be the result of sufficient substrate supply (most likely from the coaly layers) and sufficient pore space and permeability for metabolic exchange processes within the pore water.

Most reference samples far away from organic carbon-rich sediments contain no PLs. However, there are several exceptions with reference samples having PLs and transect samples with no PLs, which shows that there is still much to learn about deep microbial feeding processes and optimal life habitats for deeply buried microorganisms.

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ANALYSIS OF SPORE DIPICOLONIC ACID CONTENTS FOR ESTIMATING THE NUMBER OF ENDOSPORES IN SEDIMENTS

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Endospores are bacterial resting stages being able to remain viable for long periods of time. Consequently, they can be expected to accumulate in sediments during burial. Since they can be stained with fluorescent dyes they may contribute significantly to total cell counts emphasizing the need for a method for the estimation of endospores in sediments.

In the present study, dipicolinic acid (DPA), which is accumulated in the endospore core, was used to quantify spores in sediment samples from the backbarrier tidal flat of the island of Spiekeroog in the southern North Sea. For converting sediment DPA contents into endospore numbers, ten bacterial strains were examined for their DPA content per endospore. Six strains (*Bacillus* sp. G50II, *Bacillus* sp. G400 I, *Bacillus* sp. N300I, *Bacillus* sp. NA402, *Oceanibacillus* sp. NC301 and *Clostridiales* bacterium strain G100XIII) were isolated from the sediment sampling location. The other four strains comprised two well described *Bacillus* species from a culture collection (*B. megaterium* DSM 32^T and *B. subtilis* ssp. *subtilis* DSM 10^T) and two sulphate-reducing bacteria *Desulfosporosinus orientis* DSM 765^T and *Desulfotomaculum* sp. B2T (Sass & Cypionka, 2004). DPA contents of endospores ranged from 1.4×10^{-16} mol (*Bacillus* sp. G400I) to 1.3×10^{-15} mol (*Desulfosporosinus orientis* DSM 765^T). The observed differences in spore DPA content of the different strains apparently corresponded well with variations in spore volume (Fig. 1). Average spore volumes determined for the different strains ranged from $0.40 \mu\text{m}^3$ to $2.43 \mu\text{m}^3$, with spores of *Desulfotomaculum* sp. B2T being largest and having six times the volume determined for *Oceanibacillus* sp. NC301 spores. A rough correlation of DPA content and volume was indicated by linear regression using all data points, resulting in a DPA concentration of $0.46 \text{ mol DPA l}^{-1}$ which fits well to all strains with the exception of *Desulfosporosinus orientis* DSM 765^T. The spores of the tidal flat isolates showed little variation in size and DPA content and generally contained less DPA than endospores of the two sulphate reducers or of *Bacillus megaterium*^T. For the tidal flat strains, an average of 2.2×10^{-16} mol DPA per spore was determined and used for conversion of sediment DPA contents into spore numbers. Estimated endospore numbers were in a range of 10^6 to 10^7 endospores g^{-1} sediment and exceeded viable counts of spores determined after oxidic incubation in pasteurized MPN series

(Köpke et al., 2005) by at least three orders of magnitude, indicating that only a minor fraction of the endospores in the sediment can be detected by cultivation-dependent approaches. Since quantification on the basis of dipicolinic acid contents does neither discriminate between viable and non-viable spores nor between different physiological groups, it apparently provides a more realistic estimate of the contribution of endospores to the microbial community. For this reason, we suggest the use of DPA for determination of endospore numbers as a valuable amendment to total cell counts to reveal the importance of endospores in sedimentary microbial communities.

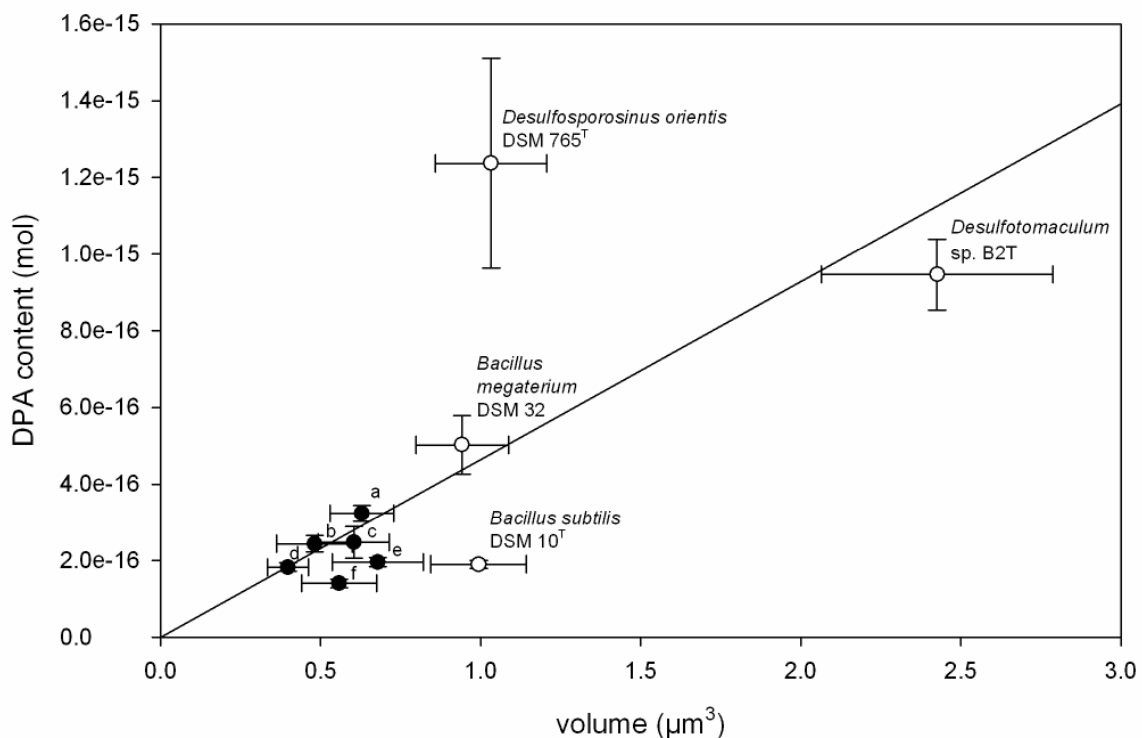


Figure 1. Plot of DPA content versus volume of the endospores analyzed in this study. The regression line (including all data points) represents $0.46 \text{ mol DPA l}^{-1}$ spore volume and fits well to most of the strains. Black dots: tidal flat isolates a) *Bacillus* sp. NA402 b) Strain G100XIII c) *Bacillus* sp. N300I d) *Oceanibacillus* sp. NC301 e) *Bacillus* G50II f) *Bacillus* G400I. White dots: sulphate reducers and aerobic freshwater bacilli.

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INTACT POLAR LIPIDS IN THE MARINE DEEP BIOSPHERE – STRUCTURAL DIVERSITY AND QUANTITIES

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The deep biosphere is thought to contain live biomass that represents up to 10% of the total carbon in live cells of our planet (Parkes et al., 2000). This vast ecosystem has become the research focus of microbiologists and geochemists to address key questions like: What types of microbes thrive in deeply buried sediments? And, what are the processes they are mediating? Recent studies have provided information on metabolic activities and quantities of deeply buried prokaryotic cells (Biddle et al., 2006; Inagaki et al., 2006; Schippers et al., 2005, 2006). However, some fundamental questions remain unresolved or even lead to highly controversial answers. For example, various techniques appear to disagree already at the domain level on WHO actually inhabits this ecosystem. Molecular biological methods like catalyzed reporter deposition - fluorescent in situ hybridization (CARD-FISH) and quantitative polymerase chain reaction (Q-PCR) suggest a predominance of bacterial over archaeal cells (Schippers et al., 2005; Inagaki et al., 2006). On the other hand FISH and intact polar lipids (IPL) suggest a predominance of archaea among live prokaryotes (Biddle et al., 2006).

Intact membrane lipids are considered to be markers for live subsurface cells (Sturt et al., 2004) since the polar headgroup is cleaved off the core lipid after cell death. Careful determination of response factors in calibration series with IPL standards shows no preferential detection of one compound class over the other. However, there are several principal difficulties that have to be overcome while analyzing environmental samples: general low abundance of target IPL compounds and the complex matrix with a high background of degraded material.

A robust analytical protocol based on high performance liquid chromatography coupled to ion trap multistage mass spectrometry (HPLC-IT-MSⁿ) was developed and applied to a set of samples from the Peru margin surface (RV Sonne SO147), ODP Legs 201, 204, 207, and IODP Expeditions 301 and 311. The major bacterial IPLs identified comprise phosphatidylglycerol (PG), phosphatidylethanolamine (PE) and phosphatidylcholine (PC) diacylglycerides with C₁₆ and C₁₈ acyl groups. The diversity in archaeal IPLs is limited to different core lipid structures, e.g. archaeol-based diether lipids vs. glycerol dibiphytanyl

glycerol tetraether (GDGT) lipids with various combinations of sugar headgroups. The composition of archaeal IPLs appears to be linked to the diversity of archaeal phylotypes.

The observed IPL concentrations were converted to cell concentrations for comparison to results from molecular biological approaches. Surface sediments are dominated by bacterial IPLs with possible admixtures of eukaryotic lipids while archaeal lipid concentrations are low. Concentrations of bacterial lipids decline rapidly to levels significantly lower than those of their archaeal counterparts. The analysis of ODP/IODP samples from deeply-buried horizons shows evidence for bacterial lipids in about 20% of samples analyzed to date. On the basis of these observations in combination with results of degradation experiments of archaeal and bacterial IPLs under typical anaerobic sedimentary conditions (Pamela Rossel et al., unpubl. data), we interpret the predominance of archaeal IPLs as evidence for a far more important role than suggested by other techniques. A composite view comprised of ~ 60 samples from both surface and deeply buried sediments on the abundance of total prokaryotic lipids in subsurface environments provides an interesting comparison to corresponding data on intact cells (cf. Parkes et al., 2000): concentrations of IPLs decline more rapidly with depth than the counts of intact cells from a global data set.

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