Jens Kallmeyer, Dirk Wagner
Microbial Life of the Deep Biosphere
Life in Extreme Environments

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Edited by
Jens Kallmeyer, Dirk Wagner

Volume 1

Microbial Life of the Deep Biosphere

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Editors
Dr. Jens Kallmeyer
GFZ German Research Centre for Geosciences
Section 4.5, Geomicrobiology
Telegrafenberg
14473 Potsdam
Germany
kallm@gfz-potsdam.de

Prof. Dr. Dirk Wagner
GFZ German Research Centre for Geosciences
Section 4.5, Geomicrobiology
Telegrafenberg
14473 Potsdam
Germany
dirk.wagner@gfz-potsdam.de

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Preface

"What are extreme environmental conditions?" Most of the answers that one gets from undergraduate students confirm a rather anthropocentric view: An environment that humans perceive as unpleasant is classified as extreme.

De facto, many of Earth's ecosystems are characterized by "extreme" environmental conditions, because they deviate from those conditions that humans would consider "normal" with regards to temperature, water availability, pressure, salinity, nutrient supply and so on. Despite being considered extreme, these habitats are colonized by a large number of organisms that thrive under the given conditions.

The definition of an extreme habitat is based on our anthropocentric view, but as a more general approach, microorganisms can be considered extremophilic when they thrive under physical and chemical conditions that destroy cellular components of most nonextremophilic organisms. In recent times, more and more scientists from various disciplines have become interested in the topic 'Life in Extreme Environments'. Through multidisciplinary research, completely new concepts were developed of how extremophiles can possibly survive and even thrive in extreme ecosystems.

Based on these recent advances, the book series 'Life in Extreme Environments' publishes topical volumes in the rapidly growing research field of microbial life in extreme environments. This includes all habitats at the edge of survivability, ranging from equatorial to polar regions, from marine to terrestrial environments and from surface to deep ecosystems. Environmental niches that are, for instance, characterized by extraordinarily hot, cold, acidic, alkaline or dry conditions, or subjected to high salinity, radiation or pressure.

The extremophilic microorganisms living in these environments represent numerous and diverse lineages from across all three domains of life: *Bacteria*, *Archaea* and *Eukarya*. Special emphasis is placed on the understanding of the structure and function of microbial communities in extreme environments, their life strategies and adaptation mechanisms as well as their reaction to changing environmental conditions.

This book series will be a useful reference for advancing our understanding of the origin of life and for exploring the biotechnology potential of these fascinating microorganisms.

The first volume of this series presents a broad overview of our current knowledge of microbial life in deep subsurface environments. Over the last decade, this so-called deep biosphere research has expanded quite dramatically. Since the early days of Morita and Zobell (1955), who set the limit of life at 7.47 meters below the sea floor, the maximum depth to which life reaches into the Earth has been set deeper and deeper and is now exceeding 3 km on land and 1.5 km in marine sediments. Active microbial communities were found in areas that were considered devoid of life, for example, the oceanic crust. Still, we have not seen the true limits of life yet. Despite major technical advances in the last few years, subsurface life exploration is still heavily de-

pending on technical improvements because quite often the abundance and activity of subsurface microbes is orders of magnitude lower than in surface sediments.

When compiling this volume, we wanted to cover the diversity of this young but rapidly growing field of research. The first section is devoted to the different major habitats. The chapter of Parkes et al. provides us with a much-awaited update of their review paper about subseafloor sediment microbiology from 2000. There is hardly any publication about deep subseafloor sediment that does not cite this classic. This chapter allows us to follow the development of the field from a small niche subject into an important research field.

Microbial life in subseafloor environments is not restricted to sediments, there are new and exciting findings of life in the oceanic crust, and these are presented by Biddle et al.

Of course, research in terrestrial subsurface environments has made a similar leap forwards, and Karsten Pedersen provides us with an update on the state-of-the-art. When searching for life elsewhere in the Universe, one should need to know what to look for. Charles Cockell argues that Earth's subsurface might be a good analogue for habitats on, or rather, in other planetary bodies.

After these more general chapters, the book focuses on several special topics that are currently under much debate. Andreas Teske's chapter about archaea in deep marine subsurface sediments gives an overview of the current knowledge about this largely uncultivated group of organisms. Although modern molecular techniques have become increasingly popular in recent years, classical cultivation still is very important. Toffin and Alain summarize recent advances in this field with specific remarks about high-pressure cultivation, and other high-tech methods that allow us to grow microbes that would otherwise remain uncultured. Not only microbes, i.e. prokaryotes live in the subsurface. Eukaryotes are also present and might play a much more important role than previously thought. Edgcomb et al. inform us about the current state of knowledge in this area.

One of the main drawbacks of molecular techniques is the apparent disconnection between phylogenetic and metabolic information. Only through novel techniques that allow measuring multiple information simultaneously from the same sample can we now actually see which microbe is doing what. Morono et al. present us their recent advances in NanoSIMS research and the challenges that are still lying ahead. This part of the book is closed by a chapter of Karen Lloyd who shows us how just minor differences in sample preparation can have huge impacts on the final results. This should be a note of caution to everybody working in this field and a friendly reminder that there are still many technical challenges ahead of us.

The subsurface biosphere is not just of purely scientific interest. As many geotechnological applications are affected by subsurface microbial activity, there is also a growing industrial interest in this field of research. Ollivier et al. introduce us to microbial activity in hydrocarbon reservoirs. Of course, not only hydrocarbons are affected

by microbes, Alawi shows us their effects on hydrothermal systems and subsurface storage of carbon dioxide.

Even with the most sensitive techniques, metabolic activity might be so low that it cannot be detected and many turnover processes occur over geologic time scales, vastly exceeding the timespan that humans can observe. While LaRowe and Amend focus on thermodynamical controls on subsurface life, DiPrimio introduces basin modeling as a valuable tool to understanding ultraslow abiotic reactions that run over geologic time scales. Røy shows us how to use actual measurements of downcore profiles to quantify metabolic rates.

We hope that this volume will provide you with a broad overview of this exciting and rapidly developing field of research and stimulates the debate on this fascinating research field in the near future.

March 2014 Dirk Wagner & Jens Kallmeyer

Contents

Contributing authors

Karine Alain
Laboratoire de Microbiologie
des Environnements Extrêmes
Institut Universitaire Européen
de la Mer Technopôle Brest-Iroise
Plouzané, France
e-mail: Karine.Alain@univ-brest.fr
Chapter 4

Mashal Alawi
GFZ German Research Centre for Geosciences
Section 4.5 Geomicrobiology
Potsdam, Germany
e-mail: malawi@gfz-potsdam.de
Chapter 10

Jan Amend
Department of Earth Sciences
Department of Biological Sciences
University of Southern California
Los Angeles, CA, USA
e-mail: janamend@usc.edu
Chapter 13

Jennifer Biddle
College of Earth, Ocean and the Environment
University of Delaware
Lewes, DE, USA
e-mail: jfbiddle@udel.edu
Chapter 2, 9

Jean Borgomano
Total CSTJF
EP/ EXPLO/ TE/ ISS/ CARB
Pau, France
e-mail: jean.borgomano@total.com
Chapter 8

Charles Cockell
Centre for Astrobiology
School of Physics & Astronomy
Centre for Astrobiology
University of Edinburgh
Edinburgh, UK
e-mail: c.s.cockell@ed.ac.uk
Chapter 11

Barry Cragg
School of Earth & Ocean Sciences
Cardiff University
Cardiff, UK
e-mail: craggb@cf.ac.uk
Chapter 1

Rolando DiPrimio
GFZ German Research Centre for Geosciences
Section 4.3 Organic Geochemistry
Potsdam, Germany
e-mail: dipri@gfz-potsdam.de
Chapter 12

Virginia Edgcomb
Department of Geology and Geophysics
Woods Hole Oceanographic Institution
Woods Hole, MA, USA
e-mail: vedgcomb@whoi.edu
Chapter 9

Fumio Inagaki
Geomicrobiology Group
Kochi Institute for Core Sample Research
JAMSTEC
Nankoku, Kochi, Japan
and
Geobio-Engineering and Technology Group
Submarine Resources Research Project
JAMSTEC
Yokosuka, Japan
e-mail: inagaki@jamstec.go.jp
Chapter 5

Motoo Ito
Geochemical Research Group
Kochi Institute for Core Sample Research
JAMSTEC
Nankoku, Kochi , Japan
and
Geobio-Engineering and Technology Group
Submarine Resources Research Project
JAMSTEC
Yokosuka, Japan
e-mail: motoo@jamstec.go.jp
Chapter 5

Sean Jungbluth
Center for Microbial Oceanography
Hawaii Institute of Marine Biology
University of Hawaii at Manoa
Honululu, HI, USA
e-mail: seanpj@hawaii.edu
Chapter 2

Douglas LaRowe
Department of Biological Sciences
Department of Earth Sciences
University of Southern California
Los Angeles, CA, USA
e-mail: larowe@usc.edu
Chapter 13

Mark Lever
Department of Bioscience
Center for Geomicrobiology
Aarhus University
Aarhus, Denmark
e-mail: mark.lever@biology.au.dk
Chapter 2

Karen G. Lloyd
Department Microbiolgy
University of Tennessee
Knoxville, TN, USA
e-mail: klloyd@utk.edu
Chapter 6

Yuki Morono
Geomicrobiology Group
Kochi Institute for Core Sample Research,
Japan JAMSTEC
Nankoku, Kochi, Japan
and
Geobio-Engineering and Technology Group
Submarine Resources Research Project JAMSTEC
Yokosuka, Japan
e-mail: morono@jamstec.go.jp
Chapter 5

Philippe Oger
Laboratoire de Geologie
Ecole Normale Supérieur de Lyon
Lyon Cedex, France
e-mail: philippe.oger@ens-lyon.fr
Chapter 8

Bernard Ollivier
Laboratoire de Microbiologie
Institute of Oceanography
Aix-Marseille Université
Marseille Cedex, France
e-mail: bernard.ollivier@univ-amu.fr
Chapter 8

William Orsi
Department of Chemistry and Geochemistry
Woods Hole Oceanographic Institution
Woods Hole, MA, USA
e-mail: william.orsi@gmail.com
Chapter 9

R. John Parkes
School of Earth & Ocean Sciences
Cardiff University
Cardiff, UK
e-mail: parkesrj@cf.ac.uk
Chapter 1

Karsten Pedersen
Department of Civil
and Environment Engineering
Chalmers University of Technology
Göteborg, Sweden
and
Microbial Analytics Sweden AB
Mölnycke, Sweden
e-mail: kap@micans.se
Chapter 3

Michael Rappe
Center for Microbial Oceanography
Hawaii Institute of Marine Biology
University of Hawaii at Manoa
Honululu, HI, USA
e-mail: rappe@hawaii.edu
Chapter 2

Erwan Roussel
School of Earth & Ocean Sciences
Cardiff University
Cardiff, UK
e-mail: RousselEG@cardiff.ac.uk
Chapter 1

Hans Røy
Department of Bioscience
Center for Geomicrobiology
Aarhus, Denmark
e-mail: hans.roy@biology.au.dk
Chapter 14

Henrik Sass
School of Earth & Ocean Sciences
Cardiff University
Cardiff, UK
e-mail: SassH@cardiff.ac.uk
Chapter 1

Andreas P. Teske
Department of Marine Sciences
University of North Carolina
Chapel Hill, NC, USA
e-mail: teske@email.unc.edu
Chapter 7

Laurent Toffin
Laboratoire de Microbiologie
des Environments Extremes, IFREMER
Technopole Brest-Iroise
Plouzané, France
e-mail: laurent.toffin@ifremer.fr
Chapter 4

Gordon Webster
School of Biosciences
Cardiff University,
Main Building,
Park Place, Cardiff, Wales
CF10 3AT, UK
e-mail: WebsterG@cardiff.ac.uk
Chapter 1

Andrew Weightman
School of Biosciences
Cardiff University,
Main Building,
Park Place, Cardiff, Wales
CF10 3AT, UK
e-mail: weightman@cf.ac.uk
Chapter 1

R. John Parkes, Henrik Sass, Barry Cragg, Gordon Webster, Erwan Roussel, and Andrew Weightman

1 Studies on prokaryotic populations and processes in subseafloor sediments – an update

This chapter provides an update of a year 2000 review of the microbiology of subseafloor sediments [1]. At the time of this review, our Geomicrobiology Group was the main group researching in this area and had been the first to propose the subseafloor biosphere [2]. At this time, the presence of a significant prokaryotic biosphere in subseafloor sediments was still contentious due to perceived low-energy supply coupled with geological time scales, resulting in the view that most microorganisms in subseafloor sediments were either inactive or adapted for extraordinarily low metabolic activity [3]. However, as predicted [2], most cells were subsequently shown to be active [4, 5]. Since the year 2000, a significant number of additional research groups have been investigating the microbiology of subseafloor sediments (> 10, e.g. [5–18]) and they have confirmed our results of the presence of a globally significant subseafloor biosphere. Here, we provide an update of our recent deep biosphere research (7 new sites), including simulation experiments, and place these into a broader context of subseafloor biosphere research.

Two aspects which need to be noted at the start of the update are: (1) The general depth trend in intact prokaryotic cells in subseafloor sediments which refers to the update of our original depth plots of acridine orange stained cells [2] which was modified for the 2000 review and (2) The organic acid acetate, which is an important anaerobic metabolic breakdown intermediate of organic matter, as well as a product of H_2/CO_2 metabolism, via acetogenesis, and changes in concentration or metabolism of porewater acetate is used as an index for general prokaryotic activity.

1.1 New sites investigated

1.1.1 Southeast Atlantic sector of the Southern Ocean (Leg 177)

Ocean Drilling Program (ODP) Leg 177 provided an opportunity to investigate prokaryotic distributions in carbonate-rich, low organic carbon Sites (1088 & 1093) in the Southeast Atlantic sector of the Southern Ocean (▶ Fig. 1.1), and to contrast these with porewater acetate concentrations [19]. Calcium carbonate concentrations at the nannofossil ooze Site 1088 (water depth 2082 m and sediment surface temperature ~2.4 °C) was high (88.2 wt%), and ~10 times higher than the deeper water, diatom ooze Site

1093 (water depth 3636 m and sediment surface temperature ~2.6 °C). Prokaryotic cell-depth distributions at both sites were lower than the general trend in deep marine sediments (▸ Fig. 1.2), but Site 1088 had the lowest cell numbers despite the much shallower water depth. It seems at these sites that the high calcium carbonate content, and hence, low organic carbon, had a greater effect on prokaryotic cell numbers (decreasing) than water column depth or latitude. This was also reflected in porewater acetate concentrations with Site 1088 having consistently low concentrations (0–15 µM), compared to acetate peaks of up to 110 µM at Site 1093, associated with localized diatom rich laminae (▸ Fig. 1.2). Geochemical data at both sites also demonstrated low levels

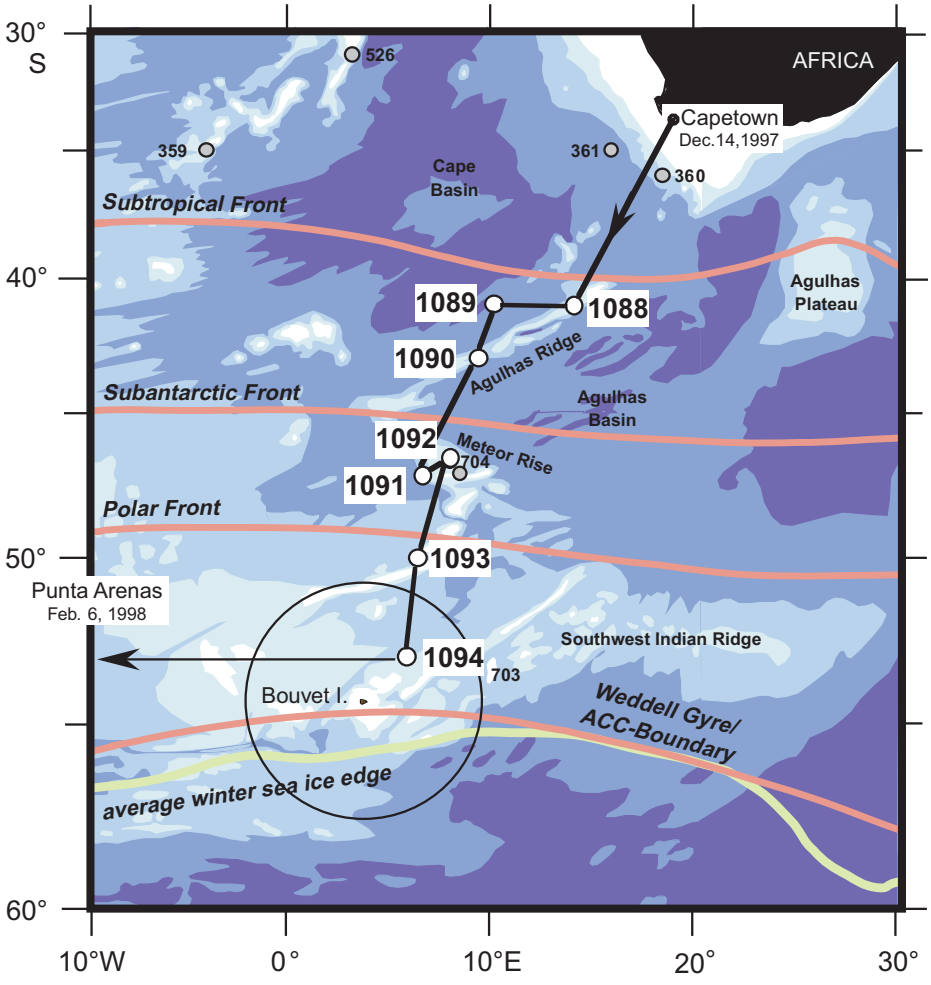

Fig. 1.1: Southern Ocean ODP Leg 177 Sites, including Sites 1088 and 1093.

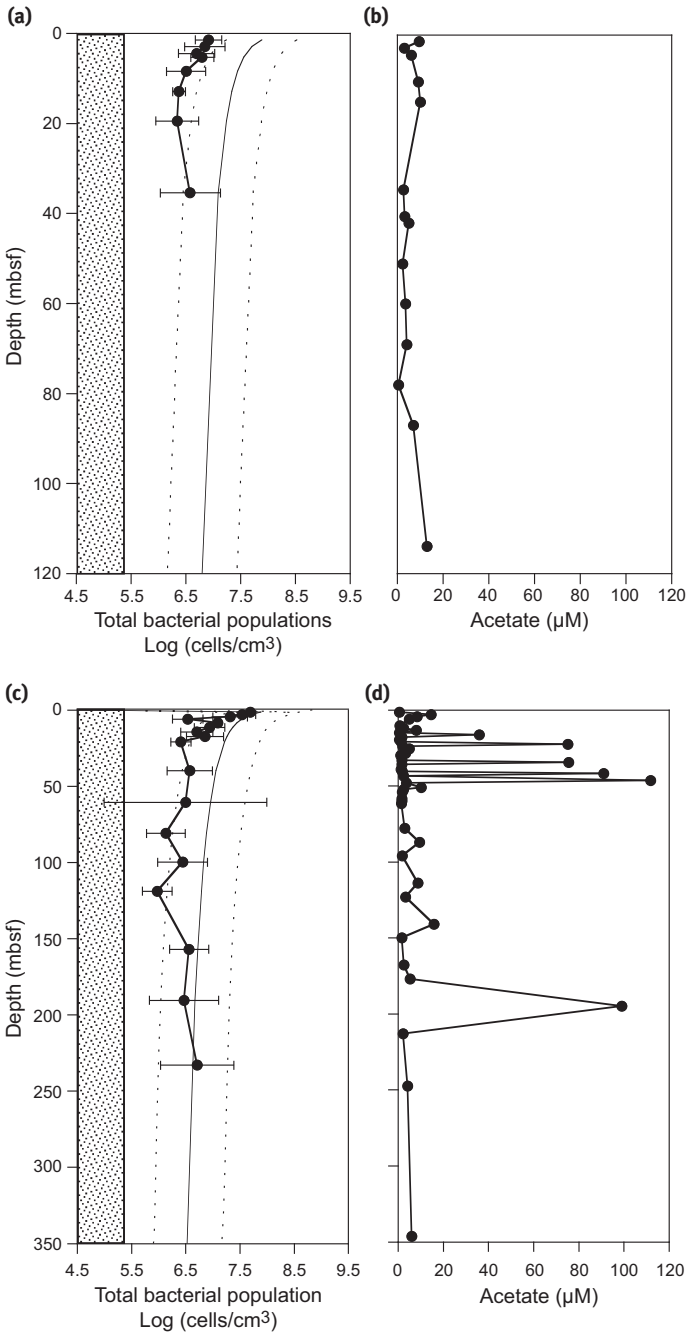

Fig. 1.2: Depth distributions of total bacterial populations and porewater acetate, Site 1088 (a,b) and 1093 (c,d) Southeast Atlantic sector of the Southern Ocean. Gray shaded area = data below the detection limit of the technique (2.23×10^5 cells/cm^3).

of prokaryotic activity with only limited sulfate removal and low methane concentrations [20].

As carbonate-rich sediments account for ~52% of global seafloor area [21], if prokaryotic cell numbers are consistently lower in carbonate-dominated sediments compared to other sediment types, this would reduce estimates of the total biomass of the subseafloor biosphere.

1.1.2 Woodlark Basin, near Papua New Guinea, Pacific Ocean (Leg 180)

Three sites were sampled at water depths from 1150 to 2303 m [22]. Two sites (1109 and 1115) had the global average thermal gradients of ~30 °C/km and were low organic carbon (~0.4%) and low organic matter sedimentation rate sites. Active prokaryotic populations (microscopic cells, culturable prokayotes [anaerobic fermentative heterotrophs, autotrophic and heterotrophic acetogens] and radiotracer activities [sulfate reduction, methanogenesis from acetate and H_2/CO_2, growth – thymidine incorporation into DNA] and geochemistry) were present to all depths sampled at these sites, maximum 801 meters below seafloor (mbsf), and ~15 million years ago (mya). In 2002, these were the deepest subseafloor sediments that the presence of prokaryotes had been detected by a range of complementary methods. Prokaryotic populations and activities were greatest near the sediment surface and decreased with increasing depth, although there were some limited subsurface peaks (▶ Fig. 1.3). Consistent with the presence of active prokaryotic populations in deeper layers, there were continuing geochemical changes (porewater sulfate removal and subsequently, methane formation) and corresponding low activity rates (up to 10,000 times lower than near-surface rates). Interestingly, however, depth integration of measurements on the full sediment depth showed the biogeochemical significance of the deeper layers, with 78% of cells, 93% of cell production, and ~90% of prokaryotic activity (methanogenesis and acetate oxidation) occurring in sediments below 20 m. The depth distribution of sulfate reduction activity, in contrast, depended on the rates that occurred, with the higher rates at Site 1109 more rapidly removing sulfate, and thus, restricting most activity to the upper 20 m (65%). Whilst at Site 1115 with lower sulfate reduction rates, sulfate penetrated deeper and sediments below 20 m were responsible for the majority of measured sulfate reduction activity (72%).

Cell counts and geochemical data alone measured at the deepest water depth site (1118, 2303 m) also provided strong evidence for significant prokaryotic populations to at least 842 mbsf (▶ Fig. 1.4). In addition, there was circumstantial evidence for deep anaerobic oxidation of methane (AOM) providing a new energy source, as fluid flow at depth (~700 mbsf) provided sulfate, and this coincided with removal of methane that had been consistently present from ~240 mbsf. Although at this site there was not an increase in prokaryotic cell numbers due to stimulation of AOM, this has occurred in other deep sediments (e.g. [14, 23, 24]). Also at this site, which had a higher

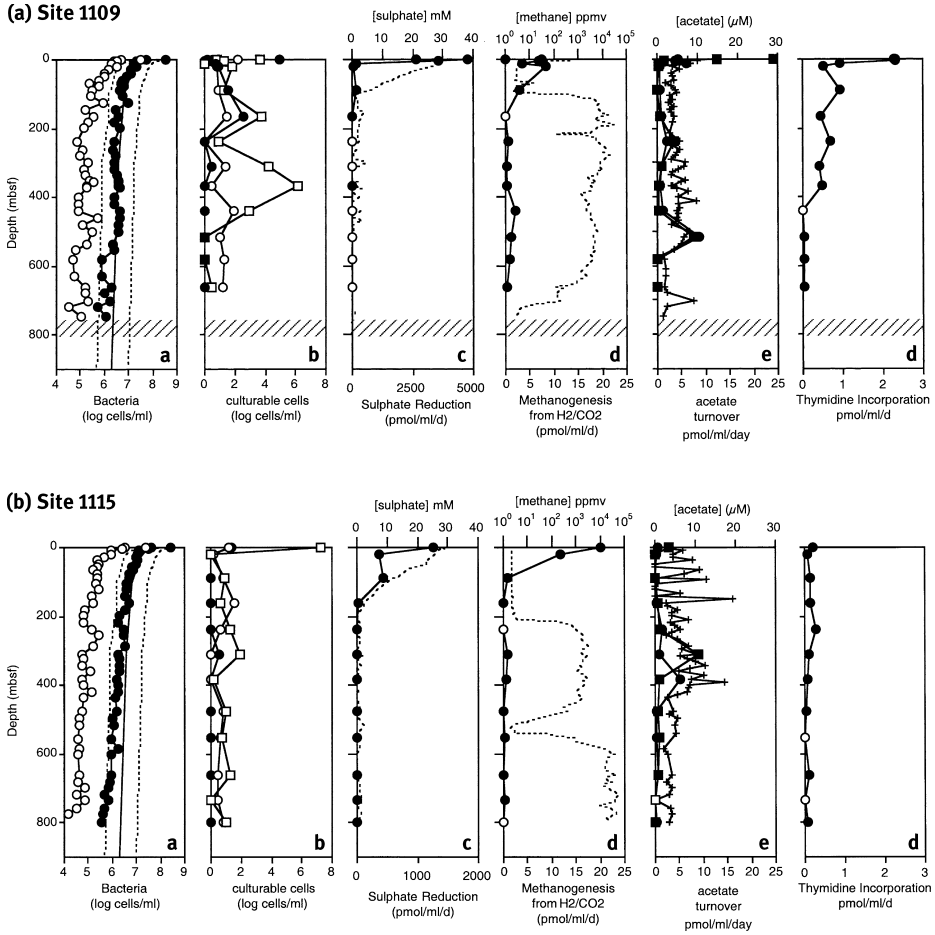

Fig. 1.3: Depth profiles of prokaryotic populations and activities in Woodlark Basin sediments (a) Site1109, (b) Site 1115. (a) Total (●) and dividing cells (○). The solid lines are Parkes' general model for cell distributions in marine sediments [1], and dotted lines represent 95%prediction limits. (b) Culturable cells from MPN enrichments; heterotrophic (○) and autotrophic acetogens, (●) and fermentative heterotrophs (□). (c) Sulfate reduction (●) and porewater sulfate (dashed line). (d) Methanogenesis from H_2:CO_2 (●) and *in situ* methane(dashed line). (e) Acetate metabolism to CO_2 (●) and CH_4 (■) and porewater acetate (+); (f) thymidine incorporation-rate into DNA (●). Hollow symbols denote zero values. For Site 1109 //// represents a dolerite layer.

thermal gradient (~63 °C km^{-1}), there were peaks in acetate concentrations at depth not present at the lower temperature sites. This could reflect temperature activation of recalcitrant organic matter [25, 26], with acetate accumulation being restricted by acetoclastic sulfate reduction. At the other sites, acetate oxidation was directly measured (▶ Fig. 1.3), but low acetate concentrations were consistently present, which demonstrates that acetate was also being produced at depth at these sites. Deep acetate for-

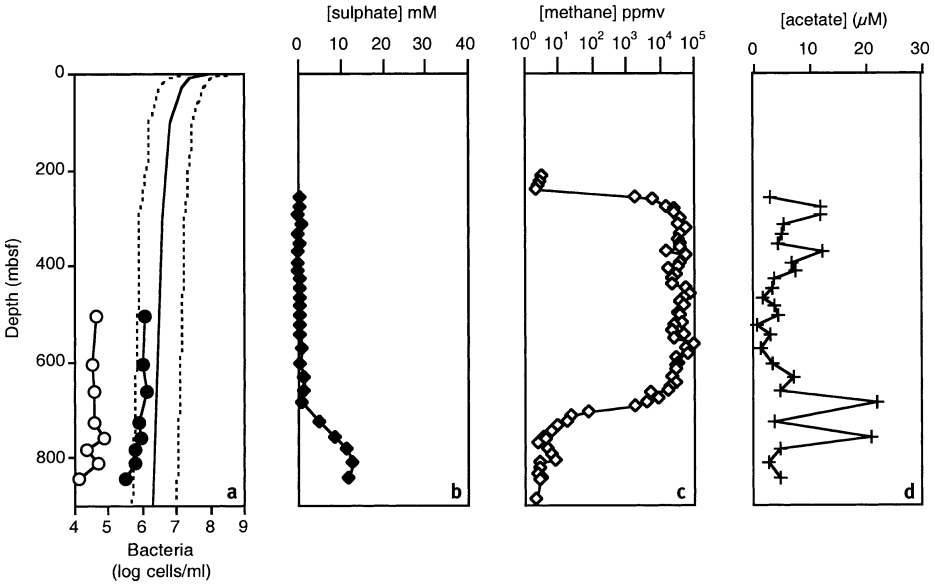

Fig. 1.4: Depth profiles of bacterial populations and activities in sediments at Site 1118 in Woodlark Basin. (a) Total bacterial populations (○) and dividing and divided cells (●). The solid line shows Parkes' general model for bacterial distributions in marine sediments [1], and dotted lines represent 95% prediction limits. (b) Porewater sulfate (◆). (c) *In situ* methane (◇). (d) Porewater acetate (+).

mation in ~15 mya sediments may seem surprising, but this has been observed in other deep subsurface environments, including Cretaceous age sediments [27] and was consistent with the presence of viable acetogens (▶ Fig. 1.3). Low molecular weight hydrocarbons (LMWH) were also detected at sites 1109 and 1115, and their downhole profiles combined with low *in situ* temperatures suggested that the LMWH components were formed *in situ* by low-temperature biological processes [28].

1.1.3 Leg 185, Site 1149 in the Izu-Bonin Trench, Western Equatorial Pacific

ODP Leg 185 was the first ODP cruise where contamination checks were conducted for microbiology [29, 30]. These tests demonstrated that the inner portion of cores, where the microbiological samples were taken from, were free from any potential sampling contamination. Bacterial populations were present in all samples (deepest at 171.2 mbsf) at this deep-water (5818 m) low-sedimentation-rate site. The highest cell numbers were near the surface (1.4 mbsf; 7.2×10^6 cells/cm^3), but then declined rapidly within the upper 10 mbsf. Below this, numbers decreased at a more gradual rate to 7.2×10^5 cells/cm^3 at 172 mbsf, a 10-fold reduction. This two-stage bacterial depth distribution has been observed at several other ODP sites (e.g. Amazon Fan [31] and Santa Barbara Basin [32]). Bacterial depth distributions at Site 1149 were well below

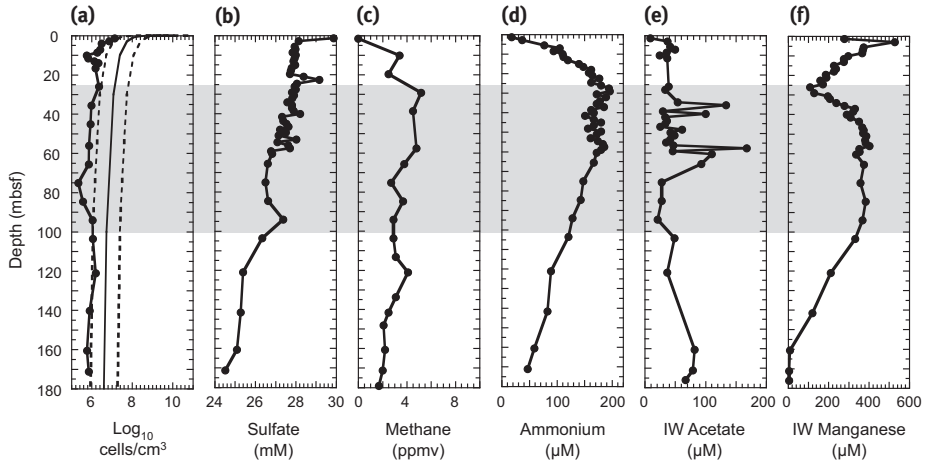

Fig. 1.5: Depth profiles at Site 1149, Izu-Bonin Trench, Western Equatorial Pacific. (a) Total prokaryotic cells. Solid sloping line is the regression line of best fit derived from previous ODP legs, dashed lines are the 95% prediction limits [2] (b) Sulfate. (c) Methane. (d) Ammonium. (e) Acetate. (f) Reduced manganese. The shaded area highlights the broad peak in bacterial manganese reduction activity between 26 and 100 mbsf.

those for other subseafloor locations and were predominantly below the lower 95% prediction limits (▶ Fig. 1.5). These low bacterial populations probably reflect the low sedimentation rates and low input of bioavailable organic matter that is characteristic for deep-water sites. Consistent with the low cell numbers, there was only limited removal of porewater sulfate, suggesting low bacterial sulfate reduction activity. Most sulfate removal was in the top ~5 mbsf, coinciding with the highest bacterial populations, the presence of small amounts of methane and an increase in porewater manganese and ammonia. In the deeper sediments, however, there was still indirect evidence of continuing low prokaryotic activity, with increases in porewater ammonia, soluble manganese (approx 26 to 100 mbsf), bioavailable acetate and decreasing sulfate. Unexpectedly, manganese reduction, sulfate reduction and a limited amount of methanogenesis seemed to be occurring simultaneously at depth in this low organic matter site, rather than in the expected depth succession. Similar situations were subsequently shown at other deep sediment sites (e.g. [33, 34]).

1.1.4 Nankai Trough (Leg 190), subduction zone/accretionary prism, Pacific Ocean

Nankai Trough is a deep trench formed at a subducting plate boundary where there is also active sediment accretion producing a large accretionary prism [35, 36]. Three deep-water sites were analyzed (4751–4844 m), which had relatively low organic carbon concentrations (mean 0.35–0.45% w/w) but steep temperature gradients (base-

ment temperatures at Sites 1173 and 1174 were above 100 °C and at Site 1177 were < 70 °C). Depth distribution of prokaryotic cell numbers at Site 1177 and above about 400–500 mbsf at Sites 1173–1174 were similar to other subseafloor sediment sites, but deeper samples at Sites 1173–1174 were very low (< 10^5 cells cm^{-3}). It was, therefore, surprising that amplifiable DNA could not be extracted from Sites 1177 or 1174. However, amplifiable DNA was obtained at three upper depths from Site 1173 (4.15, 98.29 and 193.29 mbsf). Low, but active, prokaryotic populations at these sites was supported by measured rates of methanogenesis and, for the first time, the presence of intact phospholipids (▶ Fig. 1.6), which are chemical markers for living prokaryotes [37]. Phylogenetic analysis of the extracted DNA sequences showed a wide variety of uncultured *Bacteria* and *Archaea* [35]. Sequences of *Bacteria* were dominated by an uncultured and deeply branching "deep sediment group" (now called JS1 [38], 53% of sequences). Also present were *Planctomycetes* (4%), *Cyanobacteria* and chloroplasts (8%), *Betaproteobacteria* (11%) and *Gammaproteobacteria* (14%). The majority of archaeal 16S rRNA gene sequences belonged to uncultured clades of the *Crenarchaeota*. There was good agreement between sequences obtained independently by cloning and by denaturing gradient gel electrophoresis (DGGE). Nankai Trough sequences were similar to those detected in other marine sediments and anoxic habitats, and so probably represent environmentally important indigenous bacteria.

Kinetic analysis of sediment heating experiments to assess hydrocarbon generation in Nankai Trough sediments [36] predicted that organic matter transformation would start at Site 1173 around 300 mbsf and this was in good agreement with *in situ* thermogenic hydrocarbon formation (e.g. ethane, ▶ Fig. 1.6). In addition, below ~400 mbsf there was an increase in rates of methanogenesis, some increases in cell numbers and detection of intact phospholipids. Similar changes occurred at Site 1174, but at depths greater than ~500 mbsf and the increase in cells was more marked. Also corresponding with the predicted increased organic matter reactivity with increasing temperature were increases in porewater acetate and hydrogen (▶ Fig. 1.6), which are both important substrates for anaerobic prokaryotes. Overall, however, H_2/CO_2 methanogenesis was the dominant methanogenic process in these sediments, whilst acetate and methanol were also important substrates in some samples. Analysis of a functional methanogen (*mcrA*) gene at Site 1173 showed that both the 4.15 and 193.29 mbsf samples were dominated by *Metanobacteriales* methanogens, capable of H_2/CO_2 methanogenesis, whereas at 98.29 mbsf *Methanosarcinales* methanogens, which can utilize acetate or methylated compounds, were the dominant sequences. These results show that in deep, sub-surface sediments, thermal activation of buried organic matter can release low molecular weight substrates which can stimulate prokaryotic activity, as suggested from laboratory experiments (e.g. [25, 26], and below). These experiments also showed sulfate production at elevated temperature and this occurred in Nankai Trough subsurface sediments (▶ Fig. 1.6 and [39]), and could further stimulate deep prokaryotic activity. In addition, Nankai Trough results clearly demonstrate overlap and interaction between biogenic and thermogenic processes in

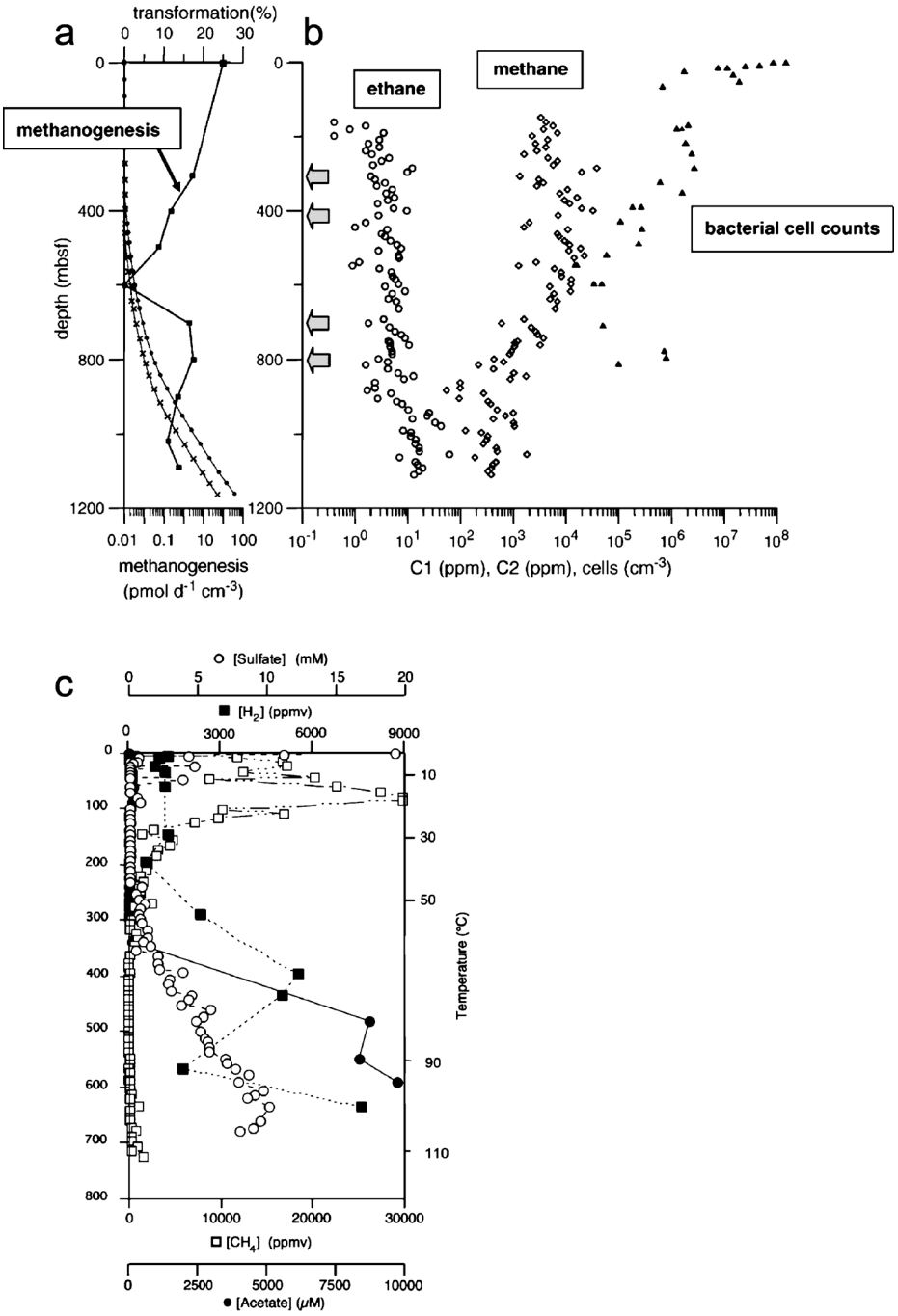

Fig. 1.6: Nankai Trough Site 1173 geomicrobiology and biogeochemistry summary. (a) Generation curves from kinetic modeling and experimentally determined rates of potential methanogenesis [36]. (b) Gas concentrations in ppm [71] for methane (diamonds) and ethane (circles), and total cell counts in $\log^{10}$ cm^{-3} (triangles), light arrows mark depths where intact phospholipids (PL) were detected [37]. (c) Increase in bacterial metabolites with temperature [26].

deep, subseafloor sediments (▶ Fig. 1.6), which may have important consequences for our understanding of fossil fuel formation [40], and sustain the deep biosphere up to its upper temperature limit (122 °C, [41]).

1.1.5 Eastern Equatorial Pacific and Peru Margin Sites 1225–1231 (Leg 201)

Leg 201 was the first dedicated "Deep Biosphere" Drilling Leg (27 January–29 March 2002) [4, 5, 24, 33, 42]. However, active deep bacteria had been detected at several of these sites on previous drilling Legs [43, 44] and repeat sampling would provide unique information about the consistency of deep biosphere populations, as well as more detailed information about these populations. At some sites, the complete sediment column was sampled plus the upper most part of the basaltic basement (1225, 1226, 1231) and prokaryotic cells were present at all sediment depths, although cells were not clearly stimulated at the sediment-basement interface despite evidence for fluid flow through this interface [33]. As previously found for other deep sediments [1], cell populations increased as water column depth decreased, presumably due to higher organic matter quantity and quality at shallow water sites (▶ Fig. 1.7). The only exception was the deep-water gas hydrate Site 1230, which had higher cell numbers than other deep-water sites. However, it has been previously shown that gas hydrate containing deep sediments can be particularly biogeochemically active [1]. This water depth trend also strongly suggests that the majority of cells are active, and not dead or dormant cells being buried, as had been previously suggested [3]. This was confirmed at some of these sites by detection of ribosomal RNA in cells (CARD-FISH) and by real-time polymerase chain reaction quantification of 16S rRNA genes (qPCR, [4]). Interestingly, the qPCR results indicated that *Bacteria* rather than *Archaea* were the dominant prokaryotes within these sediments.

16S rRNA gene libraries and DGGE analysis of Site 1229 Peru Margin sediments, which had a deep brine incursion, and hence, unusually, a deep methane-sulfate interface (~90 mbsf), in addition to the more normal sulfate-methane interface (~30 mbsf), showed marked changes in bacterial diversity and increases in total cells at these interfaces (▶ Fig. 1.7). However, changes in archaeal diversity were limited [24]. This further suggests that *Bacteria* are the major active prokaryotes in these subsurface sediments, with clear activity and diversity changes over geological time scales (e.g. 90 mbsf equals ~0.8 Myr). The dominant *Bacteria* were *Gammaproteobacteria* at 6.7 and 86.67 mbsf and *Chloroflexi* at 30.2 and 42.03 mbsf, with the common subseafloor biosphere phylum JS1 [38] being a minor component. Methanogenic *Archaea*, however, were detected in both 16S rRNA gene libraries (42.03 mbsf in the methane zone) and by methanogen-specific genes (*mcrA*, all 4 depths, 6.7, 30.2, 42.03, 86.67 mbsf). This was consistent with measured low rates of active methanogenesis from both H_2/CO_2 and acetate.

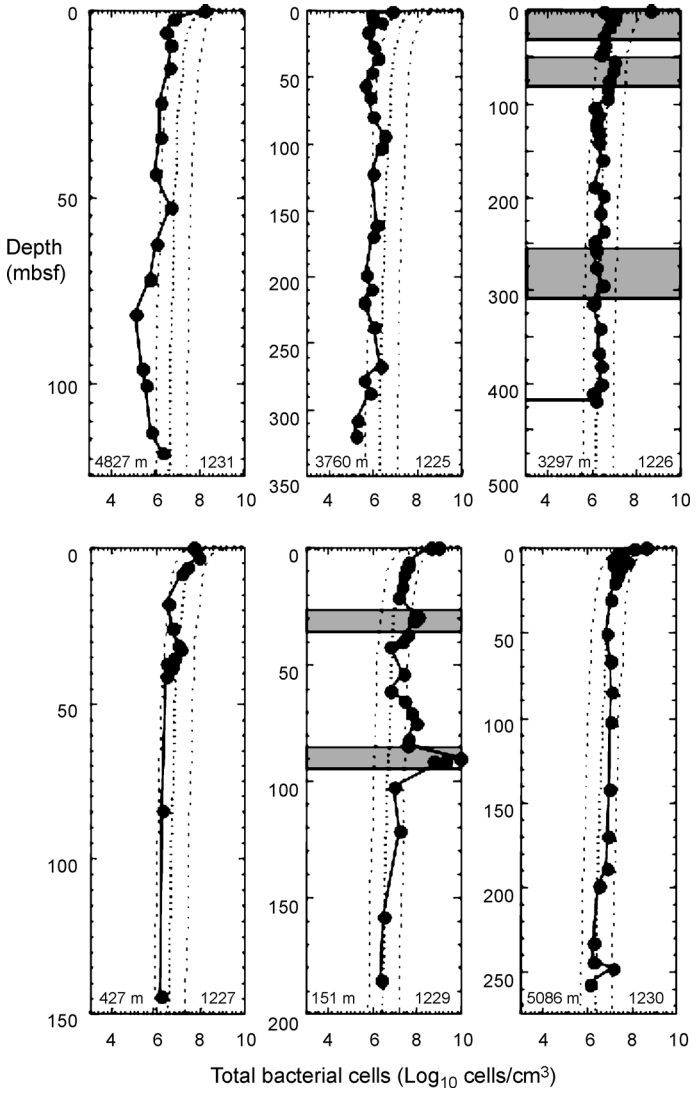

Fig. 1.7: Eastern Equatorial Pacific and Peru Margin Sites, Leg 201. Total cell numbers compared to cell depth profiles at other sites [1]. Cell populations increase as water column depth decreases, except for the deep-water gas hydrate Site 1230. Subsurface increases in cell numbers are highlighted by shaded areas in Sites 1226 and 1229.

Similar stimulation of prokaryotes occurred at an open ocean Site (1226, ▸ Fig. 1.7), but in association with repeated lithological depth changes and allied high diatom content. In the three diatom-rich layers between the surface and about 400 mbsf, there was a consistent stimulation of prokaryotic activity (sulfate reduction, growth – thymidine incorporation into DNA) and total cell numbers and/or the proportion of divid-

ing and divided cells [24]. It may be that diatomaceous organic matter is considerably less reactive than other sedimentary organic matter and as a consequence can fuel low, but continuing, prokaryotic activity over long periods. The deepest layer (~250 to 320 mbsf) was 7–11 Myr, which markedly extended the known time scale for stimulation of subsurface prokaryotic processes [1]. Furthermore, the diatom layers are controlled by Milankovitch scale cycles via oceanographic variability, intriguingly this links the depth distribution of the deep biosphere prokaryotes in some marine sediments to Earth's orbital forcing [45].

Furthermore, in the top and bottom diatom-rich layers there was an increasing concentration of dissolved manganese, indicating active prokaryotic manganese reduction in deep sediments. Manganese reduction would normally be expected to be restricted to near-surface layers, but here, due to a combination of high input of minerals and their slow reduction, continuing activity occurred in deeper layers. These sediments also deviate from expected diagenetic sequences in terms of sulfate (brine incursion), iron-reduction, methane formation in sulfate containing layers and oxidized fluids at the sediment-basement interface [33].

1.1.6 Newfoundland Margin (Leg 210)

Newfoundland Margin deep sediments are ancient and record the rifting of the North Atlantic Ocean, and thus were an important target to investigate the subseafloor biosphere in old and deep sediments. To enable deep samples to be obtained in the drilling time available, drilling occurred through the top 800 mbsf without coring, then coring was conducted from 800 to 1739 mbsf with excellent recovery (average 85%). The sedimentary succession consisted of background hemipelagic mudrocks with various proportions of interbedded gravity-flow deposits and terminated in diabase sills. Nine samples from Site 1276 were microbiologically analyzed with ages from 46 to 111 My [46]. Prokaryotic cells were present at all depths and distribution was similar to other marine sediments (▶ Fig. 1.8). The presence of dividing and live cells indicated that some of these cells were active, and this was supported by the extraction and amplification of archaeal 16S rRNA genes. Resulting 16S rRNA gene libraries showed a low diversity of *Archaea* with thermophilic *Pyrococcus* dominating the 958 m depth, and then as soon as methane increases above background concentrations, potential anaerobic methane-oxidizing archaeal (ANME) sequences became dominant. This continued until 1626 mbsf, with temperatures between 60 and 100 °C and high methane concentrations, where *Pyrococcus* and *Thermococcus* sequences dominated. This change may reflect the upper temperature limit for ANME prokaryotes [47] and thus other *Archaea* adapted to higher temperatures, and possibly able to use thermogenic higher hydrocarbons that accumulated below the diabase sill developed. These data provided direct evidence that significant prokaryotic populations are present in subseafloor sediments to greater than kilometer depths and as old

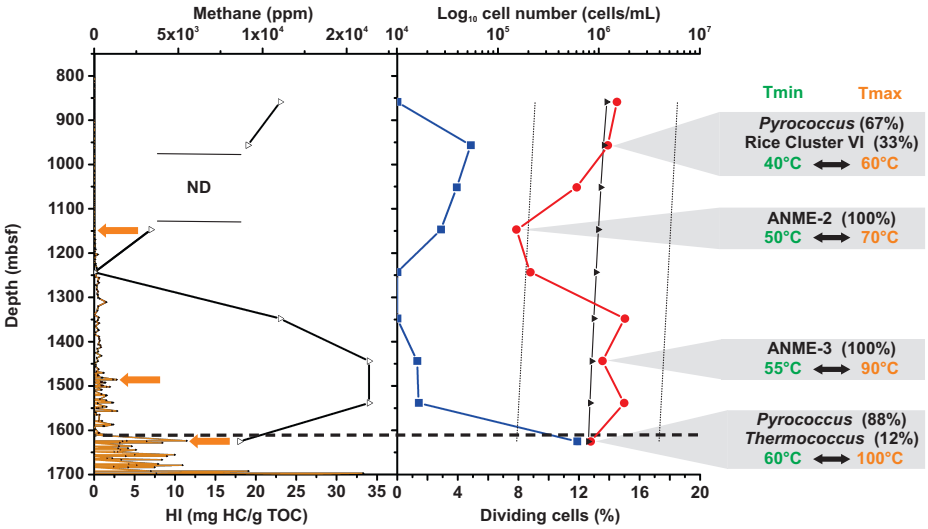

Fig. 1.8: Newfoundland Margin, Leg 210 [46]. Depth profiles of methane (black dots with orange line), prokaryotic cells (red circles), and percentage dividing cells (blue squares). Regression line for prokaryotic cells in other marine deep sediments (solid triangles), prediction limits (…) [1]. Orange arrows show local increases in methane. Hydrogen Index (HI open triangles) measured as mg of hydrocarbon (HC) per g of total organic carbon (TOC). ND, not determined. Dominant archaeal 16S rRNA gene sequences and *in situ* temperature range are on the right at the depths obtained. The diabase sill is shown as a bold horizontal dashed line.

as 111 My. Considering the 122 °C upper temperature limit for some prokaryotes [41], temperature alone would not limit prokaryotes until much deeper depths.

1.1.7 Carbonate mound (IODP Expedition 307)

The Challenger Mound (water depth 781–815 m water depth, ▶ Fig. 1.9) is a prominent mound structure (155 m high), which is partially buried with sediment and dead coral rubble on the Southwest Irish continental margin [48, 49]. Two mound sites, Flank (IODP site U1316) and Mound (IODP site U1317), were compared with a nonmound Reference site (IODP site U1318) upslope from the Challenger Mound [48]. This was the first carbonate mound to be drilled (~270 m) and analyzed in detail for microbiology and biogeochemistry (catalyzed reporter deposition-fluorescence *in situ* hybridization [CARD-FISH], qPCR [16S rRNA and functional genes, *dsrA* and *mcrA*], and 16S rRNA gene PCR-DGGE for prokaryotic diversity, and this was compared with the distribution of total and culturable cell counts, radiotracer activity measurements and geochemistry). There was a significant and active prokaryotic community both within and beneath the carbonate mound. As found in the Eastern Equatorial Pacific and Peru Margin Sites, prokaryotic activity at Expedition 307 Sites was quite diverse and activities

did not follow the expected depth distributions based on a sequence of reactions providing decreasing energy yield. Although total cell numbers at certain depths were lower than the global average for other subseafloor sediments and prokaryotic activities were relatively low (iron and sulfate reduction, acetate oxidation, methanogenesis) they were significantly enhanced compared with the Reference site. In addition, there was some stimulation of prokaryotic activity in the deepest sediments (Miocene, > 10 Ma), including potential for anaerobic oxidation of methane activity below the mound base. Both *Bacteria* and *Archaea* were present, with neither really dominant (overall 50% and 34%, respectively, with considerable variability in proportions between these geographically close sites, ▸Tab. 1.1). These were related to sequences

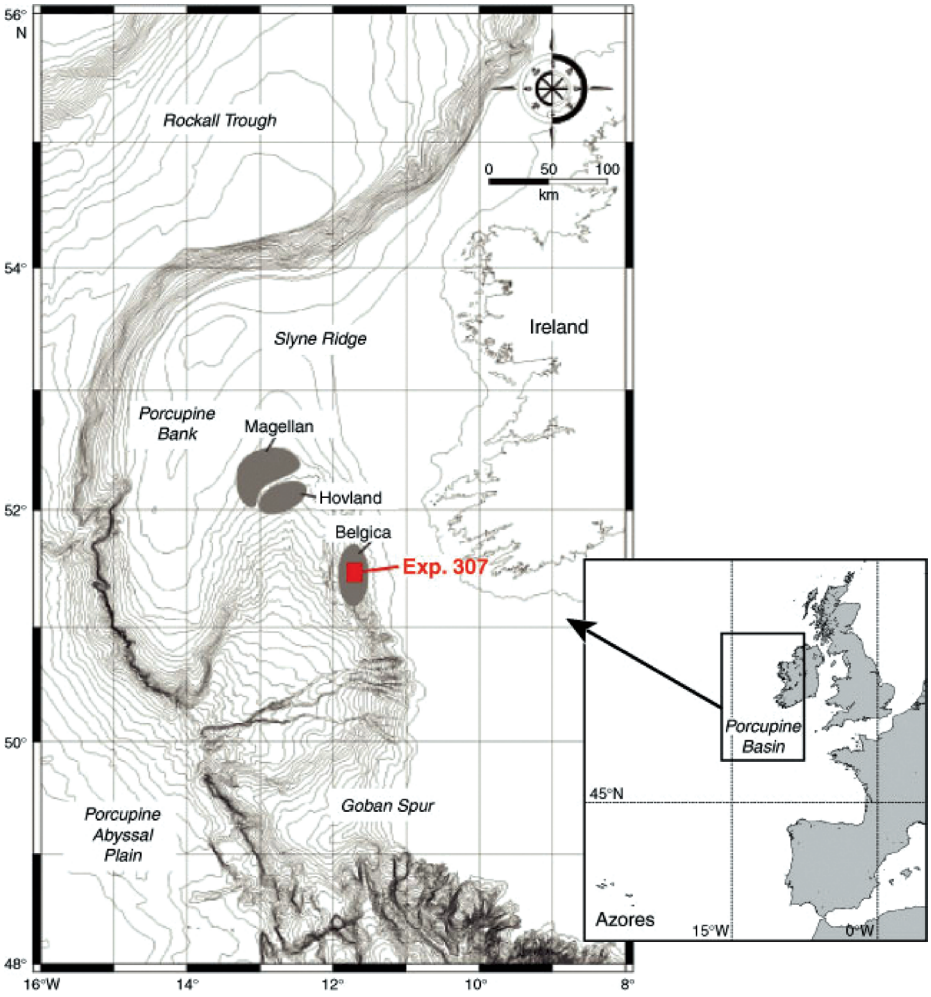

Fig. 1.9: Location of the Challenger Mound Site IODP Expedition 307.

commonly found in other subseafloor sediments (*Gammaproteobacteria*, *Chloroflexi*, JS1, SAGMEG, MBG-D and MCG). Overall, fewer prokaryotic sequences were detected at depth at all sites despite some activities being elevated in deeper layers. However, the majority of these sequences were mainly related to uncultured groups of prokaryotes from a range of different environments, and therefore, it is unclear what metabolisms are responsible for the measured deep elevated thymidine incorporation or acetate oxidation, particularly at the mound sites, and in the apparent absence of significant iron and sulfate reduction.

However, there were some contradictions within the molecular diversity data, for example no *Archaea* were detected by CARD-FISH, but they were detected by qPCR, PCR-DGGE and indirectly by the presence of archaeal methanogenesis at the mound Sites. In addition, the functional methanogen *mcrA* gene was not detected at these mounds Sites, yet was detected at the Reference site which had no detectable methane or methanogenesis. Such discrepancies may help to explain some of the differences in prokaryotic diversity at the same deep sediment locations by different research groups, for example, dominance of either *Archaea* or *Bacteria* at Leg 201 Sites [4, 5]. Despite these problems, active subseafloor prokaryotic populations were elevated in Mound sites compared to the Reference Site and with an estimate of some 1600 mounds in the Porcupine Basin alone, carbonate mounds may represent a significant prokaryotic subseafloor habitat.

1.2 High-pressure cultivation – DeepIsoBUG, gas hydrate sediments

Despite the ubiquitous presence of prokaryotic cells in subseafloor sediments and their large biomass, only a very small proportion of this population can be cultured (e.g. 0.1%, [33]). In addition, there is often a major discrepancy between the prokaryotes detected by molecular genetic approaches and culturing. Also, many phylotypes in clone libraries are unrelated to cultured sequences. Therefore, there is a large prokaryotic diversity in subseafloor sediments which has not been cultured and this severely limits our understanding of this major prokaryotic habitat. A key feature of subsurface environments is elevated pressure, e.g. ~70% of the ocean is at a pressure of 38 MPa or above [51], plus there is up to 10 km (~100 MPa) of sediment in some locations. Thus, the majority of subseafloor prokaryotes live under, and are likely to be adapted to, high pressure, which could be essential for culturing representative subseafloor prokaryotes.

We, therefore, developed a new system, Deep-IsoBUG [50], which can maintain sediments under elevated pressure (max 25 MPa) for enrichment, growth and isolation of prokaryotes at pressures up to 100 MPa. When this system is coupled with pressurized subsurface cores obtained using the HYACINTH drilling and core storage

Table 1.1: A comparison of bacterial and archaeal phylogenetic groups found in Porcupine Seabight (IODP Expedition 307) sediments.

Site/Sample depth (mbsf)	Major phylogenetic groups in 16S rRNA gene libraries or DGGE analysis (%) *Bacteria*															Total number of sequences/ DGGE bands
	Proteobacteria															
	Alpha	*Beta*	*Gamma*	*Delta*	*Chloro-flexi*	*Actino-bacteria*	*Firmi-cutes*	*Nitro-spirae*	*Spiro-chaetes*	JS1	OP1	OP8	OP11/ OD1	NT-B6	Others	
Mound flank site U1316																
18.9	–	–	4.8	23.8	23.8	–	–	–	–	9.5	–	–	–	9.5	28.6	21
90.0	–	25	25	25	–	25	–	–	–	–	–	–	–	–	–	4
98.8	–	–	–	–	–	100	–	–	–	–	–	–	–	–	–	1
Mound site U1317																
4.9	5.6	2.8	4.7	5.6	16.8	4.7	2.8	2.8	–	–	14	–	30.8	–	9.4	21,588
4.9 coral	9.8	5.7	6.6	4	14.7	9	8.2	3.2	–	–	12.3	–	18	–	8.4	19,729
20.9	10.8	3.6	6.3	4.5	23.4	5.4	9	6.3	–	–	3.6	–	16.2	–	10.8	26,617
20.9 coral	27.8	8.7	8.7	2.6	15.7	7	10.4	3.5	–	–	0.9	–	4.3	–	10.4	24,173
39.0	–	–	5.6	27.7	16.7	5.6	–	–	–	11.1	–	5.6	–	5.6	22.1	18
106.4 coral	19.5	8.8	8.8	0.9	15.9	8.8	12.4	4.4	–	–	5.4	–	9.7	–	5.4	27,678
146.6	–	50	50	–	–	–	–	–	–	–	–	–	–	–	–	4
219.9	28.6	14.3	42.8	–	–	–	–	–	–	–	–	–	–	–	14.3	7
Reference site U1318																
22.0	–	9.1	–	13.6	18.2	–	–	–	4.5	31.8	–	4.5	–	–	18.2	22
221.0	–	80	–	–	–	–	20	–	–	–	–	–	–	–	–	5

	Archaea								
		Crenarchaeota			Euryarchaeota				
	Thaum-archaeota MG1	MCG	MBG-B	SAGMEG	MBG-D	Methano-microbiales	Halo-bacteriales	Others	
Mound flank site U1316									
18.9	–	–	–	75	25	–	–	–	4
Mound site U1317									
4.9	8.6	54.3	4.3	20	–	2.9	5.7	4.2	20,855
4.9 coral	–	67.7	1.6	22.6	–	6.5	1.6	–	17,780
20.9	–	38.5	32.1	17.9	–	1.3	10.2	–	19,934
20.9 coral	–	89.5	–	8.8	–	1.7	–	–	19,428
39.0	–	20	–	80	–	–	–	–	5
106.4 coral	–	77.6	–	14.3	–	–	8.1	–	16,010
219.9	–	–	–	100	–	–	–	–	2
Reference site U1318									
22.0	–	66.6	–	–	33.3	–	–	–	3
221.0	–	100	–	–	–	–	–	–	1

Data from Webster et al. [48] and Hoshino et al. [70]; calculated from prokaryotic community profiles shown in Hoshino et al. [70].
JS1, OP1, OP8, OP11, OD1 = candidate divisions JS1, OP1, OP8, OP1 and OD1; NT-B6 = novel bacterial group NT-B6.
MG1 = Marine Group 1; MCG = Miscellaneous Crenarchaeotal Group; MBG-B = Marine Benthic Group B (or Deep Sea Archaeal Group); SAGMEG = South African Gold Mine Euryarchaeotal Group; MBG-D = Marine Benthic Group D; Others = other lineages, unaffiliated sequences and/or unsequenced DGGE bands.