

**A test of viability measures in starved, sedimentary,  
anaerobic bacterial isolates and in a temperature stressed  
estuarine sedimentary microbial community:  
insights for deep biosphere studies**

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## Summary

Marine sediments harbour vast and diverse prokaryotic communities. With ongoing burial and ageing of respective sediment layers, however, available organic matter becomes more recalcitrant. Thus, sedimentary microorganisms face starvation and ultimately death. Nonetheless, live and active cells are present in old and deeply buried sediments, up to 111 Ma (Roussel *et al.*, 2008). During IODP Leg 307 an organic-matter poor, cold-water, buried coral carbonate mound was sampled. Nineteen isolates, mainly *Proteobacteria*, were obtained from the mound and surrounding sediments. Additionally, one putative new species belonging to the genus *Ornithinimicrobium* (*Actinobacteria*) was isolated. Strains were subsequently phylogenetically and phenotypically characterised.

Selected isolates and other sedimentary bacteria were subsequently subjected to anaerobic starvation-survival experiments and their responses to substrate limitation were compared to those of near-surface relatives. All strains survived long periods of starvation (incubated up to 3 years). This was confirmed by constant total cell counts and only slowly increasing proportions of dead cells (20% after one year). Culturability and FISH detectability decreased with time but radiotracer experiments conducted after starvation confirmed viability and potential metabolic activity of many strains. No significant correlations between FISH detectability and other viability measures occurred. Instead starvation time was significantly positively correlated with percentages of dead cells and inversely with culturability.

Pure culture starvation experiments were complemented by a study on an estuarine, surface-sediment microbial community, which was stressed in sediment slurry sequential heating experiments. This mimicked burial and resulted in decreasing total counts, culturability, and FISH detectability but these were still present even after heating to 90°C. Temperatures above 42°C were significantly correlated with the reduction of total cells and FISH detectability.

This project showed that marine sedimentary microbes maintain high levels of viability and culturability during long-term anaerobic starvation and during sequential heating to mimic burial; this is consistent with the large cell population in sub-seafloor sediments.

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## Abbreviations:

AODC	Acridine Orange Direct Counts (3,6-bis[dimethylamino]acridine hydrochloride)
AOM	Anaerobic oxidation of methane
CARD-FISH	Catalysed Reporter Deposition Fluorescence <i>in situ</i> Hybridisation
DAPI	4',6'-diamidino-2-phenylindole dihydrochloride
DNA	Deoxyribonucleic acid
FISH	Fluorescence <i>in situ</i> Hybridisation
MPN	Most Probable Number
OTU	Operational Taxonomic Unit
PBS	Phosphate Buffered Saline
PI	Propidium iodide
ROS	Reactive oxygen species
RNA	Ribonucleic acid
S/GI	Sybr <sup>®</sup> Green I
Sol.	Solution
<i>sp. nov.</i>	<i>species nova</i> , new species
SRB	Sulphate reducing bacteria
TE	Tris EDTA Buffer
VBNC	Viable but nonculturable

# **Chapter I:**

## **Introduction**

### **1. Microbial Ecology as an emerging scientific field**

Microbial capacities were used by mankind for the production of wine, beer and bread for thousands of years, but it was not recognised for a long time that these processes are mediated by unseen little helpers, microorganisms. These small life-forms were first discovered and described by Robert Hooke and Antonie van Leeuwenhoek in 1665 and 1676 (Gest, 2004), respectively. Much later, in the second half of the 19<sup>th</sup> century, Koch was the first who linked bacteria to diseases and was able to prove it ([www.rki.de](http://www.rki.de)). In France, Louis Pasteur worked on lactic and alcoholic fermentation and used sterile culture medium for his experiments. This is said to be the “origin of all microbiological techniques” ([www.pasteur.fr](http://www.pasteur.fr)). Furthermore, he studied infectious diseases (e.g. rabies) and subsequently discovered vaccines to prevent them. At the same time the industrial use of microorganisms for purposes like the production of chemicals (e.g. acetone and citric acid) began. Thus, in the early days microbiology was mainly an applied science or focussed on medical aspects.

In addition, microorganisms are of great importance to humans not only for the reasons mentioned above. At the end of the 19<sup>th</sup> century scientists started to investigate the role of microbes within the environment, for example their role in geochemical cycles. Winogradsky, a Russian microbiologist, worked on geochemically important bacteria isolated from the environment and described the phenomenon of lithotrophy (Schlegel, 1996). A new science developed: microbial ecology. Since that time much research has been done in the field of microbial ecology due to the importance of microorganisms for ecosystems and their effects on the environment. They are involved in all biochemical cycles (e.g. carbon, sulphur, manganese, iron etc.) of which many reactions are exclusively driven by prokaryotes and, thus they affect our lives in many ways.

## 2. The deep biosphere

### 2.1 Discovery of the deep biosphere

In the beginning of the 20<sup>th</sup> century researchers investigated soils in regard to the indigenous bacterial population to identify potential pathogens (Schmidt and Weiss, 1902). They found a decrease in plate counts with increasing depth (from  $10^5$  cells  $\text{cm}^{-3}$  sediment near the surface to  $10^2$  cells  $\text{cm}^{-3}$  sediment at 4.5 metres below the surface) and concluded that soil is bacteria-free in depths below five metres. Additionally, since mostly spore-forming bacteria were enriched they concluded that bacteria were only present in an inactive form in greater depths.

Bastin *et al.* (1926) were among the first who investigated the widespread occurrence of sulphate-reducing bacteria in oil producing wells. They addressed the question whether the microorganisms were indigenous or introduced into the reservoirs by drilling water due to oil mining operations. Scientists assumed that the deep subsurface was sterile and thus bacteria within the oil reservoirs were introduced by human activities (Magot *et al.*, 2004).

Morita and ZoBell (1955) studied pristine marine sediments and concluded that the marine biosphere ended at the depth of 7.47 m below the surface. They based this conclusion on their inability to cultivate microbes from deeper layers. At that time microscopic detection of microbial cells in sediments was impossible. Later, the use of fluorochromes like acridine orange enabled the detection of microorganisms in a microscopic sample and made them visible to the human eye (Kepner and Pratt, 1994). Because of these findings the deep-sea was referred as a “microbiological desert” (Jannasch *et al.*, 1971) and was even thought to be sterile. This assumption was supported by results of Jannasch *et al.* (1971). Their study of food (soaked with seawater) within a sunken submarine vessel revealed that rates of microbial decomposition were 10 to 100 times lower than in control experiments under similar conditions.

However, geologists were interested in the geosphere below the oceans. In 1968 the Deep Sea Drilling Project (DSDP) started. During these scientific journeys researchers made observations within the sediment that could not be explained by geological processes alone (e.g. changes in porewater chemistry, production of gas

etc.; Parkes and Sass, 2007). These geochemical characteristics were indirect evidence for the occurrence and activity of microbes in deeper sediments. Thus, microbiologists were integrated within these legs.

The use of acridine orange, a dye that predominantly stains double-stranded DNA, allowed Parkes *et al.* (1990) to detect bacteria within Peru margin sediments. Cell numbers decreased slightly with increasing depth (deepest layer sampled: 80 mbsf, which is a factor of ten deeper than previously investigated), but they assumed that cells would be present in even deeper layers. Additionally, they observed dividing cells and furthermore were able to cultivate different physiological groups from most depths. Thus, they concluded that cells were viable and active within the subsurface. In 1994, Parkes *et al.* showed similar results for five different sites in the Pacific Ocean with viable cells up to 500 mbsf. The investigations of Rochelle *et al.* (1992) who were able to extract and amplify undegraded DNA via PCR from Japan Sea sediments supported the findings of Parkes *et al.* (1994) that active bacterial communities are present within the deep subsurface. Today we know that, for example, marine sediments harbour a deep biosphere up to 1626 metres below the seafloor (Roussel *et al.*, 2008). Furthermore, it was estimated by Whitman *et al.* (1998) that half of the Earth's prokaryotes could be found in this hostile environment. Following their calculations this corresponds to approximately 60% of the Earth's microorganisms in which up to 300 billion tonnes of carbon would be stored.

## 2.2 Current knowledge of the deep biosphere

After many years of research focussing on microbial communities and processes within the ocean's sediments, researchers have gained considerable knowledge about this hostile environment. In this section current understandings and scientific results will be presented. It is important to note that each drilling site has its own specific lithological and geochemical setting (i.e. differences in depth profiles of sulphate, methane, temperature etc.) and biological parameters (i.e. cell counts, community composition). Despite these site specific variations, general patterns were observed and will be summarized below.

Furthermore, it needs to be highlighted that sediment cores obtained during cruises of the Ocean Drilling Program (ODP) and the Integrated Ocean Drilling Program (IODP), which were used for microbiological purposes are generally of high quality and almost contamination-free (Smith *et al.*, 2000, Lever *et al.*, 2006). This is essential for studying and understanding the effects of microorganisms on geochemical processes occurring in the deep subsurface.

Researchers have addressed questions about the physiological state of the microorganisms living in the deep biosphere (Parkes *et al.*, 2000; D'Hondt *et al.*, 2004; Schippers *et al.*, 2005). It is not fully understood whether the indigenous prokaryotes are active, dormant or dead and which of these fractions is dominant. Luna *et al.* (2002) compared two different protocols for the determination of the dead, dormant, and active bacterial fractions in surface sediment cores. Bacteria with an intact membrane were considered to be alive and accounted for only 26% to 30% of the total bacterial cell counts. Their results further suggested that dead cells represent the dominant fraction (70%-74%). Additionally, they reported that among the bacteria with an intact membrane only 4% contained a nucleoid. Only nucleoid-containing cells are supposedly growing and thus metabolically active. According to their findings only a minor part of the near-surface bacteria is alive and active. This contradicts (not only) the results of Schippers *et al.* (2005) who reported that a large fraction of the prokaryotes is alive and active even in 16 million year old sediments (> 400 mbsf).

The ongoing debate of microbial physiological states and activities in marine subsurface sediments is also fuelled by the variety of methods available today. Different techniques and approaches investigating cellular viability (e.g. FISH/CARD-FISH versus LIVE/DEAD<sup>®</sup> staining) might lead to different conclusions as they target different macromolecules essential for survival (ribosomes and cell membrane, respectively). Potential microbial activities can be determined in many different ways, for example, by the utilization of electron-acceptors or substrates, the formation of metabolic products, the activity of extracellular enzymes (Bachofen *et al.*, 1998), or the incorporation of radioactively labelled substances into cell constituents. All of these approaches address certain aspects of activity which are most likely very different on a cell-to-cell basis within a mixed prokaryotic community. Nonetheless, the results obtained by such investigations give an idea

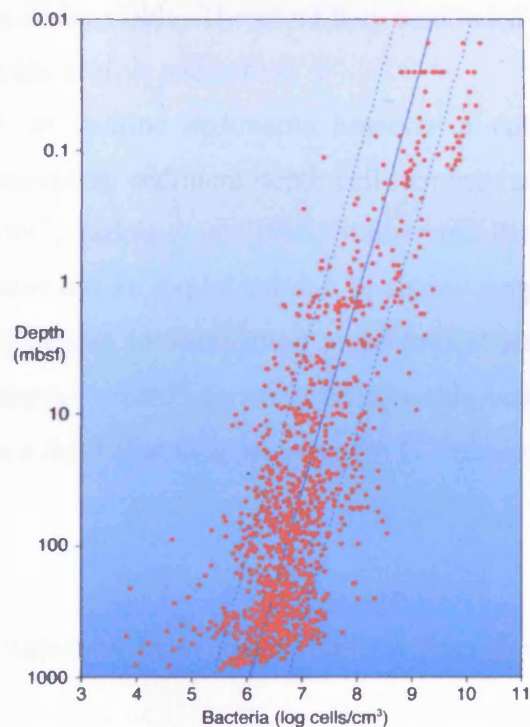
about the metabolic processes occurring in the subsurface, turnover-rates, and doubling times, all of which help gauge the degree of activity and thus viability of the deep biosphere.

### 2.3 Extent and maintenance of the deep biosphere

Counting the indigenous prokaryotic community provides fundamental information about a habitat. Linking cell numbers to measured activities leads to implications about e.g. energy fluxes, turnover times, dividing rates etc. Therefore, assessing the total cell count of a sample is a standard procedure in microbial ecology, but it can be achieved in different ways. The most established method is the acridine-orange direct count (AODC), which is used by many microbiologists. Parkes *et al.* (1994), using AODC, reported significantly decreasing cell numbers with increasing sediment depth (Fig. 1.1). Counts decreased from about  $10^9$  cells per  $\text{cm}^3$  sediment in the top few centimetres to circa  $10^6$  cells per  $\text{cm}^3$  at about to 500 mbsf. However, it was reported that AODC might yield higher counts than DAPI in seawater samples (Newell *et al.*, 1986). Furthermore, it is evident that both AODC and DAPI technique cannot differentiate between living and dead cells (Kepner and Pratt, 1994) because they target nucleic acids and even bacterial spores can be stained (Cragg and Sass, unpublished). Apparently, a dead cell could still contain its genomic DNA and hence would be counted as “live” (Kepner and Pratt, 1994).

Decreasing cell numbers with increasing depth are most likely linked to the rapid microbial depletion of easily degradable organic matter thus only leaving more recalcitrant organic compounds (i.e. humic acids, long-chain fatty acids; Parkes *et al.*, 2000). With ongoing burial and associated ageing of the respective sediment layer, microorganisms, which are not able to utilize this recalcitrant organic matter, face starvation and subsequently death. However, humic substances might also support microbial metabolism as they can be used as electron acceptors for the anaerobic oxidation of organic compounds and hydrogen (Lovley *et al.*, 1996). The electrons are shuttled to Fe(III)-oxides, which are then reduced. A recent report by Nielsen *et al.* (2010) extends this extracellular electron transfer over more than one millimetre. They observed that oxygen consumption at the sediment surface was linked to hydrogen sulphide and organic carbon oxidation within the anoxic zone of

the sediment. These processes are apparently coupled by electric currents conducted via bacterial nanowires, pyrite, soluble electron shuttles, and outer-membrane cytochromes. Thus, seemingly spatially separated chemical and biological processes might actually be linked, which “adds a new dimension to our understanding of biogeochemistry and microbial ecology” (Nielsen *et al.*, 2010). Furthermore, it has been reported by Wellsbury *et al.* (1997) that with increasing depth and temperature the recalcitrant organic matter might become available in the form of acetate, which is released from it. Other thermogenically produced compounds like methane (Whiticar, 1999) or hydrogen, microbially produced from minerals (Parkes *et al.*, 2011) or by the radiolysis of water (Lin *et al.*, 2005), also nourish the deeply buried microbial community constantly aiding their survival and activity.



**Fig. 1.1: Depth profile of prokaryotic abundance in marine sediments.** Adapted from Parkes *et al.*, (1994)

To detect cells within the marine subsurface Schippers *et al.* (2005) used ribosomal RNA as a target for catalyzed reporter deposition-fluorescence *in situ* hybridization (CARD-FISH; Pernthaler *et al.*, 2002). Ribosomes are essential for protein synthesis and the cellular ribosome content has been linked to activity (Bremer and Dennis, 1987; Bremer and Dennis, 1987; Stahl and Amann, 1991; Lee *et al.*, 1993; Kemp *et al.*, 1993), hence mono-labelled fluorescent probes have proven to be a reliable tool for the detection of active cells. CARD-FISH enables detection of cells with a low

ribosomal content and supposedly detects less active cells. Schippers *et al.* (2005) showed that one-tenth (for ocean-margin site) to one-third (for open-ocean site) of the sub-seafloor prokaryotes is active, even in very old (16 million years) and deep (>400 mbsf) sediments. Surprisingly, they reported that bacterial numbers determined by CARD-FISH did not significantly decrease with depth, in contrast to AODC. They explained this phenomenon with the ongoing degradation of cells with age and depth. According to their results the number of active prokaryotes might be an order of magnitude lower than the number of total prokaryotes previously estimated with AODC (Parkes *et al.*, 1994). However, Schippers *et al.* assumed that they only detected a minimum of active cells with CARD-FISH, as some cells might contain even fewer ribosomes than required for CARD-FISH detection (Hoshino *et al.*, 2008) yet might still be viable. Therefore they concluded that a large fraction of sub-seafloor prokaryotes is alive and active.

For most of the marine sediments however a common trend could be observed that with increasing sediment depth cell numbers are decreasing (Fig. 1.1; Cragg *et al.*, 1990, 1997; Parkes *et al.*, 1990; Mather and Parkes, 2000). Exceptions to this general situation can be explained due to energy supplies like crustal fluids (Cowen *et al.*, 2003) or due to stimulation at geological interfaces (Parkes *et al.*, 2005). The current depth “record” for detectable, viable cells in marine sediments lies at 1626 mbsf, in a layer that is approximately 111 million years old (Roussel *et al.*, 2008).

## 2.4 Processes mediated by microorganisms within the marine subsurface

Despite the fact that the marine subsurface is inhabited by the major fraction of Earth's microorganisms (Whitman *et al.*, 1998) the metabolic activities in this environment are relatively low (D'Hondt *et al.*, 2002) due to the lowest energy fluxes measured so far (Chapelle and Lovley, 1990). Within the sediment column usually a vertical succession of respiration processes occurs. The electron acceptor allowing the highest energy yield is oxygen. It is dissolved in seawater and penetrates the sediment up to a few millimetres or centimetres (Canfield *et al.*, 1993). It is readily consumed by aerobic microorganisms. After oxygen is depleted nitrate, Mn(IV) and Fe(III) reduction occur, followed by a zone of sulphate-reduction. It was found that



these processes dominate carbon mineralization, but their relative significance varies depending on the concentrations of each molecule and the type of sediment (Canfield *et al.*, 1993). Below the sulphate reduction zone methanogenesis, acetogenesis and fermentation are the major processes but these also occur in shallower layers.

It was generally accepted that microbes in marine sediments consume organic matter derived from photosynthesis (organotrophic) and therefore are depending on sunlight. This probably applies to most organisms living in marine sediments. An alternative *modus vivendi* are so called subsurface lithoautotrophic microbial ecosystems (SLiMEs). The possibility of a hydrogen-driven SLiME was first described by Stevens and McKinley (1995). Later SLiMEs were defined to be energetically powered by processes within the geosphere. These geological processes have to supply both the energy source (electron donors, i.e. H<sub>2</sub>) and the oxidants (electron acceptors, i.e. CO<sub>2</sub>) to refer to a “real” SLiME (Nealson *et al.*, 2005). One example for this is the hydrothermal fluid flow, which can stimulate microbial activity within the deep biosphere (Cowen *et al.*, 2003; Engelen *et al.*, 2008).

Another intriguing process, which has gained much attention in recent studies, is the anaerobic oxidation of methane (AOM). It often occurs in sediments where Sulphate-Methane-Transition-Zones are found (Reeburgh, 1980; Iversen and Jorgensen, 1985; Valentine and Reeburgh, 2000). The physiological reaction is supposed to be carried out by a consortium consisting of sulphate-reducing *Bacteria* and methanogenic *Archaea* (Boetius *et al.*, 2000). Methane in form of gas hydrates occurs in deep-sea sediments (Kennicutt *et al.*, 1993). Cragg and Kemp (1995) and Cragg *et al.* (1996) observed that discrete layers of methane hydrate stimulated bacterial populations and activities in deep Cascadia margin sediments (ODP Leg 146). Methane oxidation was linked to increases in cell numbers and to the re-appearance of sulphate-reducers, which were otherwise undetectable below 10 mbsf.

## 2.5 Drilling sites with special characteristics

Probably the best-investigated ODP sites in regard to microbial ecology are those sampled during Leg 201. This was the first cruise dedicated to deep biosphere research (D'Hondt *et al.*, 2003) and aimed to “study life within the ocean’s sediments”. The drilling sites chosen were similar to many other habitats found

beneath the oceans and therefore should “represent the general range of subsurface environments that exist in marine sediments throughout most of the world’s oceans.” (D’Hondt *et al.*, 2003). Samples were obtained from organic-poor, low-activity sediments (central oceanic basins) and organic-rich, coastal sediments (upwelling areas; Teske, 2006). Because of its multidisciplinary approach the results of this particular expedition provide a comprehensive picture about microbial community composition and activity.

A large number of publications deals with findings of this cruise, only a few can be summarized here. D’Hondt *et al.* (2004) found a high diversity of isolates and metabolic activities. They discovered, for example, that methanogenesis occurred in zones where sulphate- and metal ion-reduction also took place. This unusual phenomenon can be explained by the use of non-competitive substrates by different physiological groups of microorganisms (Mountfort *et al.*, 1980; Oremland and Polcin, 1982). Additionally, it was also revealed that at open-ocean sites redox-zones that occur at the water-sediment-interface are mirrored at the sediment-basement-interface (Fig. 1.2). Presumably, electron-acceptors diffuse upwards from water circulating through the underlying basalt. Therefore, D’Hondt *et al.* (2004) stated that microbial energy-gaining processes within the environment are complex and cannot necessarily be predicted from knowledge of shallow marine sediments.

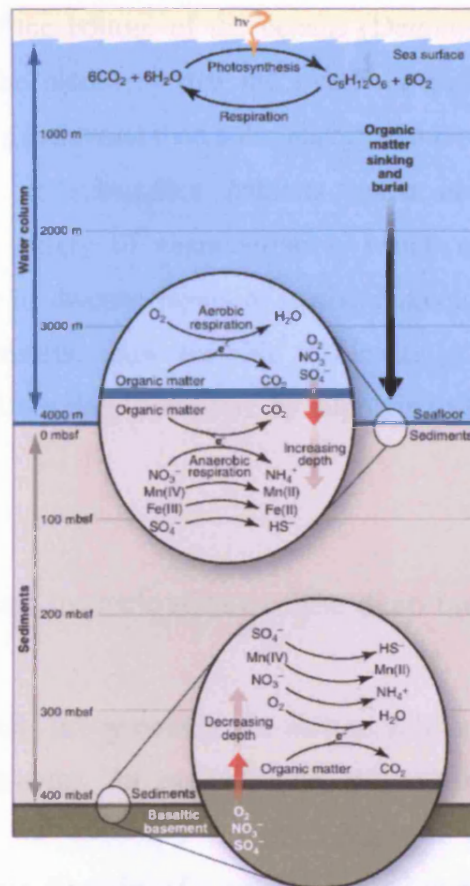
Despite the wide microbial metabolic diversity within marine sediments metabolic activity is low (D’Hondt *et al.*, 2002), but it can be stimulated in organic-rich layers (Coolen *et al.*, 2002) or at lithological interfaces as shown by Parkes *et al.* (2005). They investigated two sites of Leg 201 (open ocean- and ocean margin-site) and discovered elevated microbial activities at discrete sedimentary interfaces. They also emphasized the stability of these stimulated microbial communities as the same sites had been studied previously (Parkes *et al.*, 1990).

An ongoing debate between scientists is which prokaryotic group is predominant in marine sediments. Schippers *et al.* (2005) using CARD-FISH found *Bacteria* to represent the majority of microorganisms within the sediment column whereas Biddle *et al.* (2006) detected mostly *Archaea* using FISH. Teske (2006) summarized the microbial diversity revealed by molecular and cultivation surveys. For *Archaea* it was shown that the uncultured representatives of the Marine Benthic Group B (MBG-B) are predominant in both, organic-poor and organic-rich sediments. Other important clusters are the Miscellaneous Crenarchaeotal Group

(MCG), the South African Goldmine Euryarchaeotal Group (SAGMEG) and the Marine Group I (MG-I). Webster *et al.* (2006a) investigated bacterial lineages within Peru margin sediments. They found that dominating sequences were affiliated to Chloroflexi and *Gammaproteobacteria* using both, PCR-DGGE and cloning. In contrast to these findings isolates obtained from these open-ocean and ocean-margin sites were mostly related to *Firmicutes* and  $\alpha$ -*Proteobacteria*, whereas  $\gamma$ -*Proteobacteria* were only found at ocean-margin sites (D'Hondt *et al.*, 2004). Batzke *et al.* (2007) were able to cultivate a broad spectrum of phylotypes with *Rhizobium* and *Bacillus* being the most frequent representatives. This reflects the common discrepancy when assessing prokaryotic community composition using cultivation and cultivation-independent methods (Webster *et al.*, 2006a).

Not only at open-ocean sites of Leg 201 but at several ODP drilling sites where the basaltic basement was sampled, it was observed that fluids seeping from the basalt into the overlying sediment fuel the deep biosphere e.g. the *Juan de Fuca* Ridge (JFR; Cowen *et al.*, 2003). Seawater enters the porous basement (for JFR at the "Baby Mount") containing electron acceptors that elsewhere diffuse upwards into the sediment. It was shown that these fluids also support microbial growth by providing hydrogen and/or organic molecules (e.g. short- and long-chain *n*-alkanes) that derive from abiotic reactions of seawater with the basalts (Cowen *et al.*, 2003). As mentioned earlier, fluids also provide an electron acceptor chain that mirrors the situation at the sediment surface (Fig. 1.2; D'Hondt *et al.*, 2004). In addition to the fact that fluids support microbial activity and growth within deepest sediment layers, they themselves contain microbial communities with species typical for subsurface environments (Huber *et al.*, 2006). This indicates the existence of a hot deep biosphere even in the underlying crust (Gold, 1992).

In the Eastern Mediterranean Sea organic-rich sediment layers, called sapropels, can be found. These formed periodically approximately every 20,000 years (Rohling, 1994), contain high concentrations of organic material and harbour metabolically active chemoorganotrophic prokaryotes (Cragg *et al.*, 1998; Coolen *et al.*, 2002). In



**Fig. 1.2: Depth distribution of electron-acceptor use in marine sediments.** At the seafloor-sediment interface a zonation of electron-acceptor use can be found according to the energy obtained due to their reduction. Oxygen delivers highest energy yields and hence is depleted rapidly followed by nitrate, Mn(IV), Fe(III) and sulphate. As shown by D'Hondt *et al.* (2004) a mirrored series can be found at the sediment-basalt interface of certain drilling sites. Source: DeLong (2004)

comparison to the organic-poor intermediate layers, sapropels exhibit elevated cell numbers and increased activities of hydrolytic enzymes (Coolen *et al.*, 2002). Parkes *et al.* (2000) argued that despite the presence of vast amounts of organic matter generation times of the active population are very long (~105,000 years). The organic matter within sapropels is recalcitrant and therefore only allows very slow growth. These and other data indicate that even in the oldest investigated sapropels microorganisms are metabolizing the highly recalcitrant organic matter buried within the sediment (Coolen *et al.*, 2002; Süß *et al.*, 2004).

Another interesting habitat found in the deep sea are hydrothermal vents, which were discovered in the 1970's. Being named "windows to the deep biosphere" (Deming and Baross, 1993) scientists learnt a lot from these formations. One example is the extension of what was believed to be the upper temperature-limit of growth from below the boiling point of water at atmospheric pressure to about 113°C

at pressures present at the bottom of the oceans (Deming and Baross, 1993). But more important was the discovery that the microbial populations associated with vents rather depend on geothermal than solar energy (Jannasch and Mottl, 1985).

The examples of subsurface habitats listed above only give a brief introduction into the variety of characteristics, which can be found below the seafloor. The presence of discrete layers of methane, geological interfaces or fluids emanating from the basaltic crust are able to elevate microbial activity and cell numbers and thus, fuel the deep biosphere by supplying new energy sources and/or electron acceptors.

## 2.6. Cultivation-based investigations of the deep biosphere

Currently microbiologists are generally not able to cultivate all prokaryotes from a single habitat. For seawater, for example, the culturability often ranges between 0.001% and 0.1% of the total cell counts (Amann *et al.*, 1995). Although the phylogenetic prokaryotic diversity of a sampling site can be estimated by molecular approaches this knowledge alone does not reveal what the microorganisms might do *in situ*. With isolates it is possible to assess the capabilities of microbes and consequently conclude their role and activities within and their influence on the environment. Therefore, the study of these isolates and their physiological properties help to reveal the potential ecological role of these microorganisms within the environment (autecology).

### 2.6.1 Different cultivation approaches

The cultivation medium has a profound influence on cultivation success (Köpke *et al.*, 2005). Defined media containing a variety of different substrates can be expected to stimulate growth of a variety of metabolic types. These media are useful for MPN-series (most-probable number) to evaluate the number of culturable cells in a sample. More specific growth media are used to isolate members of certain physiological groups (e.g. sulphate-reducing prokaryotes, methanogens etc.). Therefore, it is important to apply different growth media to a single sampling site to ensure that all important physiological groups are covered. Using this strategy Köpke *et al.*, (2005)

achieved cultivation efficiencies up to 23% of total cell counts for a single medium and a remarkable high diversity among the isolates obtained.

A different approach showed that the addition of signal molecules like cAMP or homoserine lactones to growth medium significantly raised the number of prokaryotes stimulated to growth in water samples from the Baltic Sea (Bruns *et al.*, 2002). Unfortunately, attempts to apply this strategy to sediment samples did not result in elevated culturability (H. Sass 2007, personal communication).

Most microbes are likely to be adapted to low substrate concentrations due to their oligotrophic environment. Postgate and Hunter (1962) showed that sudden exposure to high substrate concentrations can lead to a “substrate-accelerated death”. To avoid such a substrate-shock (Straskrabova, 1983), it is helpful to use substrate concentrations at a sub-millimolar level (Süß *et al.*, 2004) or to use gradient tubes for cultivation (Köpke *et al.*, 2005). In the latter the cells are trapped in the surrounding agar. Substrate is supplied by diffusion from a reservoir below, thus the substrate-concentration is low at the beginning of the incubation but potentially increasing. Hence, some oligocarbophilic microorganisms can adapt to higher concentrations without dying or concentrations remain low if consumption is quicker than diffusion.

It has to be questioned whether prokaryotes that were obtained from nutrient-limited environments by using substrate-rich cultivation media play an important role within their oligotrophic habitat. They could have been dormant or inactive but were resuscitated after transfer into growth medium from which they were subsequently isolated. Such an opportunistic microorganisms would follow the ecological r strategy, rapid growth when resources are abundant (allochthonous or zymogenous) while others are K strategists would be autochthonous (indigenous) according to the definition of Winogradsky (1924).

## 2.7 Molecular-based investigations of the deep-biosphere

Molecular approaches enabled microbiologists to obtain knowledge about the microbial community composition of a habitat without cultivation. The applications range from quantifying abundances of specific phylogenetic groups (oligonucleotide probes targeting ribosomal sequences) via identification to the species level (cloning and sequencing of 16S rRNA) and the identification of environment-specific genes

(metagenomics) to direct *in situ* measurements of activities with the help of isotopes and their incorporation into biomarkers (e.g. DNA, RNA, lipids) or their metabolism (radioactively labelled compounds).

### 2.7.1 Phylogenetic analyses of microbial communities

In the middle of the 20<sup>th</sup> century, researchers developed the theoretical framework to identify organisms via DNA sequencing (Zuckerandl and Pauling, 1965). Sanger *et al.* (1977) developed a rapid and accurate sequencing technique, which in combination with the later invented amplification of genes (PCR, Mullis and Faloona, 1987) from the environment led to extraordinary results and changed our way of understanding biodiversity and evolution. One example is the discovery of the three domains of life (Woese *et al.*, 1990), another is the use of the sequences obtained as a molecular clock (Woese, 1987) to follow the processes of evolution (Zuckerandl and Pauling, 1965). The separation of the amplified genes via denaturing gradient gel electrophoresis (DGGE; Muyzer *et al.*, 1993) or temperature gradient gel electrophoresis (TGGE; Rosenbaum and Riesner, 1987) followed by advanced sequencing (Lane *et al.*, 1985) allowed researchers to affiliate them to known, cultivated microorganisms. Thus, the community composition of a habitat could be rapidly revealed.

Today the 16S rRNA is the molecule of choice when phylogenetical analyses are conducted. It replaced the 5S rRNA, which was originally used for phylogenetic analyses, because the 16S rRNA not only contains more nucleotides than the 5S rRNA but also shows regions of higher variability additionally to the well preserved ones. This is useful to calculate phylogenetic distances (Pace *et al.*, 1986). However, many other genes have also gained attention. In environmental microbiology this is especially true for *dsrAB*- and *mcrA*-genes. The *dsrAB*-gene is present in all known sulphate-reducing prokaryotes. The *mcrA*-gene codes for an essential enzyme for methanogenesis and AOM. The detection of these genes in a sample shows the presence of these physiological prokaryotic groups in the environment. Additionally, their sequences are used for the construction of phylogenetic trees. Furthermore, evolution of these genes and horizontal gene transfer among species can be followed (shown for *dsrAB*-gene: Wagner *et al.*, 1998).

### 2.7.2 FISH and CARD-FISH

With the knowledge that certain 16S rRNA sequences are specific for different phylogenetic groups fluorescent rRNA-targeted oligonucleotide probes can be designed. These probes are used to detect and quantify the abundance of phylogenetic groups in environmental samples via whole-cell-hybridization without cultivation (Amann *et al.*, 1990). Another apparent advantage of this method is that the spatial arrangement of cells can be observed. For example, Boetius *et al.* (2000) found a consortium consisting of methanogenic *Archaea* surrounded by sulphate-reducing *Bacteria*. Maixner *et al.* (2006) investigated activated sludge flocs and observed population structures with cell patterns that deviate from random community assemblies. However, the fluorescence *in situ* hybridization-technique requires a relative high number of ribosomes to give a detectable signal (Hoshino *et al.*, 2008). As activity of prokaryotes is relatively low in oligotrophic environments potentially not many ribosomes are present within single cells. Catalyzed-Reporter-Deposition-FISH (CARD-FISH) enhances the oligonucleotide-signal using tyramide signal amplification and a horseradish peroxidase. This enables detection of cells with a ribosome content too low for FISH detection (Bobrow *et al.*, 1989). Pernthaler *et al.* (2002) adapted this method for the marine environment.

### 2.7.3 Linking *in situ* activity to phylogenetic identity

While phylogenetic approaches merely prove the presence of microorganisms in an environmental sample, FISH detects cell that are presumably active because of their ribosomal content. However, neither of these techniques actually links *in situ* activity with phylogenetic identity.

#### a) Stable isotope probing (SIP)

The use of stable isotopes makes it possible to identify active phylogenetic groups (Dumont and Murell, 2005). For this purpose deuterium- ( $^2\text{H}$ -) or  $^{13}\text{C}$ -labeled substrates like methane, acetate or glucose are used. The isotopes are incorporated into biomolecules, which are later extracted and analysed. Thus, incorporation not only proves microbial activity but can reveal the identity of the microorganisms



(Radajewski *et al.*, 2000). Boschker *et al.* (1998), for example, used the incorporation of  $^{13}\text{C}$ -labelled acetate into polar lipid fatty acids to investigate the composition of a microbial community responsible for the consumption of acetate in coastal sediments and of methane in freshwater sediments. Webster *et al.* (2006a) furthermore investigated incorporation of the isotopes into DNA, which was then separated from non-labelled DNA by CsCl-ethidium bromide density gradient ultracentrifugation. Subsequently they used PCR-DGGE of the 16S rDNA and the *dsrA* gene to identify the active microorganisms. Dumont and Murell (2005) stated that combining SIP with other technologies offers promising applications for the future.

#### b) Microautoradiography combined with FISH

Another approach linking activity with identity is to combine microautoradiography with the recently developed fluorescence *in situ* hybridisation (MAR-FISH, Nielsen *et al.*, 1999). The importance and wide applicability of microautoradiography has been recognised very early on by Brock and Brock (1966). This technique uses radioactively labelled substances such as  $^3\text{H}$ -leucine,  $^3\text{H}$ -thymidine, or  $^{14}\text{C}$ -glucose, which are incorporated into cellular structures by active microorganisms. If the radioactively labelled specimen is brought into contact with a radiosensitive emulsion containing silver bromide and subsequently photographically developed, cells that have incorporated the radioactive signal will be surrounded by silver grains (Nielsen *et al.*, 1999). If FISH targeting certain phylogenetic groups is performed on these specimens, cells that exhibit both a fluorescent and a radioactive signal are thus being identified as metabolically active and in regard to their phylogeny. Lee *et al.* (1999) applied this technique to complex activated sludge microbial communities. They hybridised cells prior to the microautoradiographic development procedure and used confocal laser microscopy of cryosectioned samples for single-cell resolution. They concluded that the combination of these techniques provides unique insights into the structure and function of mixed microbial communities.

### 3. Starvation-survival of prokaryotes

Starvation survival of prokaryotes is of great interest. Many publications, not only in microbial ecology, deal with the question of how microorganisms cope with limited substrate supply and overcome periods of starvation. This, for example, is also important for public health. Knowledge about survival of pathogens and maintenance of pathogenesis under starvation conditions is crucial. This introduction, however, tries to focus on environmental prokaryotes that are exposed to starvation.

The majority of habitats on Earth is considered oligotrophic. Open ocean waters, for example, are limited in dissolved organic carbon with often less than 0.5 mg carbon per litre (Menzel and Ryther, 1970), therefore not much substrate is available to heterotrophic prokaryotes. Hence, it can be assumed that the organisms suffer from starvation. The same is true for the oceans' sediments. Although much higher total organic carbon is present it is less available for microbial degradation.

There are different ways how bacteria cope with substrate limitation. Those, which are capable of forming spores, can do this in response to starvation to survive long periods of time until conditions become more favourable (Driks, 2002). Others form so-called "dwarf"-cells to evade predation (Andersson *et al.*, 1986) and to increase their surface to volume ratio (Kjelleberg *et al.*, 1982) to survive periods of substrate limitation. Bacteria, which cannot cope with the harsh conditions, die and potentially serve as substrate for the remaining microorganisms.

In the scientific literature the nomenclature of describing different states of prokaryotes is often equivocal. The terms viability and culturability, for example, are often equated (e.g. viable cell count often refers to the number of culturable cells). However, I find that there is a difference between these two. A viable cell detected, for example, by LIVE/DEAD<sup>®</sup> staining or FISH has the potential to grow and divide under certain conditions but not necessarily in the cultivation medium provided. Culturability, however, is the actual act of growth in the used medium. Hence viability is a prerequisite for culturability. If starving cells that used to grow in a specific medium subsequently lose their culturability, they are called viable but nonculturable (VBNC). Often when the VBNC state is observed cells are referred to as dormant or inactive. However, this is not necessarily the case as the cells might well be active – they "just" do not grow anymore and frequently this state is

temporarily. Throughout this thesis I will try to distinguish between these terms in this way.

### 3.1 Mechanisms of starvation-survival

In their review Kjelleberg *et al.* (1987) pointed out that marine non-growing prokaryotes are not necessarily dormant but still active and taking up substrates. These exogenous but also endogenous substrates, for example, storage molecules (poly-hydroxybutyrate) or cell constituents (e.g. proteins, RNA) enable cellular maintenance and the starvation survival response (e.g. *de novo* synthesis of proteins; Amy and Morita, 1983a) during periods of limitation but do not enable growth. Therefore nongrowing cells still contribute to the carbon flow.

Joux *et al.* (1997) investigated *Salmonella typhimurium* under starvation conditions and proposed a succession of physiological states that cells enter upon nutrient limitation. These states are: 1. culturable cells; 2. nonculturable cells still able to respire; 3. cells without respiration but potential respiration maintenance; 4. cells without any respiration but maintenance of membrane permeability; 5. cells with loss of membrane permeability but cell integrity without changes in DNA; 6. cells showing integrity but with DNA change leading to lysed or non-nucleoid-containing cells.

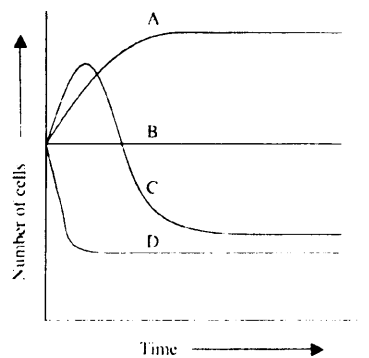
The intracellular processes that occur at the onset of starvation differ greatly between species and some will be discussed below. It seems, however, that a reduction in cell size is the most common response to starvation.

#### 3.1.1 Reductive cell division versus “dwarfing”

Nyström (2004) summarised the behaviour of starving *E.coli* cells in the stationary phase. He clearly differentiated between reductive cell division and dwarfing. He stated that reductive division is the completion of rounds of DNA replication and cell division when cells enter the stationary phase. Therefore it is not a process induced by starvation but nonetheless an adaptation that might be advantageous during substrate limitation because it results in a better surface/volume ratio. Dwarfing instead is induced by starvation and is a continuing process with ongoing substrate

limitation after reductive cell division is completed. Cells utilise parts of their own macromolecules such as the cytoplasmic membrane or the cell wall to obtain energy for survival and therefore decrease in cell size; they “self-digest”. The reduction of cell size offers another advantage for marine bacteria as shrinking might reduce the risk of predation (Andersson *et al.*, 1986). Therefore, despite not being able to multiply species avoid extinction, as grazers prefer larger prey (Kjelleberg *et al.*, 1987). Torrella and Morita (1981) proposed the term ultramicrobacteria to refer to small/dwarfed cells formed due to starvation to not confuse this phenomenon with small cells of *E. coli* that lack DNA (Adler *et al.*, 1967).

Morita (1985) described four different patterns of changes to total cell numbers in pure cultures in response to starvation (Fig. 1.3). Pattern A, which shows an increase in cell numbers with subsequent stabilisation, was found by Amy and Morita (1983b). Line B represents findings in nitrifying bacteria (Jones and Morita, 1985) where cell numbers remained constant over the complete starvation period (21 weeks). Behaviour of *Vibrio* sp. Ant-300 (Novitsky and Morita, 1976) and other marine strains (Kurath and Morita, 1983; Kjelleberg *et al.* 1982) is represented in curve C. Pattern D was also shown by Amy and Morita (1983b) after they starved freshly isolated marine bacteria. Morita (1985) stated that this pattern likely represents bacteria grown under relative energy-rich conditions, which then had been released into the marine environment.



**Fig. 1.3: Patterns of total cell number development during starvation.** Reproduced from Morita (1985).

### 3.1.2 Energy supply during starvation

Self-digestion or autolysis of microorganisms during starvation is due to endogenous metabolism. It was defined by Dawes and Ribbons (1962) as “metabolic reactions that occur within the living cell that when it is held in the absence of compounds

which may serve specifically as exogenous substrates". By using their own macromolecules cells regain part of the energy that was used for the build-up. This energy is then used to alter cellular processes. It was observed, for example, that cells produce new proteins when entering starvation, which is energy consuming, whereas other proteins disappear (Amy and Morita 1983a). Energy is also needed for substrate uptake (Stouthamer, 1973). It was shown that bacteria maintain and regulate their uptake systems (Kjelleberg *et al.*, 1987). These systems show low substrate specificity but possess a high affinity (Akagi and Taga, 1980; Poindexter, 1981), a crucial feature for marine bacteria using a variety of substrates. By using one system to transport a variety of different substances via the membrane, energy is saved and furthermore energy-delivering molecules are acquired. Energy is present within the cell in form of adenosine triphosphate (ATP). It was shown that ATP levels increase at the beginning of starvation in a marine *Vibrio* (Amy *et al.*, 1983) and *Pseudomonas* (Kurath and Morita, 1983) and are maintained at a high level throughout starvation. In contrast to that Oliver and Stringer (1984) observed a decrease of 59% in cellular ATP content in the same marine *Vibrio* strain as Amy *et al.* (1983) did.

### 3.1.3 Fate of intracellular nucleic acids

DNA and RNA are macromolecules rich in energy. Hood *et al.* (1986) were able to show that concentrations of intracellular DNA decreased with ongoing starvation in *Vibrio cholerae*. Similar effects were seen by Novitsky and Morita (1977) during starvation of *Vibrio* sp. Ant-300. Decreasing DNA concentrations can be explained by degradation of not fully replicated genomes. However, without the entire genome or even parts of it cells would not be able to recover from starvation. Lebaron and Joux (1994) investigated *Salmonella typhimurium* and its ability to change the topological state of DNA thereby preserving it, in order to enable regrowth after starvation. Despite these observations it does not seem the "normal" case that DNA is degraded during starvation. Boylen and Ensign (1970) observed increases in DNA concentrations in *Arthrobacter crystallopoietes*, which then remained constant over a 30 day period. They explained this occurrence with the finishing of replication rounds. Replication was not reinitiated and neither division occurred due to limitation of amino acids, which are necessary for protein synthesis.

Boylen and Ensign (1970) also observed degradation of RNA in *Arthrobacter crystallopoietes*. After 30 days rods had lost 85% of their initial RNA contents. RNA is mainly present in form of mRNA and rRNA. Both of which are used readily in cells and seem to be the preferred macromolecules to transform usable energy (Morita, 1997).

#### 3.1.4 Protein degradation during starvation

During starvation the *de novo* production of proteins decreases rapidly at near-logarithmic rate compared to starvation time (Rockabrand *et al.*, 1995). Throughout the time course of this experiment, however, this *de novo* synthesis in starving cells amounted to twice their mass, which highlights the “tremendous degree of protein processing or transformation...during the starvation period” (Blum, 1997). However, already synthesised cellular proteins that are not essential for cell maintenance represent a pool of possible energy and of amino acids for *de novo* protein synthesis. Investigations have shown that not only the *de novo* synthesis but also the intracellular concentrations of proteins decrease during nutrient limitation (Boylen and Ensign, 1970).

Morita (1997) summarised that cells under nutrient deprivation use their own intracellular components for maintenance. Nonessential carbohydrates are used first followed by RNA and DNA. Decreases in the amino acid pool, adenine nucleotides, and mRNA were observed at the beginning of starvation stabilised later on. Morita (1997) also claimed that when half the RNA is degraded, cells begin to die.

#### 3.1.5 Molecular basis of starvation-survival

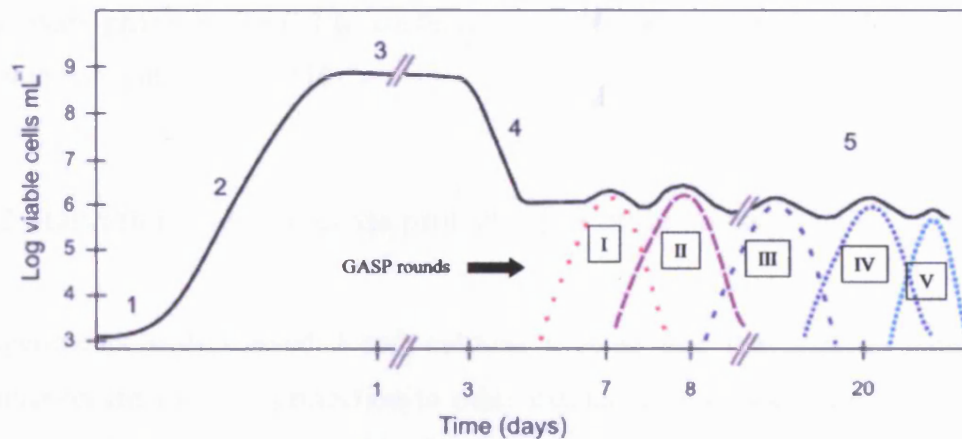
So far mainly starvation-induced phenotypic changes in environmental isolates were presented. The following section comprises a brief introduction into the underlying molecular and genetic changes that happen during starvation, mainly investigated in cultures of *E. coli*.

The transcription factor *RpoS* (also known as sigma factor,  $\sigma^S$ , *KatF*) has been linked to play a critical role in the expression of a variety of stationary phase proteins such as *BolA* and *DnaK* (Blum, 1997). *BolA* is a so-called morphogene (Aldea *et al.*,

1989) and influences the rate of peptidoglycan synthesis and degradation. It is thus likely involved in the morphological changes observed during starvation by modifying the structure of the bacterial cell wall (Blum, 1997). *DnaK* (also known as HSP70) is a chaperone and belongs to the heat-shock protein family. It plays an important role during reductive cell division (Rockabrand *et al.*, 1995) and its expression is also regulated by the sigma factor *RpoS*.

Zambrano *et al.* (1993) discovered that mutations in the gene of sigma factor *RpoS* lead to a competitive growth advantage of stationary phase cells (GASP phenotype) when “older” *E. coli* cells (10 days) were mixed with a “younger” culture (1 day). A mutation that caused a frame shift during translation resulted in a protein with a longer amino acid sequence (39 amino acids) than the wildtype protein. These additional amino acids probably influence the promoter recognition of the RNA polymerase of starvation-induced proteins. How the “older” cells outcompete the “younger” is unclear but it does not seem to be linked to production of toxins or antibacterial agents (Zambrano *et al.*, 1993). The authors discovered a second, independent mutation also conferring the GASP phenotype and even a mutation that gave resistance to “young” cells from being killed by the “older” culture. Zambrano and Kolter (1996) proposed that several, successive round of GASP mutations could arise during continued incubation (Fig. 1.4, dotted lines) and thus one population is replaced by strains that more effectively utilise nutrients released by dying cells.

A similar phenomenon to GASP was observed by Lemonnier *et al.* (2008). They found that *E. coli* cells with a mutation in the *glgC* gene (coding for ADP-glucose pyrophosphorylase, a regulatory enzyme that catalyses the first reaction of bacterial glycogen synthesis, Ballicora *et al.*, 2003) inhibited growth of cells they derived from by physical contact between the mutated cells and their ancestors. Ancestral cells were not killed but concerted to the VBNC state during stationary phase, thus the name stationary phase contact-dependent inhibition (SCDI). The inhibition is most likely caused by the overproduction of glycogen but underlying mechanisms have not yet been fully understood especially as glycogen is not known for any bactericidal activity. SCDI differs from GASP by a variety of characteristics (for full list refer to Lemonnier *et al.*, 2008) but the most important one is that the *rpoS* mutation was absent in cells exhibiting SCDI.



**Fig. 1.4: Stages of bacterial growth.** Adapted from Llorens *et al.* (2010) who adapted it from Finkel (2006); *E. coli* cells growing in optimal laboratory conditions, rich media, 37°C with aeration. 1, lag phase; 2, exponential phase; 3, stationary phase; 4, death phase; 5, long-term stationary phase. Dotted lines represent the continuous growth and taking over of the different mutants that appear within a deep stationary phase population (GASP cycles I-V; Zambrano and Kolter, 1996; Finkel, 2006).

The ecological significance of the GASP and SCDI phenotype, if they occur in the environment, is the apparent killing or inhibition of competitors for substrates. The prokaryotic community composition could be highly influenced by these phenotypes that occur once (logarithmic) growth has ceased.

Signal molecules play an important role during starvation response. Many of them have an impact on the gene expression of sigma factors (e.g. *RpoS*). High levels of guanosine tetraphosphate (ppGpp), for example, cause a global change of gene expression (Blum, 1997) as part of the stringent response (Cashel and Rudd, 1987) restricting the production of parts of the protein synthesis apparatus. It also facilitates the utilisation of alternative sigma factors (i.e. *RpoS*, Shiba *et al.*, 1997; Srivatsan and Wang, 2008), controls the growth rate in *E. coli* (Potrykus *et al.*, 2011), and redirects transcription of genes for growth and proliferation to genes important for starvation survival (Magnusson *et al.*, 2005). Intracellular cyclic AMP and homoserine lactone but also extracellular weak acids such as acetate influence the gene expression of *RpoS* (Zambrano and Kolter, 1996). Interesting here is that homoserine lactone is known to be a signal molecule during quorum sensing in high density populations (Meighen, 1991; Choi and Greenberg, 1992) and is involved in sensing the stationary phase (Huisman and Kolter, 1994).

The response to (impending) starvation on a molecular level is manifold and influenced by many parameters, genes, and molecules. One might be forgiven for assuming that no changes occur after growth has ceased, however, “if incubation in



stationary phase is allowed to continue ... what ensues is anything but stationary” (Zambrano and Kolter, 1996).

### 3.2 Starvation-induced cross protection to other stresses

Experiments with starved *E.coli* cultures showed that the proteins induced by starvation led to cross protection to other extracellular stresses like heat or H<sub>2</sub>O<sub>2</sub>-treatment (Jenkins *et al.*, 1988). Similar phenomena were observed for bacteria isolated from oligotrophic environments. Givskov *et al.* (1994b) revealed that starvation-induced cross protection to H<sub>2</sub>O<sub>2</sub> and ethanol treatment showed a higher level of protection than other pre-treatments (i.e. growth at lower temperatures) of *Pseudomonas putida* cells. A starvation period of only one day already led to almost complete survival of the cells to the chosen treatments. 2D-PAGE-analysis revealed that several proteins induced by other stresses were already identified as starvation-induced proteins (Givskov *et al.*, 1994a).

Nyström *et al.* (1990) reported the discovery of some starvation-induced proteins to be similar to proteins induced by other stresses. However, they emphasized that proteins induced by one treatment are not necessarily the same induced by the response to other stresses. Although there seems to be an interconnecting stress regulation for a few specific proteins (regulated by the same sigma-factor) the majority of stress-induced proteins differ from treatment to treatment.

### 3.3 Longevity of prokaryotes

Examples of microorganisms surviving extremely unfavourable conditions over long periods of time can also be found in the literature. Vreeland *et al.* (2000) reported of the recovery of a halotolerant bacterium from a 250 million-year-old salt crystal. The isolated bacterium was affiliated to the genus *Bacillus* and able to form spores. Although the authors applied various procedures to avoid contamination during sampling the results remain doubtful (Hazen and Roedder, 2001). Upon their observations, Parkes (2000) raised the question if spores could effectively be

immortal and which biochemistry allows them to survive for such a long time. Grant *et al.* (1998) reviewed the occurrence of halobacteria in many brine incursions as evidence for their longevity. They suggested halobacteria to be the best candidates for long-term survival because of their internal ion concentration that almost avoids the degradation of life-based polymers such as DNA.

Other scientists were also able to isolate bacteria from ancient samples. Cano and Borucki (1995) obtained a spore-forming bacterium from a 25 to 40 million-year-old Dominican amber (also affiliated to the genus *Bacillus*). Also from amber of that age, Lambert *et al.* (1998) isolated a new species belonging to the genus *Staphylococcus*. *Micrococcus* related strains were revived by Greenblatt *et al.* (1999) from 120 million year old amber.

### 3.4 The viable but nonculturable state and resuscitation of dormant prokaryotes

A major problem for microbiologists is the phenomenon of the viable but nonculturable (VBNC) state in bacteria. Roszak and Colwell (1987) have described this as a physiological state in which the cells show detectable metabolic function but cannot be grown in commonly used cultivation media (Rahman *et al.*, 1994) but resuscitation might be possible (Oliver, 2005). Many investigations concerning clinical aspects of this phenomenon have shown that bacteria when introduced into a new environment often enter this state, for example when human pathogens are released from the body (Colwell, 2000). Although apparently no growth occurs viable but nonculturable cells were shown to retain their membrane integrity (Lloyd and Hayes, 1995), to respire (Rahman *et al.*, 1994) and to produce mRNA (Ileo *et al.*, 2000, Ileo *et al.*, 2001, Heim *et al.*, 2002). Boaretti *et al.* (2003) investigated the effects of the *rpoS* gene, on the VBNC state. They showed that RpoS<sup>-</sup> mutants of *E. coli* reached the nonculturable state approximately 12 days earlier and showed restricted resuscitation capability. But still little is known about the genetic mechanisms that lead to the VBNC state (Boaretti *et al.*, 2003).

It was mentioned before that stimulation of growth in environmental microorganisms can be quite difficult. Due to environmental conditions

microorganisms occur in different physiological states within the environment (starving, dormant etc.) and thus might have to be resuscitated. For spores it is known that so called germinants cause the germination after times of inactivity (Gould, 1969), maybe a similar system exists for non-spore forming microorganisms.

One factor helping to resuscitate bacteria is temperature. Studies of *Vibrio vulnificus*, a human pathogen occurring in seawater revealed that culturing success depends on the season. While during warmer months the cells easily grew on solid media they failed to grow in times of cooler weather (Rice *et al.*, 2000).

Another possible reason for unsuccessful cultivation is the lack of nutrients in oligotrophic systems. The organisms suffer from starvation and enter a state of unculturability. Mukamolova *et al.* (1998) starved cultures of *Micrococcus luteus* in a prolonged stationary phase and stored them at room temperature for different periods of time. They were able to resuscitate cells by adding sterilized supernatant of the same batch culture sampled previously during the logarithmic growth phase. Apparently the supernatant contained a "pheromone-like" substance synthesized by the active cells of *M. luteus* (Kaprelyants *et al.*, 1996), which was needed to regain the ability of growth. They also showed that with prolonged time of starvation the success of resuscitation decreased. This phenomenon was also observed by Tappe *et al.* (1999) who starved cultures of *Nitrosomonas europaea* and *Nitrobacter winogradskyi*.

An example for the application of resuscitated microbes is a study by Lappin-Scott *et al.* (1988). To boost oil recovery they injected carbon-deprived *Klebsiella pneumoniae*, which showed reduced cell size, into sandstone cores. Their aim was to increase the depth of bacterial penetration into the sandstone to plug high-permeability zones already drained of oil. Subsequently this should increase oil recovery from low-permeability-zones. The cells regained their original size and colonised the whole column after nutrients were added and thus successfully plugged high-permeability rock strata to potentially improve oil recovery.

#### 4. Summary

The deep biosphere is an extremely oligotrophic environment with overall extremely low substrate availability and low energy fluxes. However, microorganisms are able

to cope with these conditions and are growing and even multiplying remote from photosynthetically derived organic material. Pure cultures obtained from the deep biosphere are essential for our understanding of the processes occurring within the subsurface. But the way they are cultured does not reflect the situation *in situ*. They are adapted to nutrient limitation and thus starvation.

Recently developed methods, such as FISH, are frequently applied to mixed microbial communities in deep subsurface sediments. However, results are often contradictory. So far no study has conducted starvation survival experiments with representative deep biosphere isolates and tested these commonly applied methods on pure cultures to investigate the variety of responses that might be exhibited. This would help to understand data gathered from the deep biosphere.

## **5. Aims and objectives of this project**

From the introduction given above the lack of knowledge in regard to starvation survival of marine sedimentary microbes should be apparent, yet mixed microbial communities in the deep biosphere are studied with an array of techniques that have not been tested on pure starving cultures from this environment. Hence, the main objectives of this project were:

- To isolate novel deep biosphere prokaryotes from one of the most organic matter-poor, marine habitat (Challenger Mound, IODP Leg 307).
- To then investigate the survivability of the obtained isolates alongside other representative deep biosphere strains as well as selected near-surface relatives in long-term, anaerobic starvation survival experiments.
- To assess any potential differences in the starvation responses of the pure cultures applying commonly used methods of cell detection, viability, and activity used in deep biosphere research and to test their effectiveness.
- To furthermore investigate the changes in viability and culturability of a near-surface microbial community subjected to increasing temperature stress as it occurs during burial in deep marine sediments with the same methods applied to pure cultures.

## **Chapter II:**

### **Material and Methods**

This chapter contains all the material and methods applied during the course of this project. It also comprises site descriptions of sampling campaigns along with all prokaryotic species and strains investigated. All methods described were conducted by the author unless stated otherwise.

#### **1. Sampling Sites**

Sediments from two different habitats were used during this project. Samples from the Porcupine Seabight Challenger Mound were collected during IODP Leg 307 and were used for cultivation and isolation of deep biosphere prokaryotes. Sediment from the Tamar Estuary was collected in June 2009, analysed biogeochemically and with molecular biological techniques before using it in heating experiments.

##### **1.1 Porcupine Seabight Challenger Mound (IODP Leg 307) site description**

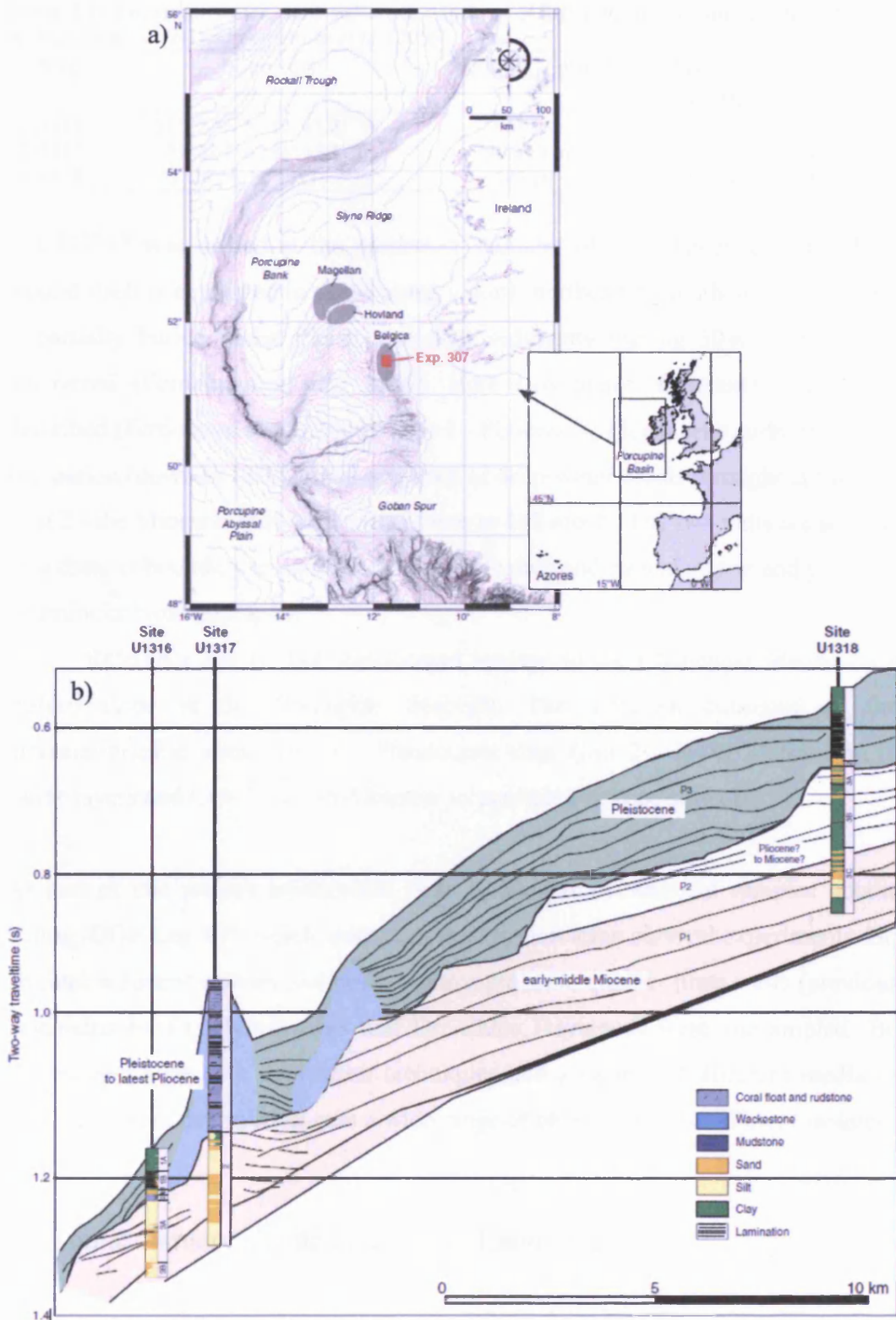
The first carbonate mound to be investigated in great detail microbiologically and biogeochemically was drilled during IODP Leg 307 (Webster *et al.*, 2009). The Challenger Mound is part of the Belgica Mound Province in the Porcupine Basin southwest of the coast of Ireland just off the continental margins of Europe (Wheeler *et al.*, 2007). The Challenger mound is just one of 1600 carbonate mounds in the Porcupine basin alone and is classified as a modern cold-water coral mound. It rises approximately 155 m high and is located 600-900 m below the sea surface (Table 2.1), partially buried with sediment and dead cold-water coral (*Lophelia pertusa*) (Ferdelman *et al.*, 2006). Webster *et al.* (2009) reported a significant and active prokaryotic community within and beneath the mound and concluded that carbonate mounds may represent a significant prokaryotic habitat within the deep biosphere.

Sediment cores were taken from two mound sites (Flank, site U1316 and Mound centre, site U1317) and a reference site (site U1318; Fig. 2.1).

During Leg 307 different coring systems were used to reach the target depth (Table 2.1): advanced piston corer (APC), extended core barrel (XCB), and rotary core barrel (RCB; Ferdelman *et al.*, 2006). These different techniques are often combined to recover cores from the same hole but regularly result in core-recovery gaps between successive cores (Ferdelman *et al.*, 2006). Hence, for each site a number of holes were drilled to ensure complete recovery of the sediment column down to target depth. To achieve this, cores from different holes were stratigraphically correlated and subsequently depth-shifted relative to one another (Ferdelman *et al.*, 2006). For sites U1316 and U1317, holes from the same drilling site resulted in different depths compared with the mound base. Therefore, Webster *et al.* (2009) standardised the sediment depth to “metres above mound base” (mamb) rather than the commonly used “metres below seafloor” (mbsf), thus enabling recovered cores from different holes of the same site to be compared with each other.

The Challenger Mound is located on a sharp erosional boundary, which can be found at all three drilling sites. At mound sites U1316 and U1317 it cuts into the middle Miocene strata (Fig. 2.1). At the reference site 1318 it separates Miocene sediments (10 Ma) from Middle Pleistocene or younger sediments (Kano *et al.*, 2007; Loowye *et al.*, 2008). The sediments beneath the mound are made up of glauconitic and silty-sandstone originating from the middle Miocene (Van Rooij *et al.*, 2003; Loowye *et al.*, 2008). Kano *et al.* (2007) reported that the growth of Challenger Mound began at 2.6 Ma and continued rapidly at a rate of 24 cm k.y.<sup>-1</sup> until it stopped at 1.7 Ma. After 0.7 Ma growth continued for 0.2 Ma.

Site U1316 is located in the downslope sediment deposits ~700 m southwest of Challenger Mound. The surface sediments are mainly made of rippled sands littered with dropstones (Foubert *et al.*, 2007). Ferdelman *et al.* (2006) divided this site into three distinct lithostratigraphic units: Unit 1: Pleistocene, Unit 2: late Pliocene-Pleistocene (present at Carbonate Mound), and Unit 3: early to middle Miocene origin (present at Reference site). These units are based on sediment composition and corresponding age in addition to colour reflectance and natural gamma radiation (Ferdelman *et al.*, 2006).



**Fig. 2.1: Drilling location of the Challenger Mound, IODP Leg 307.** a) Location map. b) Lithostratigraphy of the three sites projected on the seismic profile of Challenger Mound along a north-northwest to south-southeast transect. P1–P3 refers to seismic units as defined by Van Rooij *et al.* (2003). Lithostratigraphic units are numbered next to the lithologic column. Based on the lithostratigraphic data, seismic Unit P3 is younger (<0.26 Ma) than the upper mound succession. Source: Ferdelman *et al.* (2006) and [http://publications.iodp.org/preliminary\\_report/307/](http://publications.iodp.org/preliminary_report/307/)

**Table 2.1: Location, water, and sediment depth of IODP Leg 307 sampling sites.** Data from Webster *et al.* (2009) and Ferdelman *et al.* (2006).

Site	Location	Water Depth	Maximum Sediment Depth recovered
U1316	51°22.56'N, 11°43.81'W	965 m	143.1 mbsf
U1317	51°22.8'N, 11°43.1'W	790-840 m	270.0 mbsf
U1318	51°26.16'N, 11°33.0'W	409 m	244.6 mbsf

Site U1317 was drilled at the northwest shoulder of the Challenger Mound. The mound itself is elongated in shape along a north-northeast to south-southwest axis. It is partially buried under Pleistocene drift sediments leaving 30 m of the mound uncovered (Ferdelman *et al.*, 2006). Here two major sedimentary units were described (Ferdelman *et al.*, 2006): Unit 1 - Pliocene to Pleistocene carbonate mound succession (down to 130.1 mbsf; presence of deep-water corals throughout this unit); Unit 2 - the Miocene drift sediments (down to 268 mbsf). The two units are separated by a distinct boundary as indicated by seismic data and nanoplankton and planktonic foraminifer biostratigraphy.

Reference site U1318 was located upslope of the Challenger Mound on the eastern slope of the Porcupine Seabight. The sediment consisted of three lithostratigraphic units. Unit 1 - Pleistocene clay, Unit 2 - lower Pleistocene thin sandy layers and Unit 3 - lower Miocene to possible Pliocene silty clay.

As part of this project prokaryotes were isolated from sediment samples obtained during IODP Leg 307, which were then used in starvation survival experiments. Both original sediment slurries and positive most probable number plate wells (previously inoculated by Dr. Henrik Sass and Dr. Chloe Heywood) were subsampled. Both aerobic and anaerobic cultivation techniques and a variety of different media (see Section 3) were deployed to gain a wide range of physiologically different isolates.

## 1.2 Tamar Estuary site description and sampling

### 1.2.1 Site description

The Tamar Estuary is situated west of Plymouth, Cornwall, UK (Fig. 2.2) and has been subject to a variety of biogeochemical (Wellsbury and Parkes, 1995; Wellsbury



*et al.*, 1997; Parkes *et al.*, 2007) studies in the past. It represents a typical medium sized coastal estuary, which comprises extended tidal flats and mud banks (silt and clay) and opens into the western end of Plymouth Sound (Watson *et al.*, 1985). The three main tributary rivers, which form the ria system are the Tamar, the Tavy, and the Lynher. The entire catchment area is around 1500 km<sup>2</sup> to which the river Tamar contributes about 924 km<sup>2</sup> (Monbet *et al.*, 2009). Ria systems are funnel shaped estuaries at a river mouth, formed by submergence of the lower portion of the river valley. These are commonly very irregular and may possess several branching tributaries. A typical characteristic is the widening funnel shape and steadily increasing depth seaward resulting in increasing tidal ranges within the estuary. This tidal effect can be seen along the 31 km length of the estuary resulting in varying water depths in the main channel ranging between two and eight metres below mean spring high-water in the inner estuary (Monbet *et al.*, 2009). Stephens *et al.* (1992) reported less than 10% organic content in the sediment throughout the estuary. During summer months large populations of freshwater algae, mainly diatoms, have been observed (Jackson *et al.*, 1987)

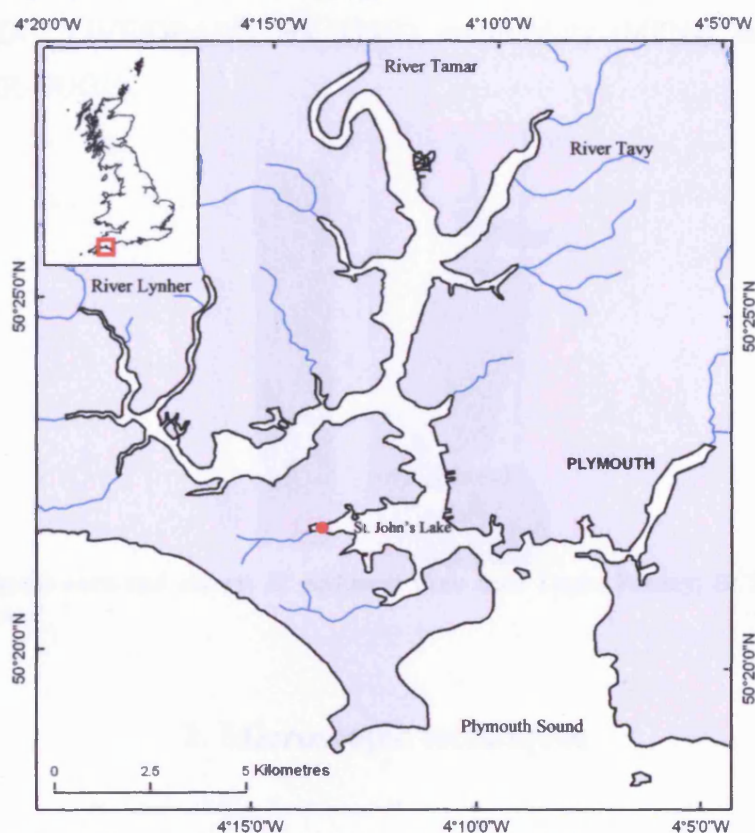
### 1.2.2 Sampling of Tamar Estuary sediment

Samples were collected from St. John's Lake of the estuary approximately three kilometres south-west of Plymouth (50°21.823'N, 4°13.847'W) in June 2009 (Fig 2.2). This site is relatively free of anthropogenic influences and hence representative for other estuarine sediments. Sampling was undertaken using round, plastic hand corers (approximate length of corer 80 cm, maximum core length ca. 30 cm; Fig 2.3). The cores were subsequently transported to the laboratory in Cardiff within four hours after sampling in an upright position using frozen ice packs for cooling. In Cardiff cores were stored at 4°C in an upright position until processing.

### 1.2.3 Subsampling of the sediment cores

Subsampling of the cores was undertaken as aseptically as possible. One core was used for biogeochemical, molecular, and microbiological analysis. The sediment was

pushed from the bottom towards and above the top of the corer. Then slices (~2 cm thick) were cut using sterile aluminium slides (furnaced at 450°C for 2 h). Syringes were used for sampling, which had been modified by removing their ends prior to sterilisation. Samples (~1 cm<sup>3</sup>) were either transferred into vials for cell counts containing a fixative, for storage (-20°C for later molecular biological analysis), or for immediate use for cultivation of anaerobic prokaryotes (MPN dilution series [this project] and further enrichment cultures targeting methanogenic *Archaea* [Andrew J. Watkins, PhD project, Cardiff University]).



**Fig. 2.2: Map of the Tamar Estuary.** The map was produced by converting the GPS data into National Grid coordinates and plotting them onto a map from the Ordnance Survey using the Geographical Information Systems (GIS) software (map courtesy of Dr. Jennifer L. Moss)

#### 1.2.4 Setup of sediment slurry experiments

The top 12 cm from two sediment cores were used to produce two 25% slurries for heating experiments (75% anoxic artificial seawater [ASW], for composition see Section 3.3.1). The anoxic ASW was prepared without addition of vitamins or substrates. Sediment and ASW were mixed and poured into gas-tight conical incubation vessels (Fig 2.3) and subsequently incubated under a N<sub>2</sub>/CO<sub>2</sub> (80/20 v/v)

atmosphere. The vessels had two sampling accesses, one at the bottom for liquid (slurry) sampling and one at the top for gas phase sampling (Wellsbury and Parkes, 1995; Parkes *et al.*, 2007). Sediment slurries were initially incubated at 15°C (close to *in situ* temperature of sediment at day of sampling) for the first eight weeks. Subsequently the incubation temperature was increased at a rate of 3°C per two weeks until 90°C were reached. Every two weeks prior to increasing the temperature sediment slurries were sampled for gas analysis and pore water geochemistry. At selected temperatures additional sampling was conducted including samples for cell counts (AODC, LIVE/DEAD<sup>®</sup>, and FISH), culturability (MPNs), and molecular analysis (PCR-DGGE).



**Fig. 2.3: Sediment core and slurry.** A: Sediment Core from Tamar Estuary; B: Sediment Slurry Experiment Vessel

## 2. Microscopic techniques

### 2.1 Acridine Orange Direct Counts (AODC)

To determine total cell counts of pure cultures and environmental samples the acridine orange direct count-method was used as described by Fry (1988) with minor modifications. Samples were fixed with 0.2  $\mu\text{m}$  filtered (Vacuap 0.2  $\mu\text{m}$  bottle-top vacuum filter unit, Pall Corporation, New York, USA) formaldehyde solution (2% end concentration; filtered through a 0.2  $\mu\text{m}$  filter; 3.5% NaCl was added for marine samples and strains, 0.8% NaCl for freshwater strains) in particle free, glass vials and stored in the dark at room temperature until further processing. For counting, a subsample of fixed cell suspension was taken and diluted in 10 ml formaldehyde

solution (2%, filtered through 0.1 µm filter [inorganic membrane filters, Anotop 25, 0.1 µm, Whatman, Kent, UK]) and mixed with 50 µl acridine orange (1 g×l<sup>-1</sup>, membrane filtered [0.2 µm]). After staining for three minutes samples were filtered onto black polycarbonate filters (GE Water and Process Technology, Pennsylvania, USA) using a glass funnel. Subsequently filters were washed with 10 ml formaldehyde solution (2%) to reduce background fluorescence. Filters were mounted onto glass slides using paraffin oil and inspected under the microscope (Axioskop I, Zeiss, Oberkochen, Germany) with the appropriate filterset (Blue 450-490; Set number 487909; Excitation Filter BP 450-490; Chromatic Beam Splitter FT 510; Barrier Filter LP 520). Counts were done in duplicates (or triplicates when necessary) and at least 200 cells or 20 fields of view per filter were counted. Sample concentrations were adjusted so that approximately 70% of the field of view area was covered with sediment particles or 20 cells per field of view for pure cultures when counting. When sediment samples were inspected cells located on and off particle were accounted for separately as described by Goulder (1977). Furthermore dividing and divided cells were counted. The total cell number of a sample was calculated (Equation 1) by using an Excel spreadsheet designed by Dr. Barry Cragg.

Total bacterial numbers are calculated from: Numbers of dividing and divided cells are calculated from the same equation with the omission of the terms “2C<sub>ON</sub> + C<sub>OFF</sub>” at the start of the equation, and the substitution of B<sub>D</sub> for B<sub>T</sub>. The percentage of dividing and divided cells is calculated from the numbers of dividing and divided cells expressed as a percentage of the total bacterial numbers. Where total bacterial numbers approach the detection limit (10<sup>5</sup> cells g<sup>-1</sup> sediment), or numbers of cells counted approach the number of cells observed in the blanks, then this calculation becomes unreliable.

$$\text{Equ. 1: } C_{\text{count}} = \frac{2C_{\text{ON}} + C_{\text{OFF}} + C_{\text{DG}} + 2C_{\text{DD}} + \frac{C_{\text{ON}}}{2C_{\text{ON}} + C_{\text{OFF}}} \cdot (C_{\text{DG}} + 2C_{\text{DD}})}{\text{VIEW}} \cdot A \cdot B_T \cdot \frac{1000}{V_{\text{CF}}} \cdot D$$

Key:

C<sub>ON</sub> and C<sub>OFF</sub>: Number of cells counted ON and OFF particles. For the purposes of this calculation transparent particles e.g., diatom frustules, are not particles.

$C_{DG}$  and  $C_{DD}$ : Numbers of cells observed DIVIDING (a cell with an invagination) and DIVIDED (two adjacent cells of identical morphology with a distinct space between them). Cells counted in these two categories are not also tallied under ON and OFF particle categories but are corrected for the relationship of ON and OFF cells in the respective sample.

VIEW: The total number of fields of view observed during a cell count on a filter.

A: Filter area ratio. Total countable area of the filter divided by the area of filter observed for one field of view.

$B_T$  and  $B_D$ : Blank correction terms for the total cell number ( $B_T$ ) and the dividing and divided cell numbers ( $B_D$ ). Calculated from counts of blank membranes and using the same equation with the omission of the correction term. In this instance  $V_{CT}$  will equal 10050 (10 ml of formaldehyde +50  $\mu$ l of acridine orange) and D will equal 1.

$V_{CT}$ : Volume of formaldehyde-preserved sample that is used ( $\mu$ l)

D: Dilution factor of original sample expressed as a proportion, e.g., 1  $cm^3$  of sediment in 9 ml of formaldehyde will give  $D = 0.1$

## 2.2 LIVE/DEAD<sup>®</sup> staining of cells

The LIVE/DEAD<sup>®</sup> *BacLight*<sup>™</sup> *Bacterial Viability Kit* 1.7007 was purchased from Molecular Probes (Invitrogen Corporation, Carlsbad, USA). It contains the components A (Syto<sup>®</sup>9 dye, 1.67 mM and propidium iodide, 1.67 mM, 300  $\mu$ l solution in DMSO), B (Syto<sup>®</sup>9 dye, 1.67 mM and propidium iodide, 18.3 mM, 300  $\mu$ l solution in DMSO), and C (mounting oil). Syto<sup>®</sup>9 is able to penetrate living bacterial cells and emits a green signal under UV light (Excitation 480 nm/ Emission 500 nm) whereas propidium iodide (PI) only enters cells with a compromised cell membrane and appears as a red signal (Exc. 490 nm/ Em. 635 nm). Red fluorescing cells are considered dead because a damaged cell membrane would lead to the breakdown of trans-membrane gradients and subsequently cell energetics and therefore, the cell loses its viability. Upon receipt components A and B were mixed (1:1) and diluted in 0.85% NaCl solution, aliquoted, and stored at -20°C (working

solution). Staining of cells was conducted following the manual provided by the manufacturer.

Samples for LIVE/DEAD<sup>®</sup> count were taken and stained immediately without fixation. A subsample of cell culture or sediment (2-200 µl) was added to 800-998 µl TRIS-HCl (10 mM, pH 7.4, 0.85% NaCl for freshwater bacteria, 3.5% NaCl for marine strains). To each millilitre of sample at least 3 µl of diluted working solution were added and incubated for at least 20 minutes in the dark at room temperature. Samples were filtered onto black polycarbonate filters (Millipore, Billerica, USA) and washed with 5 ml of TRIS-HCl buffer (of appropriate salinity) to remove excess dye and reduce background fluorescence. Cells were counted under the microscope (Axioskop I, Zeiss, Oberkochen, Germany) using the appropriate filterset (Blue 450-490; Set number 487909; Excitation Filter BP 450-490; Chromatic Beam Splitter FT 510; Barrier Filter LP 520). Counts were done in duplicates (or triplicates when necessary) and at least 200 cells or 20 fields of view per filter were counted. Calculation of cell numbers was done following Equation 2.

Equ. 2:

$$Count = \frac{2C_{L\_ON} + C_{L\_OFF} + C_{L\_DG} + 2C_{L\_DD} + C_{DEAD} + C_{D\_DG} + 2C_{D\_DD} + \left( \frac{C_{L\_ON}}{2C_{L\_ON} + C_{L\_OFF}} \right) \cdot (C_{L\_DG} + 2C_{L\_DD} + C_{DEAD} + C_{D\_DG} + C_{D\_DD})}{VIEW} \cdot A \cdot B_f \cdot \frac{1000}{V_{L\_L}}$$

Key:

$C_{L\_ON}$  and  $C_{L\_OFF}$ :

Number of “live” cells counted ON and OFF particles. For the purposes of this calculation transparent particles e.g., diatom frustules, are not particles.

$C_{L\_DG}$  and  $C_{L\_DD}$  /  $C_{D\_DG}$  and  $C_{D\_DD}$ :

Numbers of “live” or “dead” cells, respectively, observed DIVIDING (a cell with an invagination) and DIVIDED (two adjacent cells of identical morphology with a distinct space between them). Cells counted in these two categories are not also tallied under ON and OFF particle categories.

$C_{DEAD}$  Number of “dead” cells on and off particles.

VIEW: The total number of fields of view observed during a cell count on a filter.

- A: Filter area ratio. Total countable area of the filter divided by the area of filter observed for one field of view.
- $B_T$  and  $B_D$ : Blank correction terms for the total cell number ( $B_T$ ) and the dividing and divided cell numbers ( $B_D$ ). Calculated from counts of blank membranes and using the same equation with the omission of the correction term. In this instance  $V_{CT}$  will equal 10050 (10 ml of formaldehyde +50  $\mu$ l of acridine orange) and  $D$  will equal 1.
- $V_{CT}$ : Volume of formaldehyde-preserved sample that is used ( $\mu$ l)
- $D$ : Dilution factor of original sample expressed as a proportion, e.g., 1  $\text{cm}^3$  of sediment in 9 ml of formaldehyde will give  $D = 0.1$

### 2.3 Fluorescence *in situ* hybridization (FISH)

Samples were fixed in phosphate buffered saline (PBS) solution (1xPBS: 145 mM NaCl, 1.4 mM  $\text{NaH}_2\text{PO}_4$ , 8.0 mM  $\text{Na}_2\text{HPO}_4$ , pH 7.4) containing 4% formaldehyde and 3.5% NaCl as described by Glöckner *et al.* (1996 and 1999), Perntahler *et al.* (2002) and Ishii *et al.* (2004). Fixed samples were washed three times with 1xPBS by centrifugation at  $15.800\times g$  for seven minutes after each wash (Centrifuge 5415, Eppendorf AG, Hamburg, Germany). Samples were resuspended in PBS/Ethanol (1:1) and stored at  $-20^\circ\text{C}$  until further processing.

For hybridisation samples were transferred onto white polycarbonate filters (Isopore Membrane filters type 0.2  $\mu\text{m}$  GTTP, Millipore, Billerica, MA, USA). Filters were cut into sections to allow hybridisation with different probes (EUB338I and NON-EUB338, Table 2.2.) purchased from Biomers (Ulm, Germany). For dehydration filter sections were put into Ethanol (50% for 5 min, 80% for 1 min, and 90% for 1 min) and dried at room temperature. 1  $\mu\text{l}$  of Probe (50  $\text{ng ml}^{-1}$ ) was mixed with 8  $\mu\text{l}$  of hybridisation buffer (0.9 M NaCl, 20 mM Tris/HCl [pH 7.4], 35% formamide, and 0.01% SDS). Sections were placed onto glass slides and incubated in an equilibrated chamber (Falcon tubes containing a small piece of tissue and 1.5 ml hybridisation buffer) at  $46^\circ\text{C}$  for two hours without shaking (Hybridisation Oven S101HS, Stuart Scientific, Staffordshire, UK). Afterwards filter sections were transferred into preheated washing buffer (70 mM NaCl, 20 mM Tris/HCl, pH 7.4, 5 mM EDTA, and 0.01% SDS) and incubated for 20 minutes at

48°C. Subsequently filter sections were covered with 50 µl DAPI solution (1 µg ml<sup>-1</sup>) and stained for 10 minutes in the dark at room temperature. Sections were gently washed in ddH<sub>2</sub>O and dried. To mount sections on a glass slide Citifluor AF1 (Citifluor Ltd., London, UK) and at a later stage *SlowFade*<sup>®</sup> Gold (Molecular Probes, Invitrogen Corporation, Carlsbad, USA) was used. Cell numbers were calculated as described for AODC (see 1.1).

**Table 2.2: Probe sequences**

Probe Name	Sequence	Melting Temperature	Reference
EUB 338	5'- GCT GCC TCC CGT AGG AGT -3'	55°C	Amann <i>et al.</i> , 1990
NON EUB338	5'- ACT CCT ACG GGA GGC AGC -3'	55°C	Wallner <i>et al.</i> , 1993

## 2.4 Gram staining

Gram staining was performed according to Gerhardt *et al.* (1994) and ready-made solutions were used (BDH Laboratories, Poole, Dorset, UK). Bacterial cells were heat-fixed on glass slides. The fixed cells were covered with crystal violet staining solution for one minute and washed with an indirect and gentle stream of ddH<sub>2</sub>O. Then Gram's iodine was applied for one minute and thoroughly washed with ethanol (indirect stream) before the counter-stain safranin was added (for 10 seconds). Before observing cells under the microscope (bright-field) cells were once more washed with ddH<sub>2</sub>O and dried.

## 2.5 Flagella staining

Flagella were stained using the method described by Ryu (1937). The working solution was prepared by mixing one volume of stock solution II (crystal violet [1.2% in methanol]) to 10 volumes of stock solution I (10 ml of a 5% phenol solution, 2 g tannic acid, and 10 ml of AlK(SO<sub>4</sub>)<sub>2</sub>×2H<sub>2</sub>O).

Cell suspensions were left to dry on a microscopic slide and covered with working solution for 5 to 15 minutes. Slides were rinsed with ddH<sub>2</sub>O and observed under the microscope (bright-field).



## 2.6 Microphotography and cell size measurements

Microphotographs of fluorescently stained sediment samples and stained or non-stained pure cultures were taken using a digital camera (Nikon). For cell size measurements glass slides were covered with hot agarose solution (1.2%) and left to solidify. Then a small volume of culture was transferred onto the solid agarose and a cover slip was placed on top. Cell size measurements were conducted using Scion Image (Scion Corporation, Frederick, Maryland, USA). For scale a microscopic slide with a stage micrometer (100×0.01 mm; Graticules Ltd., Tonbridge, Kent, UK) was used.

## 3. Cultivation of microorganisms and phenotypical testing

The cultivation and isolation of microorganisms is an important aspect in microbial ecology. It allows assessment of *in situ* roles and functions of the obtained strains or species. This chapter contains recipes for aerobic and anaerobic media used in the cultivation and isolation of deep biosphere prokaryotes from sediment samples of IODP Leg 307. These media are based on basal medium (buffered artificial seawater) (Oxic: HEPES-buffered, Coolen and Overmann, 2000; anoxic: carbonate-buffered, Süß *et al.*, 2004; see 2.8) with supplements depending on the physiological group of microorganisms targeted (see 2.4-2.14). The anoxic basal medium was also used for starvation survival experiments of marine strains. Freshwater strains were starved in their respective but nutrient-free media. For this purpose neither vitamins nor substrates were added.

In addition, media recommended by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) are listed, which were used for initial resuscitation of purchased strains. Later those strains were also grown on the above-mentioned media.

### 3.1 Microbial strains used for starvation-survival experiments

To cover a wide range of physiological diversity in starvation experiments a variety of deep-subsurface and near-surface prokaryotes was used (Table 2.3). Strains were

obtained from a range of sources. Some well described species were purchased from the DSMZ. Near-surface strains isolated from tidal flat sediment in the North Sea were provided by Dr. Henrik Sass. Dr. Gerard Sellek kindly provided a *Marinilactibacillus* sp. G8a3 which was also tested as well as selected isolates obtained from IODP Leg 307 sediments.

### 3.1.1 Setup of starvation-survival experiments

Microbial strains were grown in suitable media (see below) and incubated until fully grown as indicated by a high level of turbidity. Cells for starvation treatments "A" and "B" (Fig. 2.4) were harvested by centrifugation at  $4500\times g$  for 20 minutes (Rotanta 460 R, Hettich Zentrifugen, Tuttlingen, Germany), which in addition removed potential leftover substrate and toxic or growth-inhibiting endproducts. Cell pellets were resuspended in substrate- and vitamin-free medium (ca. 5 ml) using a syringe and needle (Sterican 0.8x120 mm, B. Braun Melsungen AG, Melsungen, Germany). Subsequently, cells were injected into gas-tight transfusion bottles (Ochs, Bovenden-Lenglern, Germany) containing ASW (see Chapter 2.8) supplemented with resazurine ( $0.5 \text{ mg ml}^{-1}$ ). The headspace gas was replaced by a  $\text{N}_2/\text{CO}_2$  atmosphere (80/20 v/v) and cultures were incubated at  $10^\circ\text{C}$  (*Photobacteria*) and at  $25^\circ\text{C}$  (all other strains.) In those cases where resazurine changed colour indicating presence of oxygen, a few crystals of sodium-dithionite were dissolved in ASW and one to two millilitre of the solution was added to the experiments until colourless.

Treatment "A" remained unamended, to treatment "B"  $15 \mu\text{M}$  substrate was added, whereas the "C" version represented the unchanged continued incubation of the culture, the only treatment being the transfer into blood infusion bottles but without harvesting/washing.

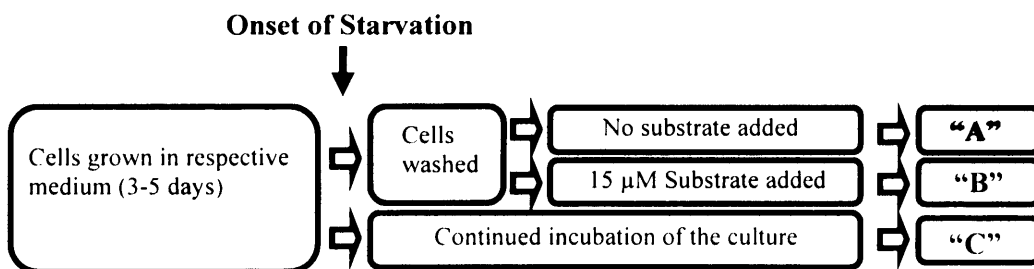


Fig. 2.4: Flow chart of starvation-survival experimental setup

**Table 2.3: Bacterial strains used during starvation survival experiments.** Sources: HS, Dr. Henrik Sass; GAS, Dr. Gerard Selleck. n.a. not applicable

Phylogenetic affiliation	Metabolic Property	Species name/ Strain	Source (No.)	Origin	Water depth [m]	Sediment depth [mbsf]
<i>Actinobacteria</i>	Aerobic	<i>Sermicoccus marinus</i>	DSMZ (15273)	East Sea, Korea	surface	n.a.
	Aerobic/fermenting	<i>Ornithinimicrobium</i> sp. F181V		IODP Leg 307	423	22.9
<i>Firmicutes</i>	Acetogenic	<i>Acetobacterium malicum</i> <sup>T</sup>	DSMZ (4132)	Freshwater ditch sediment	unknown	unknown
	Acetogenic	<i>Acetobacterium</i> sp. R002 (terrestrial subsurface)	HS	DEBITS project	n.a.	144
	Fermenting	<i>Marimolactibacillus</i> sp. G8a-3	GAS	Indian Continental Margin, Natural Gas Hydrate Project	1049	77
<i>Gammaproteobacteria</i>	Fermenting	<i>Photobacterium</i> sp. SAMA2	HS	North Sea Tidal Flat Sediment	intertidal	> 0.05
	Fermenting	<i>Photobacterium</i> sp. NA42	HS	North Sea Tidal Flat Sediment	intertidal	> 0.05
	Fermenting	<i>Photobacterium</i> sp. S10	HS	Mediterranean Sea Sediment	2154	3.75 - 4.6
	Fermenting	<i>Photobacterium</i> sp. S11	HS	Mediterranean Sea Sediment	2154	0.35–2.50
	Fermenting	<i>Photobacterium</i> sp. 67TD	HS	Mediterranean Sea Sediment	2154	-
	Fermenting	<i>Photobacterium</i> sp. F181		IODP Leg 307	423	4.25
	Fermenting/ Mn reducing	<i>Shewanella</i> sp. F18III		IODP Leg 307	423	4.25
	Fermenting/ Mn reducing	<i>Shewanella</i> sp. F17V		IODP Leg 307	781-815	96.25
	Fermenting	<i>Shewanella</i> sp. SCSA3	HS	North Sea Tidal Flat Sediment	intertidal	> 0.05
	Fermenting	<i>Vibrio</i> sp. F17IV		IODP Leg 307	781-815	96.25
	Fermenting	<i>Vibrio</i> sp. SCSA1	HS	North Sea Tidal Flat Sediment	intertidal	> 0.05
<i>Deltaproteobacteria</i>	Sulphate reducing	<i>Desulfovibrio acrylicus</i> <sup>T</sup>	DSMZ (10141)	North Sea Tidal Flat Sediment	intertidal	> 0.1
	Sulphate reducing	<i>Desulfovibrio desulfuricans</i> subsp. <i>desulfuricans</i> <sup>T</sup>	DSMZ (642)	Tar and sand mix around corroded gas man. UK	unknown	unknown
	Sulphate reducing	<i>Desulfovibrio profundus</i> <sup>T</sup>	DSMZ (11384)	Japan Sea Sediment	900	500
	Sulphate reducing	<i>Desulfovibrio</i> sp. F16I		IODP Leg 307	965	4.25
<i>Epsilonproteobacteria</i>	Fermenting	<i>Arcobacter</i> sp. F17IX		IODP Leg 307	781-815	254.17
	Fermenting/ DMSO reducing	<i>Arcobacter</i> sp. NA105	HS	North Sea Tidal Flat Sediment	intertidal	> 0.05

## 3.2 Aerobic media for the cultivation of microorganisms

### 3.2.1 Oxidic YPGL-medium

The oxidic YPGL medium is based on the basal medium published by Coolen and Overmann (2000). This medium was used for cultivation of aerobically growing isolates (Table 2.4). In addition to the basal medium it contains yeast extract, peptone, glucose and lactate.

The ingredients were added to ddH<sub>2</sub>O and stirred until fully dissolved. The pH was adjusted to 7.2-7.4 using NaOH. The medium was autoclaved for 60 minutes at 121°C. Vitamins and substrates were added from sterile stock solutions after the autoclaved medium was cooled to room temperature.

**Table 2.4: Composition of oxidic YPGL medium based on basal medium**

Added before autoclaving			
ddH <sub>2</sub> O	1000 ml	HEPES	2.38 g
NaCl	24.32 g	KBr (0.84 M)	1 ml
MgCl <sub>2</sub> · 6H <sub>2</sub> O	10.00 g	H <sub>3</sub> BO <sub>3</sub> (0.4 M)	1 ml
CaCl <sub>2</sub> · 2H <sub>2</sub> O	1.50 g	SrCl <sub>2</sub> (0.15 M)	1 ml
KCl	0.66 g	NH <sub>4</sub> Cl (0.4 M)	1 ml
Na <sub>2</sub> SO <sub>4</sub>	4.00 g	KH <sub>2</sub> PO <sub>4</sub> (0.04 M)	1 ml
Trace element sol.	1 ml	NaF (0.07)	1 ml
Selenite tungstate sol.	0.2 ml		
Peptone	0.06 g	Yeast Extract	0.03 g
Before autoclaving pH was adjusted to 7.2 - 7.4			
Added after autoclaving from sterile stocks			
10-Vitamin Sol. (5x conc.)	2 ml		
Glucose (1 M)	1.25 ml		
Na-Lactate (1 M)	1.25 ml		

### 3.2.2 Nutrient Broth medium

Nutrient Broth No.2 was purchased from Merck (Darmstadt, Germany). Following the instructions 25 g were dissolved in one litre of ddH<sub>2</sub>O resulting in a final concentration of 10 g meat extract, 10 g peptone, and 5 g NaCl per litre. Additionally, ingredients of basal medium were dissolved (only 19.32 g NaCl). It was further processed as described above (see 3.2.1).

### 3.2.3 Marine Broth medium

Difco Marine Broth granules were purchased from BD (Oxford, UK). Ingredients (37.4 g per litre; Table 2.5) were dissolved in one litre ddH<sub>2</sub>O and processed as described above (see 3.2.1).

**Table 2.5: Composition of Marine Broth medium.**

Added before autoclaving			
ddH <sub>2</sub> O	1000 ml	NaHCO <sub>3</sub>	0.16 g
Peptone	5.00 g	KBr	0.08 g
Yeast extract	1.00 g	SrCl <sub>2</sub>	34.0 mg
Ferric citrate	0.10 g	Boric acid	22.0 mg
NaCl	19.45 g	Sodium silicate	4.0 mg
MgCl <sub>2</sub>	5.90 g	Sodium fluoride	2.4 mg
MgSO <sub>4</sub>	3.24 g	Ammonium nitrate	1.6 mg
CaCl <sub>2</sub>	1.80 g	Di-sodium phosphate	8.0 mg

### 3.2.4 Aerobic cultivation on agar plates

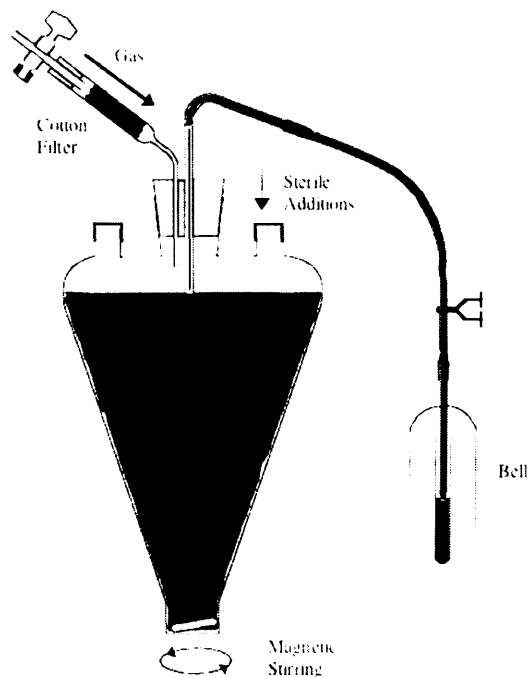
To prepare agar plates 300 ml of agar (4%, Fisher Scientific, Loughborough, UK) were added to 700 ml of medium. The agar was washed three to five times in ddH<sub>2</sub>O prior to autoclaving for 45 minutes at 121°C. The medium was preheated to 60°C in a water bath before mixing with agar and then gently poured into Petri dishes (Fisher Scientific, Loughborough, UK). The plates were left to cool down and later sealed with gas-permeable Nesco Film (Bando Chemical Ind. Ltd., Kobe, Japan) to prevent them from drying out.

## 3.3 Anaerobic media for the cultivation of microorganisms

### 3.3.1 Preparation of anoxic basal medium

To prepare anoxic basal medium a vessel designed by Widdel (1980, PhD Thesis, University Göttingen, Germany) was used (manufactured by Ochs Glasgeraetebau, Bovenden-Lenglern, Fig. 2.5). The ingredients for were dissolved in ddH<sub>2</sub>O (Table 2.6). The bell was wrapped in aluminium foil; the cotton-filled glass socket was sealed with a rubber stopper. One of the two screw caps remained slightly open to

avoid damage to the vessel due to pressure build-up during autoclaving for 60 minutes at 121°C. The medium was taken out of the autoclave at around 80°C as at this temperature almost no gas (including oxygen) is dissolved. The headspace was flushed with oxygen-free N<sub>2</sub>/CO<sub>2</sub> (80/20, v/v, pressure approximately 5 kPa) or H<sub>2</sub>/CO<sub>2</sub> (80/20, v/v) for up to 20 minutes with open screw caps. Subsequently, the screw caps were closed and the medium was cooled down whilst stirring. As overpressure was applied resulting in an outflow of sterile gas upon opening of the screwcaps airborne contamination is extremely unlikely.



**Fig. 2.5: Widdel flask for preparation of anoxic medium.** (Figure courtesy of Dr. Henrik Sass)

After the medium had cooled down vitamins, NaHCO<sub>3</sub>- solution (~ 0.5M), and substrates were added from sterile stock solutions. The medium was reduced by using either sterile sodium dithionite crystals until the redox indicator (resazurine) turned colourless or FeS (as 1.2 ml×l<sup>-1</sup> Na<sub>2</sub>S [0.8 M] and 0.5 ml×l<sup>-1</sup> FeCl<sub>2</sub> [1M]). When reducing with FeS resazurine was omitted because a colour change from black FeS to red/brown Fe<sub>2</sub>O<sub>3</sub> would occur due to oxidation upon exposure to oxygen. After 15 minutes the pH was checked and, if necessary, adjusted to pH 7.2-7.4 with either sterile NaHCO<sub>3</sub> (~0.5M) solution or sterile HCl (1M) Afterwards the medium was transferred into sterile bottles or tubes under the bell leaving only a small gas bubble in the respective vials.

**Table 2.6: Composition of anoxic basal medium.**

Added before autoclaving			
ddH <sub>2</sub> O	1000 ml	KBr (0.84 M)	1 ml
NaCl	24.3 g	H <sub>3</sub> BO <sub>3</sub> (0.4 M)	1 ml
MgCl <sub>2</sub> · 6H <sub>2</sub> O	10.00 g	SrCl <sub>2</sub> (0.15 M)	1 ml
CaCl <sub>2</sub> · 2H <sub>2</sub> O	1.50 g	NH <sub>4</sub> Cl (0.4 M)	1 ml
KCl	0.66 g	KH <sub>2</sub> PO <sub>4</sub> (0.04 M)	1 ml
Na <sub>2</sub> SO <sub>4</sub>	4.00 g	NaF (0.07)	1 ml
Trace element sol.	1 ml		
Selenite tungstate sol.	0.2 ml	Resazurine (2 M)	1 ml
Added after autoclaving from sterile stocks			
10-Vitamin Sol. (5x conc.)	2 ml	Reducing agent	Sodium dithionite or FeS
NaHCO <sub>3</sub> -solution			

### 3.3.2 Anoxic medium for heterotrophic bacteria

To isolate general heterotrophic prokaryotes from drilling sites of IODP Leg 307 monomer-medium was used. First anoxic basal medium (Table 2.6) was prepared. After it cooled down the following substrates were added before distributing the medium into smaller vials (Table 2.7).

**Table 2.7: Substrates added to anoxic basal medium for general heterotrophic bacteria**

Add to 1L medium after autoclaving (add from sterile stock solutions)	
NaNO <sub>3</sub> (0.5 mmol·l <sup>-1</sup> final concentration)	
Amino acids (containing the 20 common L-amino acids; final concentration 0.1 mmol·l <sup>-1</sup> , each)	
TCA (containing lactate, malate, fumarate, succinate; final concentration 0.1 mmol·l <sup>-1</sup> , each)	
Alcohols (containing methanol, ethanol, <i>n</i> -propanol and <i>n</i> -butanol; final conc. 0.1 mmol·l <sup>-1</sup> , each)	
Fatty acids (containing formate, acetate, propionate, butyrate, valerate, caproate; final conc. 0.1 mmol·l <sup>-1</sup> , each)	
Glycerol (stock solution 1 mol·l <sup>-1</sup> , final concentration 0.1 mmol·l <sup>-1</sup> )	
Glucose (stock solution 1 mol·l <sup>-1</sup> , final concentration 0.1 mmol·l <sup>-1</sup> )	
FeCl <sub>2</sub> (1 M in 0.1 M HCl) (reducing agent)	0.5 ml
Na <sub>2</sub> S (1 M) (reducing agent)	1.2 ml

### 3.3.3 Anoxic medium for metal-reducing bacteria

In order to enrich and isolate metal-reducing bacteria from samples of IODP cruise 307 the anoxic basal medium (Table 2.6) was supplemented with the following substrates (Table 2.8). Sodium dithionite was used as a reducing agent. Amorphous iron hydroxide and manganese oxides were prepared according to Köpke *et al.* (2005). Iron hydroxide was prepared by titrating 8.0 g FeCl<sub>3</sub> · H<sub>2</sub>O into 70ml ddH<sub>2</sub>O containing NaOH (final pH 7.0). Afterwards the precipitate was washed twice in ddH<sub>2</sub>O and resuspended in ddH<sub>2</sub>O (final concentration 400 mM).

**Table 2.8: Additions to anoxic basal medium for metal-reducing bacteria**

Add to 1L medium after autoclaving (add from sterile stock solutions)	
Sodium lactate	(3 mmol·l <sup>-1</sup> final concentration)
Sodium acetate	(5 mmol·l <sup>-1</sup> final concentration)
Sodium formate	(2 mmol·l <sup>-1</sup> final concentration)
Sodium propionate	(2 mmol·l <sup>-1</sup> final concentration)
Sodium butyrate	(1 mmol·l <sup>-1</sup> final concentration)
Amorphous iron hydroxide	(20 ml·l <sup>-1</sup> , final conc. approx. 20 mmol·l <sup>-1</sup> )
Manganese oxides	(10 ml·l <sup>-1</sup> , final conc. approx. 10 mmol·l <sup>-1</sup> )

In order to prepare amorphous manganese oxides, 2 g of H<sub>2</sub>O<sub>2</sub> (35%) were dissolved in 50 ml of a MnCl<sub>2</sub> solution (1 M). Dropwise addition of 10 ml of NaOH (4 M) was necessary to start the reaction. The mixture was then centrifuged (20,000×g) for 10 minutes. Subsequently the supernatant was treated again with H<sub>2</sub>O<sub>2</sub> and NaOH. The particulate manganese oxides were eventually resuspended into ddH<sub>2</sub>O (50 ml) and autoclaved resulting in a mixture of MnOOH and MnO<sub>2</sub>.

### 3.3.4 Anoxic freshwater medium for sulphate-reducing bacteria

*Desulfovibrio desulfuricans* subsp. *desulfuricans* (DSM 642, Type strain Essex 6) is a freshwater sulphate reducer. For its cultivation the medium described by Widdel and Bak (1992) was used (Table 2.9).

**Table 2.9: Anoxic medium for freshwater sulphate reducers**

Add before Autoclaving	
ddH <sub>2</sub> O	1000 ml
KH <sub>2</sub> PO <sub>4</sub>	0.1 g
NH <sub>4</sub> Cl	0.1 g
NaCl	0.25 g
KCl	0.1 g
MgCl <sub>2</sub> · 7H <sub>2</sub> O	0.32 g
CaCl <sub>2</sub> · 2H <sub>2</sub> O	0.1 g
Resazurine sol. (2 M)	0.5 ml
Selenite tungstate sol.	0.2 ml
Trace element solution	1.0 ml
Na <sub>2</sub> SO <sub>4</sub>	1.42 g
Add after Autoclaving from sterile stocks	
1 M NaHCO <sub>3</sub> -Sol. (autoclaved)	2.5g in 30 ml
Vitamin Sol.	2.0 ml

### 3.3.6 Anaerobic cultivation on agar plates

For anaerobic cultivation on agar plates the washed agar was flushed with oxygen free nitrogen for 10 minutes and autoclaved under a nitrogen atmosphere. Both agar



and anoxic medium were transferred into an anaerobic cabinet (Modular Atmosphere Controlled System, Don Whitley Scientific, Shipley, West Yorkshire, UK) containing a nitrogen, carbon dioxide, and hydrogen atmosphere. Hot agar and medium (heated to about 60°C) were mixed and poured gently into Petri dishes and later sealed with Nesco Film (Bando Chemical Ind. Ltd., Kobe, Japan). Plates were transferred either into bags containing Anaerocult A mini (Merck, Darmstadt, Germany) and sealed with an Anaeroclip (Merck, Darmstadt, Germany) or into anaerobic jars (Oxoid, Basingstoke, UK) containing Anaerocult A (Merck, Darmstadt, Germany). In both cases an oxygen indicator (Anaerotest, Merck, Darmstadt, Germany) was added, which changes colour if oxygen is present (from white to blue).

### 3.3.7 Composition of DSMZ media

#### a) Anoxic medium for acetogens

Acetogens were used in starvation experiments. Medium was prepared according to DSMZ (Medium 135; Table 2.10).

**Table 2.10: Anoxic medium for acetogens**

Add before autoclaving			
ddH <sub>2</sub> O	1000 ml	MgSO <sub>4</sub> · 7 H <sub>2</sub> O	0.10 g
NH <sub>4</sub> Cl	1.00 g	Yeast Extract	2.00 g
KH <sub>2</sub> PO <sub>4</sub>	0.33 g	Resazurine	1.00 mg
K <sub>2</sub> HPO <sub>4</sub>	0.45 g	Trace Element Sol.	1 ml
Add after autoclaving from sterile stocks			
Vitamin solution	2.00 ml		
Cysteine-HCl · H <sub>2</sub> O sol.	25.00 ml	Na <sub>2</sub> S 9H <sub>2</sub> O	0.50 g
NaHCO <sub>3</sub> solution	100.00 ml	FeSO <sub>4</sub>	0.50 g

For cultivation of *Acetobacterium malicum* fructose was added at a final concentration of 0.01% (w/v) and the pH was adjusted to 7.2-7.4. For maintenance *A. malicum* was subsequently grown in anoxic freshwater medium (Table 2.10) with lactate (20mM).

#### b) Anoxic medium for sulphate reducers

Sulphate reducers from different origins were used in starvation experiments. For

initial cultivation of marine strains medium was prepared according to the DSMZ recipe (Medium 383; Table 2.11).

**Table 2.11: Anoxic medium for marine sulphate reducers**

Add before autoclaving			
ddH <sub>2</sub> O	1000 ml	MgCl <sub>2</sub> · 6H <sub>2</sub> O	3.00 g
NaSO <sub>4</sub>	3.00 g	KCl	0.50 g
KH <sub>2</sub> PO <sub>4</sub>	0.20 g	CaCl <sub>2</sub> · 2H <sub>2</sub> O	0.15 g
NH <sub>4</sub> Cl	0.30 g	Resazurine (2 M)	2.00 ml
NaCl	21.00 g	Selenite tungstate sol.	1 ml
Other solutions to autoclave:			
Na <sub>2</sub> SeO <sub>3</sub> · 5 H <sub>2</sub> O sol.	1 ml of stock solution (3 mg l <sup>-1</sup> in 0.01 M NaOH)		
NaHCO <sub>3</sub>	2.50 g in 50 ml ddH <sub>2</sub> O		
Add after autoclaving from sterile stocks			
Vitamin solution	2.00 ml	NaHCO <sub>3</sub> solution	50.00 ml
FeCl <sub>2</sub>	1M	Na <sub>2</sub> S	0.8 M

After autoclaving the medium was cooled down under a N<sub>2</sub>/CO<sub>2</sub> (80%/20%; v/v) atmosphere.

### 3.3.8 Composition of supplement solutions

The composition of the trace element solution (Widdel and Pfennig, 1981) is listed in Table 2.12. Ingredients were dissolved and the pH was adjusted to 7.0 using KOH. After autoclaving the solution was filled into Pfennig bottles. Ingredients for the vitamin solution (Balch *et al.*, 1979) were dissolved in ddH<sub>2</sub>O (Table 2.15) and filter sterilised into 50 ml Pfennig bottles and stored at 4°C in the dark.

**Table 2.12: Composition of trace element solution and vitamin solution**

Trace element solution		Vitamin solution	
Compound	Amount added	Compound	Amount added
Hydrochloric acid, 25%	10 ml	Biotin	10.0 mg
FeCl <sub>2</sub> · 4 H <sub>2</sub> O	1500 mg	Folic acid	10.0 mg
CoCl <sub>2</sub> · 6 H <sub>2</sub> O	190 mg	Pyridoxine-HCl	50.0 mg
MnCl <sub>2</sub> · 2 H <sub>2</sub> O	100 mg	Thiamine-HCl · 2 H <sub>2</sub> O	25.0 mg
ZnCl <sub>2</sub>	70 mg	Riboflavin	25.0 mg
Na <sub>2</sub> MoO <sub>4</sub> · 2 H <sub>2</sub> O	36 mg	Nicotinic acid	25.0 mg
NiCl <sub>2</sub> · 6 H <sub>2</sub> O	24 mg	D-Ca-pantothenate	25.0 mg
H <sub>3</sub> BO <sub>3</sub>	6 mg	Vitamin B <sub>12</sub>	0.5 mg
CuCl <sub>2</sub> · 5 H <sub>2</sub> O	2 mg	p-Aminobenzoic acid	25.0 mg
dd H <sub>2</sub> O	1000 ml	Lipoic acid	25.0 mg
		dd H <sub>2</sub> O	1000 ml

The compounds for the selenite-tungstate solution (Table 2.13; Widdel and Bak 1992) were added into 1000 ml of ddH<sub>2</sub>O. After autoclaving it was aliquoted into Pfennig bottles and stored at room temperature.

**Table 2.13:** Composition of selenite-tungstate solution

Compound	Amount added
NaOH	0.4 g l <sup>-1</sup>
Na <sub>2</sub> SeO <sub>3</sub> 5 H <sub>2</sub> O	6 mg l <sup>-1</sup>
NaWO <sub>4</sub> 2 H <sub>2</sub> O	8 mg l <sup>-1</sup>

### 3.4 Determination of culturable cells counts using the Most Probable Number method

The Most Probable Number method (MPN) was used to determine the number of culturable cells in a given sample. For this a dilution series in appropriate medium was prepared using three or five parallels in 96-deep-well plates (Beckman Coulter UK Ltd. Bucks, Buckinghamshire, UK; Süß *et al.*, 2004; Köpke *et al.*, 2005). MPN plates were incubated in the dark at the appropriate temperature for the organisms.

For anaerobic prokaryotes MPN series were prepared in an anaerobic chamber (Modular Atmosphere Controlled System, Don Whitley Scientific, Shipley, West Yorkshire, UK).

To 900 µL of growth medium 100 µL of culture (diluted if necessary) were added and subsequently diluted. Between different strains or samples at least one row of medium-containing wells was left uninoculated as negative controls and blanks for later analysis (see 3.5). The plates were covered with sterile lids (Capmat, Beckman Coulter Inc, Fullerton CA, USA), which seal each well separately to avoid cross contamination. Anaerobic MPN plates were incubated in sealed bags containing Anaerocult A mini (Merck, Darmstadt, Germany).

When the MPN technique was used for sulphate-reducing bacteria, Na<sub>2</sub>S was replaced as a reducing agent by dithionite during preparation of the anoxic basal medium (Table 2.6). As these organisms produce H<sub>2</sub>S, black FeS will precipitate and indicate their activity. Wells containing FeS were therefore counted positive.

### 3.5 MPN analysis using a Sybr<sup>®</sup>GreenI fluorescence approach

To analyse growth in MPN plates the technique described by (Martens-Habbena and Sass, 2006) was used. According to the authors it is a sensitive method to detect low but significant growth in aqueous suspension. From each MPN well 200 µl of culture were transferred into black microtiter plates (*Nunc-Immuno*<sup>™</sup> 96 *MicroWell*<sup>™</sup> Plates

F96, Nunc GmbH & Co. KG., Wiesbaden, Germany); 50 µl of Sybr<sup>®</sup>GreenI solution (Molecular Probes, Invitrogen Corporation, Carlsbad, USA; 1:2000 diluted in TE buffer [200 mM Tris-HCl, 50 mM sodium EDTA, pH 8.0]) were added resulting in a total dilution of 1:10000 for Sybr<sup>®</sup>GreenI. Plates were incubated in the dark and fluorescence was measured after 4 and 20 hours (Excitation 485 nm/Emission 530 nm) using a plate reader (Fluorocount<sup>™</sup>, Packard Biosciences Company UK) attached to a Xenon Fiberoptic Lightsource. The reading parameters were set to a read length of 0.5 seconds, photomultiplier tube (PMT) of 900 V, and a gain of 1.0. The gain allows amplification of weak signal levels and interacts with the PMT value. The PMT setting controls the voltage of the photomultiplier tube, which controls the signal intensity that is emitted from the machine. According to the manual of the plate reader a setting of gain 1.0 and PMT 1100 is optimal. For most samples analysed this resulted in a reading off the scale and hence the PMT was lowered to 900 V.

To determine growth in individual MPN wells, measured fluorescence readings of blank wells of each plate were averaged and five times the value of the standard deviation was added. This value was then subtracted from all measured sample wells. If the resulting value was greater than zero the well was accounted positive for growth. Using the tables of de Man (1983) the most probable numbers of culturable cells for the original samples could be determined.

### 3.6 Phenotypical testing of isolates

#### 3.6.1 Substrate tests

Isolates obtained from IODP Leg 307 were tested in regard to their substrate spectra. For this 59 different substrates (Table 2.14) were chosen and utilization was tested using a microtitre approach (Süß *et al.*, 2008). Substrates from sterile stock solutions were diluted in oxic basal medium and 180 µl of each mixture were transferred into 96-well plates (Fisher Scientific, Loughborough, UK).

**Table 2.14: Substrate used for substrate spectra tests.**

Substrate	Final Concentration	Substrate	Final Concentration	Substrate	Final Concentration
Peptone	0.2%	Rhamnose	10 mM	Methanol	10 mM
Casamino Acids	0.2%	Mannitol	10 mM	Ethanol	10 mM
Yeast Extract	0.5%	Sorbitol	10 mM	Propanol	5 mM
Cellulose	0.2%	Formate	10 mM	Butanol	5 mM
Starch	0.1%	Acetate	10 mM	Glycol	10 mM
Chitin	0.25%	Propionate	10 mM	Glycerol	10 mM
Xylan	0.1%	Butyrate	5 mM	Tween 80	0.05%
Laminarin	0.25%	Valerate	5 mM	Alanine	20 mM
Sucrose	5 mM	Caproate	5 mM	Arginine	10 mM
Cellobiose	5 mM	Caprylate	5 mM	Cysteine	10 mM
Maltose	5 mM	Crotonate	10 mM	Glutamine	12.5 mM
Lactose	5 mM	Malonate	15 mM	Glutamate	10 mM
Glucose	10 mM	Succinate	15 mM	Serine	20 mM
Fructose	10 mM	Fumarate	15 mM	Isoleucine	10 mM
Mannose	10 mM	Malate	15 mM	Phenylalanine	5 mM
Galactose	10 mM	Glycolate	20 mM	Tryptophane	5 mM
Xylose	10 mM	Lactate	20 mM	Methylamine	20 mM
Arabinose	10 mM	$\alpha$ -Ketoglutarate	10 mM	Betaine	20 mM
Gluconate	10 mM	Citrate	10 mM	Benzoate	2.5 mM
Glucosamine	10 mM			Salicylate	2.5 mM

For inoculation, 50  $\mu$ l cell suspensions with a cell concentration of  $\sim 10^5$  cells ml<sup>-1</sup> were used. On each plate and for each substrate one well served as a negative control. All tests were done in duplicate. Cultures were incubated in the dark at room temperature for four weeks and analysed by visual inspection (pellet formation) or microscopy.

### 3.6.2 Tests for anaerobic growth

Isolates obtained from IODP Leg 307 were tested in their ability to grow under anoxic conditions. Anoxic basal medium was supplemented with casamino acids or glucose (test for fermentation) or with electron acceptors (Table 2.15). Then 900  $\mu$ l of each mixture were transferred into 96-deep-well plates (Beckman Coulter UK Ltd. Bucks, Buckinghamshire, UK) and inoculated. On each plate one well for each substrate/electron acceptor served as a negative control. Cultures were incubated in the dark at room temperature, for four weeks and analysed by visual inspection and ion chromatography. Additionally, the usage of dimethyl sulfoxide (DMSO) and 9,10-anthraquinone-2,6-disulfonate (AQDS) was tested in screw cap glass tubes with final concentrations of 10 mM and 4 mM, respectively (Süß *et al.*, 2008). All tests were done in duplicate.

**Table 2.15: Substrates and electron acceptors for anaerobic growth tests.**

Substrate	Final Concentration	Electron Acceptor	Final Concentration
Casamino acids	0.2%	Thiosulphate	10 mM
Glucose	10 mM	Ferric citrate	10 mM
		Fe(OH) <sub>3</sub>	40 mM
		MnO <sub>2</sub>	25 mM
		NaNO <sub>3</sub>	10 mM
		Sulphur	20 mM

### 3.6.3 Tests for salinity range and optimum

Isolates obtained from IODP Leg 307 were tested in their ability to grow at different salinities (Table 2.16). A range of different salinities was produced by mixing different amounts of freshwater and marine medium with a high percentage saline solution (NaCl: 29%; MgCl<sub>2</sub>: 1.8%). The solutions were then transferred into 96-well plates (Fisher Scientific, Loughborough, UK) and inoculated. On each plate and for each salinity, one well served as a negative control. Cultures were incubated in the dark at room temperature for five days. Growth was determined by optical density (OD<sub>600</sub>) measurements using a microplate reader (MRX, Dynex Technologies, Cantilly, VA, USA). All tests were done in duplicate.

**Table 2.16.: Range of salinities tested.**

Final Salinities [%]	Final Salinities [%]
0.10	5.01
0.20	7.51
0.50	10.00
0.75	12.54
1.00	15.04
1.50	17.54
2.53	19.95

### 3.6.4 Tests for pH range and optimum

To test the pH range and optimum for growth of isolates, oxic YPGI medium was used. During preparation six buffers were added instead of the commonly used HEPES resulting in different pH of the respective assay (Table 2.17; Kaksonen *et al.*, 2006). Tests were performed in glass tubes and in duplicates. After three days of incubation OD<sub>600</sub> was determined using a spectrophotometer (Cary 50 UV-Vis spectrophotometer, Varian Inc., Palo Alto, CA, USA) with attached dipper, which

was submerged in the bacterial suspension. Readings for each tube were measured in triplicate and averaged.

**Table 2.17: Buffers used for tests of pH range and optimum**

Name	Abbreviation	Concentration [mmol]	Final pH
Morpholineethanesulfonic acid	MES	10	5.93
Piperazine- <i>N,N'</i> -bis-(2-ethanesulfonic acid)	PIPES	10	6.70
Morpholine propanesulfonic acid	MOPS	10	7.30
4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid	HEPES	10	8.06
2-Amino-2hydroxymethyl-propane-1,3-propanediol	TRIS	10	8.43
2-( <i>N</i> -cyclohexylamino)ethanesulfonic acid	CHES	10	9.11
3-(cyclohexylamino)-2-hydroxypropane-sulfonic acid	CAPSO	10	9.41

### 3.6.5 Antibiotic resistance tests

Isolates obtained from IODP Leg 307 were tested in regard to their antibiotic susceptibility and to determine minimum-inhibitory concentrations. For this dilute marine broth medium was used in a microtitre approach (Reeves *et al.*, 1980), which is recommended by the American Society of Microbiology (Murray *et al.*, 1995). Antibiotics were diluted (two-fold steps) from 256 to 0.00195  $\mu\text{g ml}^{-1}$  for chloramphenicol, erythromycin, gentamycin, kanamycin, streptomycin, tetracycline, and trimethoprim or from 3200 to 0.0244 U for penicillin G (all final concentrations, Sass *et al.*, 2010). These solutions were inoculated with 100  $\mu\text{l}$  of bacterial suspension ( $\sim 10^6$  cells  $\text{ml}^{-1}$ ). Cultures were incubated for five days in the dark and at room temperature. Growth was considered positive by visual inspection (pellet formation).

### 3.6.6 Tests for vitamin requirements

To test isolates for their vitamin requirements the latter were omitted during preparation of YPGL medium. Strains that were able to grow without vitamins for five consecutive subcultures were considered prototrophic. Strains that did not grow were investigated further. For this eight different assays were prepared each lacking one of the following vitamins (Sass *et al.*, 2010): *p*-aminobenzoate, biotin, folate, lipoate, nicotinate, pantothenate, pyridoxine, and thiamine. If growth in one of these

cultures did not occur (visual inspection) strains were considered auxotrophic for the particular vitamin. Tests were performed in duplicates.

### 3.6.7 Tests for temperature range and optimum

To test the temperature range and optimum for growth of isolated strains from IODP Leg 307 YPGL agar plates were prepared and incubated at different temperatures (ranging from 2°C to 42°C). Growth was checked between three days and up to two weeks of incubation and scored depending on strength of growth (“+++” for strong positive, “+” positive, or “-” no growth). The isolated *Desulfovibrio* sp. strain was incubated at the same temperatures but in liquid, anoxic SRB Medium (Table 2.11) was used.

For the species description of *Ornithinimicrobium* sp. F18IV liquid YPGL medium (with 4 mM glucose) was used to determine specific growth rates ( $\mu$ ) at certain temperatures (4°C, 10°C, 15°C, 20°C, 25°C, 30°C, and 36°C). Incubations were performed in triplicates in small Erlenmeyer-flasks, continuously shaken at 150 rpm (Orbital Shaker 3019, GFL mbH, Burgwedel, Germany) to allow sufficient oxygen supply. OD<sub>600</sub> of the growing culture was checked over time on subsamples using a spectrophotometer (Cary 50 UV-Vis spectrophotometer, Varian Inc., Palo Alto, CA, USA) using polystyrene cuvettes (semi-micro, 1.6 ml, Fisher Scientific, Loughborough, UK). Readings of each parallel were done in triplicate and averaged. When OD<sub>600</sub> reached 0.5 the subsample was diluted with original medium to decrease the cell concentration in the cuvette. Specific growth rate was calculated as follows:

$$\text{Eq. 3) } \mu = \frac{\ln x_t - \ln x_0}{t - t_0}$$

### 3.6.8 Phenotypical characterisation of isolates using solid media

For the physiological characterisation of bacterial isolates agar plates with different amendments (Table 2.18; Gerhardt *et al.*, 1994) to the above-mentioned aerobic media or commercially available tests were used. Agar plates were prepared (as described in 3.2.4), inoculated, and incubated in the dark at room temperature for



three to five days. Results of these tests were either visible after growth or certain treatments were necessary (see below).

**Table 2.18: List of YPGL media amendments for phenotypical characterisation of isolates.**

Amendment	Concentration (g l <sup>-1</sup> )	Purpose
Agarose (replacing the Agar)	1.3 g	Liquefaction of agarose
Casein	1-10 g	Extracellular caseinase activity
Gelatine (replacing the Agar)	12 g	Liquefaction of gelatine
Phenolphthalein phosphate	10 g	Extracellular phosphatase activity
Tween 20	10 g	Extracellular lipase activity
Tween 80	10 g	Extracellular lipase activity
Urea	20 g	Urease activity

○ Agarose liquefaction

To test for agarose liquefaction YPGL medium was autoclaved with 1.3% agarose and poured into Petri dishes. Strains were determined positive for liquefaction if the agarose became fluid.

○ Caseinase activity

Casein was added to YPGL medium and was visible as a precipitate within the agar plates. Extracellular enzyme activity was determined as positive if a clear zone appeared around individual colonies.

○ Gelatinase activity

Two tests for gelatinase activity were used. In the first the agar was replaced completely by gelatine and mixed with YPGL medium. When extracellular gelatinase was active this resulted in liquefaction of the gelatine. Also, a commercial test medium was purchased from Condalab (Madrid, Spain). Fifty grams of dehydrated medium were dissolved in one litre of ddH<sub>2</sub>O resulting in the following concentrations: peptone (10 g l<sup>-1</sup>), NaCl (5 g l<sup>-1</sup>), agar (15 g l<sup>-1</sup>), beef extract (10 g l<sup>-1</sup>), and gelatine (10 g l<sup>-1</sup>). To determine gelatinase presence, plates were covered with saturated ammonium sulphate solution resulting in clear zones around gelatine-liquefying colonies.

○ Lipase activity

Extracellular lipase activity was investigated using different substrates. Tween 20 and 80 were individually added to YPGL medium and poured into Petri dishes after autoclaving. If enzyme activity is present opaque halos (crystals of

calcium soaps) appear in the immediate surrounding of colonies. In addition, a commercial test (Difco™ Spirit Blue Agar) was purchased (BD, Oxford, UK) consisting of Casein (10 g l<sup>-1</sup>), yeast extract (5 g l<sup>-1</sup>), agar (20 g l<sup>-1</sup>), and Spirit Blue (0.15 g l<sup>-1</sup>; indicator of lipolysis). Lipid Reagent (containing tributyrin and polysorbate 80) was added from sterile stock after autoclaving. Here, lipase activity resulted in a halo around colonies.

○ Phosphatase activity

Dinitrophenyl phosphate (from sterile stock solution) was added to sterile YPGL medium (final concentration 0.01%) to investigate phosphatase activity. To determine presence of the enzyme, ammonia was poured into the lid of the Petri dish, which was then covered with the colonies facing down. If positive for phosphatase, the area around colonies turned into a bright pink colour.

○ Starch hydrolysing enzyme activity

Difco™ Starch Agar was purchased from BD (Oxford, UK). Following the instructions, 25 g of the dehydrated medium were dissolved in one litre of ddH<sub>2</sub>O resulting in the following concentrations of beef extract (3 g l<sup>-1</sup>), starch (10 g l<sup>-1</sup>; soluble), agar (12 g l<sup>-1</sup>) and subsequently autoclaved. After growth of colonies the plate surface was covered with Gram's iodine solution resulting a clear zone around colonies positive for starch hydrolysing enzymes.

○ Urease Activity (Gerhardt *et al.*, 1994)

Cells were grown in YPGL agar plates amended with urea (2%) and phenol red (0.001%). After incubation a red-violet colour indicated urease-positive strains.

### 3.6.9 Phenotypical characterisation of isolates in liquid media

To complement the physiological characterisation of bacterial isolates certain additions to YPGL Medium (Table 2.4) were made. The amended media were inoculated and subsequently incubated in the dark at room temperature for three to five days (Gerhardt *et al.* 1994).

○ Arginine dihydrolase activity

Basal medium in tubes for anaerobic growth was amended with 1% L-arginine monohydrochloride. Tubes were examined for a colour change (from yellow) due to an increase in the pH if activity is present. Weak reactions result in a bluish grey colour whereas positive tubes turned violet or reddish-violet.

○ Catalase activity

Colonies were transferred from agar plates into a drop of 3% hydrogen peroxide on a glass slide using a nonmetallic inoculation loop. Catalase is present if bubbles are formed.

○ Esculin hydrolysis

Cultures were grown in basal medium amended with 0.05% esculin (6,7-dihydroxycoumarin 6-glucoside) and 0.05% ferric citrate. Cultures were considered positive if medium turned brownish black due to the reaction of 6,7-dihydroxycoumarin and  $\text{Fe}^{3+}$ .

○ Hippurate hydrolysis

Basal medium was amended with sodium hippurate (1%) and transferred into screw-cap tubes. After incubation, increments of 0.1 ml of ferric chloride solution ( $\text{FeCl}_3 \times 6\text{H}_2\text{O}$  in 100 ml of 2% HCl) were added into a control tube resulting in a precipitate of iron hippurate, which during further additions dissolved. The smallest total amount of ferric chloride that caused the precipitate to dissolve was noted down. The supernatant of incubated cultures was then amended with the smallest total amount of ferric chloride determined. Cultures were considered positive for enzyme activity if a heavy, permanent precipitate of iron benzoate formed.

○ Indol production from Tryptophane (Kovac-Test)

Basal medium (free of carbohydrates and nitrite) was amended with L-tryptophane (0.1%). After incubation 0.5 ml Kovacs' reagent (3 g *p*-Dimethylaminobenzaldehyde in 75 ml pentanol or butanol and 25 ml HCl, concentrated) were added and the tube was gently shaken. If the colour of the medium changed to red, strains were considered positive for indol production.

- Lysine decarboxylase  
See arginine dihydrolase activity (above). Instead of arginine, 1% L-lysine dihydrochloride was added.
  
- Ornithine decarboxylase  
See arginine dihydrolase activity (above). 1% L-ornithine dihydrochloride was added instead of arginine.

#### **4. Molecular biological techniques**

Molecular biological techniques were employed to either determine the phylogenetic affiliation of isolates from IODP Leg 307 or to determine the phylogenetic community composition of sediment samples (Tamar Estuary sediment and subsequent slurry experiments).

##### **4.1 DNA extraction from pure cultures**

Colonies from agar plates were transferred into small reaction vials. Depending on the amount of biomass, 50  $\mu$ l to 100  $\mu$ l Chelex solution (5%, BioRad, Hemel Hempstead, Hertfordshire, UK) was added. Vials were heated in boiling water for five minutes and subsequently rapidly cooled on ice for five minutes. This “freeze and thaw” procedure was repeated three times. The cell extract served as template for DNA amplification via Polymerase Chain Reaction (PCR).

##### **4.2 DNA extraction from sediment samples**

To guarantee a successful PCR amplification of DNA extracted from sediment samples the commercial FastDNA® SPIN Kit for Soil (QBiogene, Cambridge, UK) was purchased. Following the manufacture’s protocol 500 mg of sediment were transferred into Lysing Matrix E tube and 978  $\mu$ l sodium phosphate buffer and 122  $\mu$ l MT buffer were added. The mixture was homogenized for 1 minute using a Vortex mixer and subsequently centrifuged (14,000 $\times$ g for 5-10 minutes, Centrifuge

5415, Eppendorf AG, Hamburg, Germany). The supernatant was then transferred into a clean 2 ml microcentrifuge tube and 250  $\mu$ l Protein Precipitation Solution was added. The mixture was shaken by hand for 30 seconds followed by centrifugation. Afterwards the supernatant was transferred into a clean 1.5 ml reaction vial. The binding matrix was resuspended and 1 ml of the suspension was added into the 1.5 ml vial. For binding of the DNA the vial was shaken by hand for 2 minutes and then allowed to set for 3 minutes. Subsequently 500  $\mu$ l of the supernatant were discarded and the binding matrix was resuspended in the remaining supernatant. After transferring 600  $\mu$ l of the mixture onto a SPIN<sup>TM</sup> Filter it was centrifuged for 1 minute. The catch tube was emptied and the remaining binding matrix mixture was added and centrifuged again. Afterwards the pellet was resuspended in 500  $\mu$ l of ethanol containing SEWS-M and centrifuged for 1 minute, the catch tube was emptied and again centrifuged for 2 minutes followed by replacing the used catch tube with a clean one. The SPIN<sup>TM</sup> Filter was allowed to air dry for 5 minutes at room temperature and afterwards 50-100  $\mu$ l of DNase/Pyrogen-free water was added and centrifuged to elute DNA, which was subsequently stored at -20°C. (Francis *et al.*, 2005; Mincer *et al.*, 2005)

### 4.3 Polymerase Chain Reaction (PCR)

The Polymerase Chain Reaction was invented by Mullis and Faloona (1987). It uses the bacterial enzyme DNA-polymerase to amplify specific regions of template DNA. In microbial ecology PCR is widely used to investigate the microbial community composition of samples. During this project it was used to amplify the 16S rRNA gene in the process to identify isolates from samples of IODP Leg 307 and to determine community compositions of sediment samples and slurry experiments. Therefore, specific primers were used, which determine/bracket the target gene on the DNA (Table 2.19).

The 16S ribosomal RNA gene is used to affiliate microorganisms to their closest relatives. For supposedly pure cultures primers 27f and 1492r were used (numbers correspond to the position in the gene of *E.coli*). To investigate whether still consisted of more than one strain and for community composition analysis primers 357FGC and 907R for denaturing gradient gel electrophoresis (DGGE) were

**Table 2.19: Primers used during PCR.**

Primer	Sequence
8F (Lane <i>et al.</i> , 1991)	5'-AGA GTT TGA TCM TGG CTC AG-3'
357F (Muyzer <i>et al.</i> , 1993)	5'-CCT ACG GGA GGC AGC AG-3'
907R (Muyzer <i>et al.</i> , 1998)	5'-CCG TCA ATT CMT TTG AGT TT-3'
1492R (Lane <i>et al.</i> , 1985)	5'-GGT TAC CTT GTT ACG ACT T-3'
GC clamp (Muyzer <i>et al.</i> , 1993)	5'-CGC CCG CCG CGCCCC GCG CCC GGC CCG CCG CCC CCG CCC C-3'
M13F (O'Sullivan <i>et al.</i> , 2008)	5'-GTA AAA CGA CGG CCA G-3'
M13R (O'Sullivan <i>et al.</i> , 2008)	5'-CAG GAA ACA GCT ATG AC-3'
109F (Grosskopf <i>et al.</i> , 1998)	5'-ACK GCT CAG TAA CAC GT-3'
958R (DeLong, 1992)	5'-YCC GGC GTT GAM TCC AAT T-3'
SaF (Nicol <i>et al.</i> , 2003)	5'-CCT AYG GGG CGC AGC AGG-3'
PARCH519R (Ovreas <i>et al.</i> , 1997)	5'-TTA CCG CGG CKG CTG-3'

used. One of the DGGE primers (GC351) has a “tail” of guanosine and cytosine bases. When amplified this tail becomes a GC-clamp (Muyzer *et al.*, 1993) in the double stranded DNA. Because the GC pair in the DNA strand develops three hydrogen bonds it is relatively resistant to denaturing. This characteristic is later used in the DGGE.

#### 4.4 Agarose gel electrophoresis

To check for successful DNA amplification during PCR, agarose gel electrophoresis with incorporated staining of DNA with SybrSafe<sup>TM</sup> (Molecular Probes, Invitrogen Corporation, Carlsbad, USA) was used. Agarose was dissolved in 1×TAE buffer (1.2% w/vol, Severn Biotechnology, Kidderminster, Worcestershire) and heated in the microwave for 2 minutes. The dissolved agarose solution was cooled down for a few minutes at room temperature and 10 µl of SybrSafe<sup>TM</sup> was added. The still liquified agarose was poured into electrophoresis trays. Two combs were put into the gel creating wells into which the samples were pipetted later. The gel was left to solidify for 20 minutes. The gel was loaded with DNA, 5 µl of PCR product was mixed with 1 µl of loading dye (Fermentas Inc., MD, USA) and then pipetted into the wells. A marker (Hyper Ladder, Bioline, London, UK) was added at both sides of the gel to ensure that the gel ran correctly and that the PCR product had the correct length. A voltage of 100 mV was applied for 30 minutes using Powerpack 300 (BioRad, Hemel Hempstead, Hertfordshire, UK). DNA is charged negatively and therefore migrates through the gel towards the anode in an electric field. Afterwards the gel was inspected under UV light using in GeneGenius Bioimaging System (Syngene, Cambridge, UK). Sybr<sup>®</sup>Safe<sup>TM</sup> binds to DNA and gives a clear

fluorescence signal under UV light. Pictures were taken using GeneSnap software (Syngene, Cambridge, UK) and GeneTool to estimate DNA concentrations.

#### 4.5 Denaturing gradient gel electrophoresis (DGGE)

DGGE is a molecular tool to separate PCR products of similar same length but with different sequences using a denaturing gradient (Muyzer *et al.*, 1993). The amount of GC pairs and their distribution within the DNA sequence determine at which position in the gel the dsDNA fragments will start to denature/melt. The polyacrylamide gel (8%) contains a gradient of increasing denaturing substances (urea and formamide) leading to the formation of single bands that ideally only contain copies of the same sequence, which later can be excised and sequenced. DGGE can be used for fingerprint analyses, for example, of changing bacterial communities over time or to determine whether cultures are pure or still contain different organisms.

The strength of the denaturing gradient can be adjusted. In this study a 30-60% denaturing gradient for bacterial 16S rRNA genes and a 0%-80% gradient for archaeal 16S rRNA genes were applied using the stock solutions shown in Table 2.20.

**Table 2.20: Preparation of stock solutions for DGGE-Gels.**

	Stock Solution		
	0%	30%	60%
Acrylamide (40%)	10 ml	20 ml	20 ml
Formamide	-	12 ml	24 ml
TAE buffer (50x)	1 ml	2 ml	2 ml
Urea		12.6 g	25.2 g
ddH <sub>2</sub> O	add to 50 ml	add to 100 ml	add to 100 ml

DGGE glass plates were thoroughly cleaned with SDS (1%) and rinsed with water to prevent the presence of dust particles in the gel that would interfere with the electric field and the migration of the DNA fragments. Glass plates were assembled and mounted into the D Code System from BioRad (Hemel Hempstead, Hertfordshire, UK). One millilitre of the 0% Stock solution was poured into the bottom to check for potential leaks. A gradient mixer was then filled with the 60% stock solution in one column, the 30% stock solution in the other. Stock solutions were amended with 86 µl ammonium persulphate (10%, BioRad, Hemel Hempstead, Hertfordshire, UK) and 17 µl TEMED (Severn Biotechnology, Kidderminster, Worcestershire). These

are necessary to start the polymerisation of the acrylamide. The higher concentrated stock solution was filled in first and the gradient mixer then created a continuous gradient with concentrations ranging from 60% denaturing at the bottom to 30% at the top of the gel. The gel was left overnight to polymerise. The next day the 0% denaturing stock solution was poured on top and a comb was placed on top of the gel to create wells to place samples into. PCR product subsamples (8  $\mu$ l) were mixed with 2  $\mu$ l of loading dye (Fermentas Inc., MD, USA) and pipetted into the wells. A standard was added on each side of the gel. A 100 mV current was applied for 5 hours. The gel was then taken out and stained with Sybr<sup>®</sup>Gold (Molecular Probes, Invitrogen Corporation, Carlsbad, USA) for 20 minutes and subsequently inspected under UV light using GeneGenius Bioimaging System (Syngene, Cambridge, UK). Prominent bands were excised with a sterile scalpel and transferred into 500  $\mu$ l reaction vials for washing.

#### 4.6 Reamplification of bands

After excision, bands from the DGGE gel were washed in ddH<sub>2</sub>O (50-100  $\mu$ l) for 10 minutes at room temperature to remove substances (e.g. urea) that could affect the reamplification. Water was removed and the gel was allowed to dry for a few minutes. Afterwards bands were crushed using a sterile pipette tip, ddH<sub>2</sub>O (10-20  $\mu$ l, depending on band intensity) was added, and the sample was spun down to ensure that it was collected at the bottom of the reaction vial. Crushed and washed bands were reamplified by PCR. For this a special primer pair (M13F/M13R; see Table 2.19; O'Sullivan *et al.*, 2008) was used and the annealing temperature was set lower than in the initial amplification of 16S rRNA genes from the original sample to ensure a high yield of product for subsequent sequence analysis.

#### 4.7 DNA sequencing

After estimating the concentration of DNA in the reamplified samples, it was adjusted to an optimum for sequencing (around 10 ng DNA  $\mu$ l<sup>-1</sup>). Samples were then processed by the sequencing laboratory at the School of Biosciences at Cardiff University using the primer M13F (Table 2.19) and capillary sequencing technique



(3130XL Genetic Analyser, Applied Biosystems, Foster City, CA, USA) with fluorescence labelled dNTPs.

## **5. Gas chromatography**

Gas chromatography was routinely used to monitor headspace gas concentrations during starvation experiments, during microbial growth (product formation), and during heating experiments. Furthermore, porewater gas concentrations from Tamar Estuary sediment cores were determined.

### **5.1 Preparation of sediment porewater for gas chromatography**

In order to determine gas concentrations within porewaters from Tamar Estuary sediment cores approximately, 1 cm<sup>3</sup> of sediment was added to glass vials containing 2 ml of 3% KCl solution and glass beads in a nitrogen atmosphere. The mixture was shaken vigorously for 30 minutes to free trapped or dissolved gases. Vials were stored upside down until analysis. Headspace gas from sediment slurry experiments was analysed by gas chromatography directly.

### **5.2 Gas quantification using a Natural Gas Analyser (NGA)**

For natural gas analysis a modified Perkin Elmer/Arnel Clarus 500 Gas Chromatograph (Perkin Elmer, Waltham, MA, USA) was used equipped with a Flame Ionisation (FID) and a Thermal Conductivity Detector (TCD). Argon and helium, respectively, were used as carrier gases. The FID was used for the quantitative detection of methane whereas the TCD can determine hydrogen and carbon dioxide. The oven temperature was set to 110°C with the TCD working at 150°C and the FID at 250°C. A mixture of air and hydrogen was used for combustion in the FID. The NGA was calibrated by using three different standard gas mixtures purchased from Scott Speciality Gases (Plumsteadville, PA, USA). Data was collected using the TotalChrom Navigator Software (Perkin

Elmer, Waltham, MA, USA). Prior to gas analysis one of the above mentioned standards was used to check accurate measurement by the NGA.

To measure gas concentrations a 5 ml syringe was flushed several times with nitrogen before taking a 3 ml headspace sample from, for example, starving cultures which was then injected into the NGA.

## 6. Ion chromatography

Ion chromatography was mainly applied to monitor concentrations of volatile fatty acids (VFAs) such as acetate, lactate, and formate during starvation experiments. It was also used to analyse the porewater geochemistry of sediment samples from Tamar Estuary sediment cores and subsequent sediment slurry experiments. Methods used by Webster *et al.* (2009, 2010) were applied and are briefly described below.

### 6.1 Determination of anion-concentrations using ion chromatography

Samples from pure cultures, sediment cores, or sediment slurries were centrifuged at  $15.800\times g$  for 5 minutes (Centrifuge 5415, Eppendorf AG, Hamburg, Germany). The supernatant was then 1:10 diluted in ddH<sub>2</sub>O and concentrations of VFAs, chloride, sulphate and other chemical species were determined on a Dionex ICS-2000 Ion Chromatography System equipped with an AS50 autosampler (Dionex UK Ltd). Chromatographic separation was conducted on two Ionpac AS15 columns in series and the determination of the chemical species was performed via a self-regenerating suppressor (ASRS-ULTRA II 4-mm) unit combined with a DS6 heated conductivity cell. The following gradient was used: 6 mM KOH (38 min), 16 mM KOH min<sup>-1</sup> to 70 M (17 min), 64 mM KOH min<sup>-1</sup> to 6 mM (12 min). Standards with known concentrations were measured before and after sample analysis. Anion concentrations were determined using calibration standards and the software Chromeleon 6.50.

For radiotracer experiments (see below 7.2) the same setup was used but in addition different chemical species (glucose, VFAs, bicarbonate) were collected using the Dionex Foxy JR fraction collector (Dionex UK Ltd). Fractions were collected in polyethylene vials (Perkin Elmer, Cambridge, UK) containing 0.5 ml

NaOH (1M). Retention times for each chemical species were determined prior to analysis by Dr. Erwan Roussel.

## 6.2 Determination of cation-concentrations using ion chromatography

To determine *in situ* concentrations of methylamine and other cations in Tamar Estuary sediment cores cation chromatography was applied. Samples were centrifuged as described above (see 6.1). A DX-120 Ion Chromatography System with an AS40 autosampler fitted with an Ionpac CS16 and a cation self-regenerating suppressor (CSRS-300 4 mm) in combination with a DS4-1 heated conductivity cell (Dionex UK Ltd) was used. Methanesulphonic acid (25 mM) served as an eluent. To determine cation concentrations known standards and the software Chromeleon 6.50 were used.

## 7. Radiotracer experiments using $^{14}\text{C}$ -substrates

Radiotracer experiments were conducted after long-term starvation experiments in order to see how quickly substrate deprived cells could react to available substrate and to follow the fate of the carbon, e.g. what proportion is dissimilated and assimilated. Additionally, very low concentrations were added as might appear *in situ* in deep marine sediments.

### 7.1 Calculation of radiotracer concentrations

D-[U- $^{14}\text{C}$ ]glucose and DL-[U- $^{14}\text{C}$ ]lactate were purchased from ARC (American Radiolabeled Chemicals, Saint Louis, MO, USA). The specific activity of glucose is 11.1 GBq mmol $^{-1}$ , a total activity of 1.85 MBq was received. Hence the activity of the original glucose per microlitre was

$$\text{Eq.7.1: } \frac{1.85 \text{ MBq}}{250 \mu\text{l}} = \underline{7.4 \text{ KBq}/\mu\text{l}}.$$

This original solution was diluted (1:6) resulting in the stock solution used for further experiments with  $1.23 \text{ KBq } \mu\text{l}^{-1}$  activity. The glucose concentration of this stock solution was calculated as follows

$$\text{Eq. 7.2: } \frac{1.23 \text{ KBq} / \mu\text{l}}{11,100,000 \text{ KBq} / \text{mmol}} = 1.111 \cdot 10^{-7} \text{ mmol} / \mu\text{l} = \underline{1.1 \cdot 10^{-4} \text{ nmol} / \mu\text{l}}$$

In the experiment,  $10 \mu\text{l}$  of stock solution were added to  $3 \text{ ml}$  of culture (see experimental setup below) resulting in the following concentration of glucose in the experiment:

$$\text{Eq. 7.3: } \frac{1.1 \cdot 10^{-4} \text{ nmol} / \mu\text{l} \cdot 10 \mu\text{l}}{3,000 \mu\text{l}} = \underline{3.67 \cdot 10^{-7} \text{ nmol} / \mu\text{l}}$$

In order to understand this number better the number of glucose molecules per cell was calculated.

$$\text{Eq. 7.4: } \frac{6.02 \cdot 10^{23} \text{ molecules}}{1 \text{ mol}} = \frac{6.02 \cdot 10^{23} \text{ molecules}}{1,000,000,000 \text{ nmol}}$$

$$\text{Eq. 7.5: } \frac{6.02 \cdot 10^{23} \text{ molecules}}{1,000,000,000 \text{ nmol}} \cdot 3.67 \cdot 10^{-7} \text{ nmol} / \mu\text{l} = \underline{2.2 \cdot 10^8 \text{ molecules} / \mu\text{l}}$$

For different cell concentrations the following results:

$$\sim 10^8 \text{ cells ml}^{-1} = 10^5 \text{ cells } \mu\text{l}^{-1} \rightarrow 2200 \text{ molecules per cell}$$

$$\sim 10^7 \text{ cells ml}^{-1} = 10^4 \text{ cells } \mu\text{l}^{-1} \rightarrow 22000 \text{ molecules per cell}$$

Following the above described steps with DL-[U- $^{14}\text{C}$ ]lactate (specific activity is  $2.035 \text{ GBq mmol}^{-1}$ ,  $1.85 \text{ MBq}$  received) resulted in a final concentration of lactate of  $3.8 \times 10^6 \text{ nmol } \mu\text{l}^{-1}$  in the experiments ( $\sim 23,000$  molecules per cell with a cell concentration of  $\sim 10^8 \text{ cell ml}^{-1}$ ).

## 7.2 Experimental setup for radiotracer experiments

Three millilitres of starving culture were transferred into  $10 \text{ ml}$  gas-tight serum vials (Wheaton, VWR International, Lutterworth, UK), previously autoclaved and flushed with  $\text{N}_2/\text{CO}_2$  (80/20, v/v). A batch of the radiotracer stock solution was 1:10 diluted and  $50 \mu\text{l}$  were added to each vial, which were then incubated in the dark (stored upside down) at  $10^\circ\text{C}$  for *Photobacterium* strains and  $25^\circ\text{C}$  for other strains. Assays were incubated for 0h, 2h, 5h, 10h, 24h, 48h, 10 days, and 30 days.

For each timepoint the following procedure was conducted. A 1.5 ml subsample was taken using a syringe and transferred to a small reaction vial. To the remaining volume 0.5 ml NaOH (2M) was added to stop bacterial activity and to trap any gaseous CO<sub>2</sub> in the liquid phase for later analysis. From the plain 1.5 ml subsample 100 µl were transferred into a glass scintillation vial (Lablogic Systems, Sheffield, UK) and 9.9 ml of InstaGel<sup>®</sup> Plus (Perkin Elmer, Cambridge, UK) were added. Another 100 µl were 1:10 diluted, centrifuged for six minutes (Sigma Laborzentrifugen GmbH, Type 113, Osterode, Germany, 13,000 rpm). The supernatant was transferred into a vial for chromatographic separation and fraction collection (see section 6.1). The fractions were collected in polyethylene vials (Packard Biosciences Company [now Perkin Elmer, Cambridge, UK]) containing 0.5 ml of NaOH (1M) and subsequently transferred into glass scintillation vials and 9.9 ml of InstaGel were added. Three times 300 µl were filtered onto Cyclopure<sup>™</sup> track etched, polycarbonate, hydrophilic filter membranes (0.2 µm, Whatman, Kent, UK) and washed with 10 ml 1xPBS to remove any radioactivity present outside the cells. The filter membranes were then transferred into plastic scintillation vials (Packard Biosciences Company [now Perkin Elmer, Cambridge, UK]) containing 10 ml liquid scintillation fluid (Scintisafe 3, Fisher Scientific, Loughborough, UK).

To account for CO<sub>2</sub> production during incubation 1 ml of the previously stopped cell suspension was transferred into gastight serum vial (Wheaton, VWR International, Luttermouth, UK) containing 4 ml HCl (1M) and a magnetic flea. The headspace in these vials was then flushed with N<sub>2</sub> whilst stirring to remove all CO<sub>2</sub> from the liquid phase and lead the gas through firstly, one vial amended with VFAs and acidified (pH below 3) and secondly, three vials with 10 ml scintillation fluid plus containing 7% phenethylamine (Sigma-Aldrich, Gillingham, UK), to capture the CO<sub>2</sub>. This procedure was also performed using just anaerobic YPGL medium amended with the radiotracer as a negative control to investigate the fate of <sup>14</sup>C-glucose with ongoing incubation.

All vials containing InstaGel or scintillation fluid were measured using a liquid scintillation analyser (TRI-CARB 2900TR, Perkin Elmer, Cambridge, UK) and two different programs. Determination of DPM in vials with InstaGel (fractions and plain sample) was conducted three times for 20 minutes, whereas the filter membranes and trapped CO<sub>2</sub> in liquid scintillation fluid were measured twice for 10 minutes.

## Chapter III

# Isolation and physiological characterisation of deep biosphere prokaryotes from sediment samples obtained during IODP Leg 307

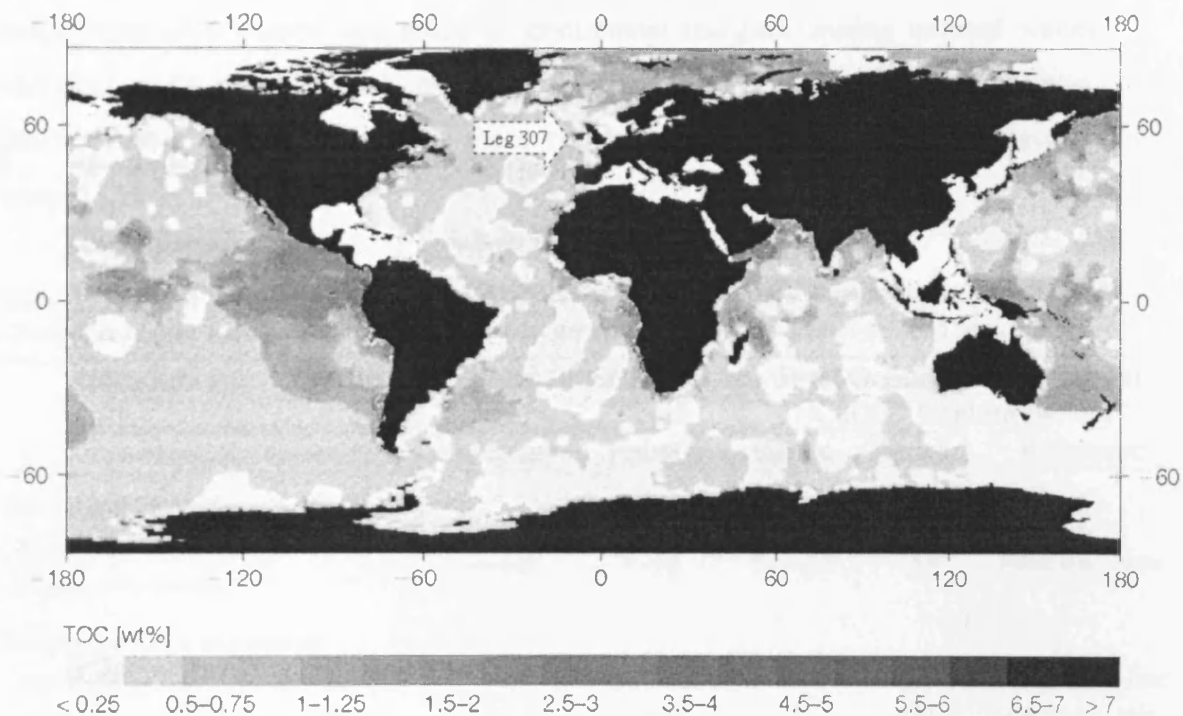
### 1. Introduction

Subseafloor sediments are considered to be the largest prokaryotic habitat on the planet (Whitman *et al.*, 1998). In general, with ongoing burial and associated ageing of sediment layers readily accessible organic material is degraded and only very recalcitrant organic matter remains (Hedges and Keil, 1995) resulting in low energy environments. The most extreme example are sediments underlying the organic matter-poor waters of mid ocean gyres (e.g. South Pacific Gyre [D'Hondt *et al.*, 2009]). These waters are very remote from the continents and receive only very little organic matter input by riverine or aeolian sources. Consequently they show the lowest primary production and calcification rates known from any marine environment mainly as a result of nitrogen limitation (Moore *et al.*, 2002). In addition, sediments underlying this oceanic desert receive only very little organic matter from the euphotic zone, most of which is degraded during its passage through the water column. This extremely low organic matter input is reflected by some of the lowest sedimentary respiration rates as indicated by almost vertical oxygen profiles (D'Hondt *et al.*, 2009).

Another example of very organic matter-poor marine sediments are cold-water carbonate mounds that occur in large clusters along the north-western European continental margin (Wheeler *et al.*, 2007) but underlie more productive seas. The situation here, however, is different from 'normal' hemipelagic or pelagic sediments as these carbonate mounds contain a significant amount of dead cold-water coral rubble and therefore more closely resemble the carbonate pavements found in the Mediterranean Sea (Aloisi *et al.*, 2000). During IODP Leg 307 the Challenger Mound, one of ~1600 mounds in the Belgica Mound Province of the Porcupine Basin southwest of the coast of Ireland was sampled and subjected to

extensive geological and microbiological investigations (Ferdelman *et al.*, 2006; Webster *et al.*, 2009), These mounds owe their existence mainly to the framework building coral *Lophelia pertusa* (Wheeler *et al.*, 2007).

One of the main objectives of IODP Leg 307 was to “establish whether the mound roots on a carbonate hard-ground of microbial origin and if past geofluid migration events acted as prime trigger for mound genesis” (Ferdelman *et al.*, 2006). Furthermore, the biogeochemistry of the Challenger Mound was to be investigated to understand if microbial activities had an impact on mound genesis and growth, thereby supporting one of the theories described below (Ferdelman *et al.*, 2006).



**Fig. 3.1: World map of surface TOC in ocean sediments.** Adapted from Introduction to Marine Biogeochemistry 2nd Edition, Ed.: Libes, 2009

There are two theories attempting to explain the existence and development of these geological features, which usually occur only in discrete provinces. The first “internal control” theory links their formation to hydrocarbon seepage and/or specific hydrodynamic conditions, which are optimal for the colonising coral *Lophelia pertusa* (Wheeler *et al.*, 2007). In addition to this, Hovland (1990) suggested that the restricted occurrence of these mounds may be explained by cold seeps stimulating activity and growth of microorganisms, which in turn serve as food for a diverse and abundant biological community (including *Lophelia*). Furthermore, these seeps potentially provide suitable conditions for microbially mediated

carbonate precipitation. Studies by De Mol *et al.* (2002) and Kiriakoulakis *et al.* (2007), however, indicated that mound growth does not depend on continuing hydrocarbon seepage although Henriet *et al.* (2001) stated that seepage may have been necessary to initiate mound growth, which subsequently continued independently.

The alternative “external control” theory trying to explain why these mounds exist in provinces is based on the deep water current hydrodynamics in areas that are favourable, especially to *Lophelia* growth. These corals prefer stronger currents and growth on exposed flanks and summits (Wheeler *et al.*, 2007). This location in conjunction with current dynamics on continental margins causing internal waves and tides might provide ideal conditions for filter feeders such as *Lophelia* (White, 2007). The growth and decay of these corals then leads to a further increase in mound size.

**Table 3.1: Total organic carbon content of selected drilling sites worldwide.** Note that the Challenger Mound harbours on average the lowest percentage of organic matter of listed sites.

Location	Site	Water Depth [metres]	Sediment Depth [mbsf]	Total Organic Carbon [wt %]		
				Range	Mean	Reference
<b>Coastal and Shelf</b>						
Neuharlingersielser Nacken (Wadden Sea, Germany)		intertidal	0 – 5.5	0.1 - >1.5	~ 0.9	Freese <i>et al.</i> , 2008a
<b>Continental Slope and Margin</b>						
ODP 128 Japan Sea	789A	900	0.4 - 141.8	0.7 - 5.44	2.4	Stein and Stax, 1992
ODP 146 Cascadia Margin	889/890	1320	0 - 472	-	0.64	Cragg <i>et al.</i> , 1996
ODP 160 Sapropel Layers	967 S7*	~2000	9.5-9.7	0.8 - 7.1	3.8	Emeis <i>et al.</i> , 1998
IODP 201 Peru Margin	1229A	150	1.4 - 156.8	0.85 - 6.09	3.2	Meister <i>et al.</i> , 2005
IODP 301 Juan de Fuca Ridge	U1301C	2650	1.5-258.8	0.05 - 0.91	0.33	Fisher <i>et al.</i> , 2005
IODP 308 Gulf of Mexico	1319A	1400	1.5 – 294.3	0.03 - 1.99	0.57	Gilhooly <i>et al.</i> , 2008
<b>IODP 307 Porcupine Basin Carbonate Mound</b>	U1317A	781 - 815	1.4 - 121.9	0.01 - 0.41 {20.8 - 70.8}	0.18 {52.5}	Leonide <i>et al.</i> , 2009 Mangelsdorf <i>et al.</i> , 2009
<b>IODP 307 Porcupine Basin Reference Site</b>	U1318A/B U1318 A/D	423	3.6 – 237.7 1.7 – 239.7	0.17 – 1.15 {4.5 - 71.7}	0.5 {25.2}	Mangelsdorf <i>et al.</i> , 2009
<b>Abyssal Plain</b>						
IODP 201 South East Pacific	1230A	5100	1.4 - 235.7	0 - 1.82	0.57	Meister <i>et al.</i> , 2005
South Pacific Gyre	SPG-7	3738	0 - 1.1‡	-	0.29	D'Hondt <i>et al.</i> , 2009
South Pacific Gyre	SPG-10	5283	0 – 21‡	-	0.56	D'Hondt <i>et al.</i> , 2009

Legend: \*ODP Leg 160 Site 967 is located on a small ridge trending west-southwest to east-northeast at the base of the northern slope of the Eratosthenes Seamount (Emeis *et al.*, 1996) and was thus collated under “Continental Slope and Margin”, data for Sapropel S7 is given, which is estimated to be 195,000 years old (Süß *et al.*, 2008 and references therein); ‡ Given is the total sediment thickness for Pacific Gyre sites. Here TOC was only determined for top 5 cm of sediment column.



In the preliminary scientific assessment, Ferdelman *et al.* (2006) stated that the theory of mound formation by microbial produced carbonate precipitation has been disproved but that the role of prokaryotes in carbonate dissolution and secondary cementation reactions may still be important. The chosen representative of these mounds, the Challenger Mound in the Porcupine Seabight, was sampled during IODP Leg 307 (cores U1316-U1318). Compared with other marine subsurface environments these carbonate mounds exhibit only very low organic matter concentrations (Table 3.1; Fig. 3.1). Nonetheless, active microbial communities were found within and around this mound proving the resilience of prokaryotes towards substrate limitation (Webster *et al.* 2009).

The aim of this part of the project was to cultivate novel microorganisms from one of the most substrate-limited marine environments known to date. Subsequently, the isolates were phylogenetically and phenotypically characterised (this Chapter) and selected strains were subjected to starvation-survival experiments (Chapter IV).

## 1.1 Results from IODP Leg 307

Results of the biogeochemical and microbiological investigation of the Challenger carbonate mound were published by Webster *et al.* (2009). A comprehensive summary of the results can be found in the Appendix while the main findings are very briefly summarised here.

Sulphate reduction in the upper sediment layers was much more pronounced at the Reference site compared with the two mound sites. Below 14 mbsf, however, sulphate concentrations increased at the Reference site while they decreased further at the two mound sites until near depletion of sulphate. No methane was detected at the Reference site and it was only present at the mound sites where sulphate concentrations were low. Total cell counts at all sites largely agreed with the global estimate (Parkes *et al.*, 2000) and no significant changes of cell counts occurred in the transition between mound and underlying sediments. The molecular investigation revealed a diverse bacterial population within the Challenger Mound, its underlying sediments as well as at the Reference site. Typical subsurface groups, such as *Gammaproteobacteria*, which often dominate clone libraries (Fry *et al.*, 2008), were

identified alongside less frequently detected sequences (e.g. *Actinobacteria*). The detection of cells using CARD-FISH, cultivation (culturable cell numbers up to  $10^4$  cells  $g^{-1}$  sediment) as well as the measured metabolic activities indicated a viable and active microbial community within the mound and underlying sediments.

## 2. Results of isolation of prokaryotes from Leg 307 sediment samples

Sediment samples obtained during IODP Leg 307 were used as inoculum for direct enrichments and for the estimation of culturable cells using MPN dilution series (see A3.2c). Despite a relatively large number of MPN subcultures (41) and direct inoculations (50) only 19 isolates were obtained (Table 3.2). This seems surprising considering the relatively high MPN counts reported by Webster *et al.* (2009) ranging between  $10^2$ - $10^4$  culturable cells per  $cm^3$  sediment for each drilling site. However, cell densities both in MPN wells and direct enrichments were generally low and this may indicate unspecific growth that could not be sustained. In addition, a number of these cultures failed to grow after transfer into fresh medium and were finally lost.

Generally, none of these cultures developed cell numbers high enough to cause turbidity and could only be monitored microscopically. Because growth was generally poor in liquid cultures and did not improve during subcultivation, samples were transferred onto agar plates for ease of handling (October 2006). In some cases a different medium was used for this (e.g. when no metal reduction occurred the respective medium was replaced, Table 3.2).

Additionally, if different colony phenotypes (morphology and/or colouration) were present on one agar plate they were separated by sub-cultivation on separate agar plates resulting in different isolates from the same original samples. In a few cases several attempts to subculture colonies on fresh agar plates failed, what also contributed to the relative low number of isolates compared to the initial high number of enrichments and positive MPN cultures. An additional problem was the time needed for cells to form visible colonies on agar plates, which in some cases took up to eight weeks. In an attempt to accelerate growth, anaerobically grown colonies were tested for aerobic growth and incubated at higher temperatures (25°C instead of 10°C). These measures proved successful for most isolates.

**Table 3.2: Origin of IODP 307 isolates.** Isolates are listed according to their isolation from which core and corresponding depth. Strains marked (M) were isolated from the Mound. Numbers given in parentheses indicate the MPN dilution step from which strains were subsampled. Initial and changes in cultivation media are shown. Abbreviations: MM: Monomer-medium; MRB: Metal-reducer medium; SRB: Sulphate-reducer medium; YPGL: Yeast extract, peptone, D-glucose, lactate medium.

Phylogenetic affiliation	Strain abbreviation	Core	Enriched from	Depth	Initial enrichment media	Isolation media
<i>Desulfovibrio</i> sp.	F16I	1316	direct	54.1 mamb	SRB; anaerobic	SRB; anaerobic
<i>Vibrio</i> sp.	F16II	1316	direct	-30.3 mamb	Basal+Propionate/SO <sub>4</sub> <sup>2-</sup> ; anaerobic	MM; then YPGL
<i>Shewanella</i> sp.	F16III	1316	MPN (10 <sup>-2</sup> )	-47.9 mamb	MM+NO <sub>3</sub> <sup>-</sup> ; anaerobic	YPGL; aerobic
<i>Shewanella</i> sp.	F16IV	1316	MPN (10 <sup>-2</sup> )	-47.9 mamb	MRB; anaerobic	YPGL; aerobic
<i>Shewanella</i> sp.	F16V	1316	MPN (10 <sup>-4</sup> )	-47.9 mamb	SRB; anaerobic	MM; then YPGL; aerobic
<i>Vibrio</i> sp.	F17I (M)	1317	direct	71.9 mamb	MM; microaerophilic	YPGL; aerobic
<i>Raoultella</i> sp.	F17II (M)	1317	direct	52.9 mamb	Acetogen Medium, anaerobic	MM; then YPGL; aerobic
<i>Shewanella</i> sp.	F17III (M)	1317	MPN (10 <sup>-1</sup> )	33.9 mamb	MRB; anaerobic	MM; then YPGL; aerobic
<i>Vibrio</i> sp.	F17IV (M)	1317	MPN (10 <sup>-1</sup> )	16.9 mamb	MRB; anaerobic	YPGL; aerobic
<i>Shewanella</i> sp.	F17V (M)	1317	MPN (10 <sup>-1</sup> )	16.9 mamb	MRB; anaerobic	YPGL; aerobic
<i>Shewanella</i> sp.	F17VI (M)	1317	MPN (10 <sup>-5</sup> )	16.9 mamb	MM+NO <sub>3</sub> <sup>-</sup> ; anaerobic	YPGL; aerobic
<i>Shewanella</i> sp.	F17VII	1317	MPN (10 <sup>-1</sup> )	-0.9 mamb	MM+NO <sub>3</sub> <sup>-</sup> ; anaerobic	YPGL; aerobic
<i>Raoultella</i> sp.	F17VIII	1317	direct	-74.1 mamb	MM; microaerophilic	YPGL; aerobic
<i>Arcobacter</i> sp.	F17IX	1317	direct	-108.0 mamb	Methanogen Medium + SO <sub>4</sub> <sup>2-</sup>	MM; then YPGL; aerobic
<i>Photobacterium</i> sp.	F18I	1318	direct	4.3 mbsf	MM; anaerobic	YPGL; aerobic
<i>Pseudomonas</i> sp.	F18II	1318	direct	4.3 mbsf	MM; microaerophilic	YPGL; aerobic
<i>Shewanella</i> sp.	F18III	1318	direct	4.3 mbsf	MM; microaerophilic	YPGL; aerobic
<i>Ornithinimicrobium</i> sp.	F18IV	1318	direct	22.9 mbsf	MM; aerobic	YPGL; aerobic
<i>Pseudomonas</i> sp.	F18V	1318	direct	79.9 mbsf	MM; aerobic	YPGL; aerobic

Despite the difficulties encountered, isolates were obtained from all three drilling sites and various depths (Table 3.2). Nine of the 19 isolates originated from the Mound site, six of these isolates came from within the mound itself and the remaining three strains from the sediment beneath the mound base. A further five isolates derived from the Flank site. No isolates were obtained from the mound itself here but from covering or underlying sediments. Only five isolates were obtained from the Reference site, which represented hemipelagic sediment. Here three strains were obtained from 4 mbsf and two isolates from deeper sediment layers. These five strains originated from direct enrichments of the sediment sample as no growth was detected in any of the MPN dilution series inoculated with sediment from this site (Webster *et al.*, 2009).

## 2.1 Phylogenetic affiliation of isolates

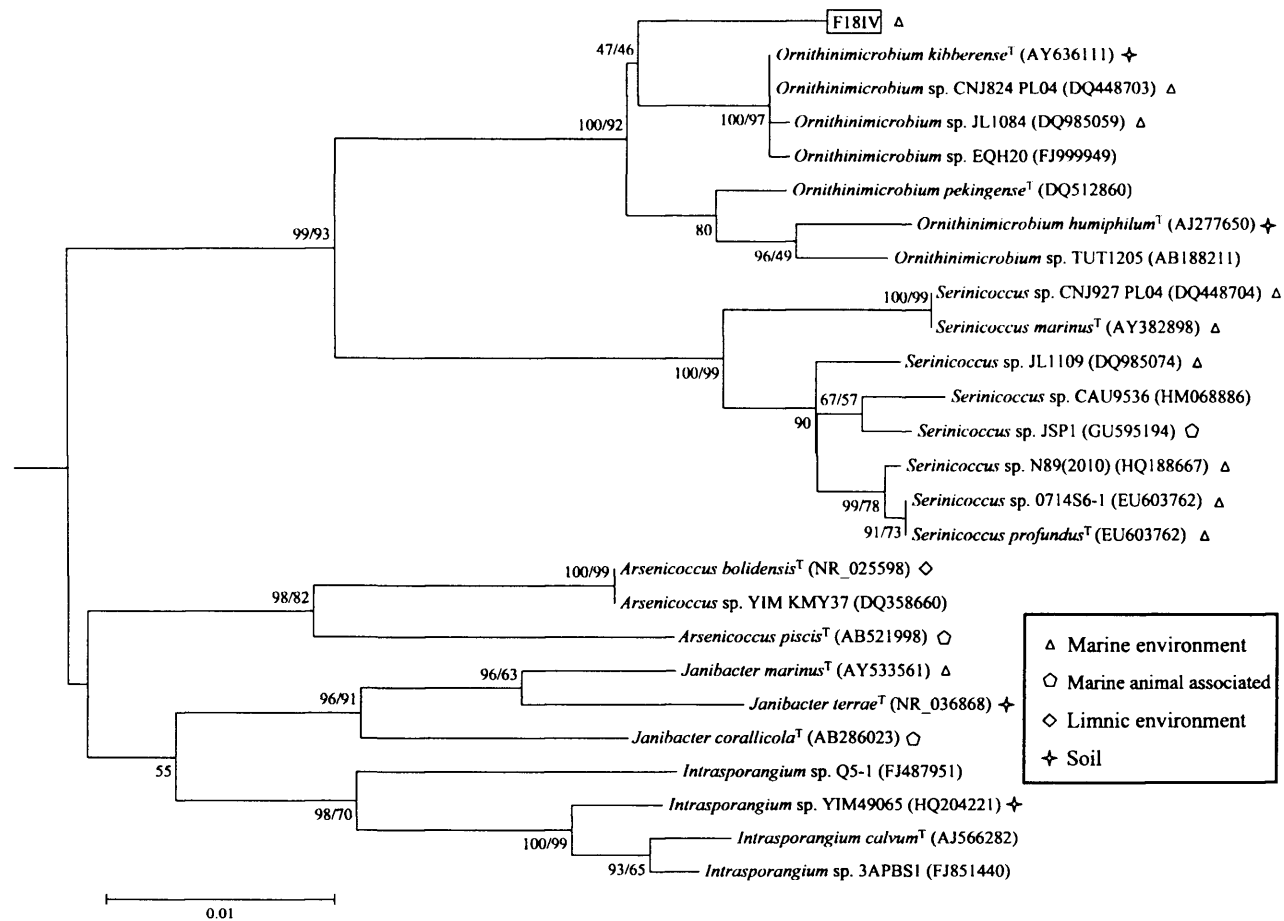
The obtained culture collection from IODP Leg 307 sediments consists almost exclusively of representatives of the phylum *Proteobacteria* amongst which the *Gammaproteobacteria* make up 16 of the total 19 isolates. *Deltaproteobacteria* and *Epsilonproteobacteria* were also isolated with one representative each. Furthermore one isolate belongs to the phylum *Actinobacteria* and probably constitutes a new species within the genus *Ornithinimicrobium* sp. (Table 3.3).

### 2.1.1 Isolates belonging to the phylum *Actinobacteria*

One of the isolates belongs to the genus *Ornithinimicrobium*, which is part of the phylum *Actinobacteria*. At the time of writing the genus comprised only three described species (J. P. Euzéby [<http://www.bacterio.cict.fr/>] accessed Jan. 2011). The type species of the genus is *O. humiphilum* (Groth *et al.*, 2001) and was isolated from garden soil. The genus name was given due to the presence of L-ornithine in the cell wall peptidoglycan. The closest cultivated relative to strain F18IV is, however, *O. kibberense* (97.8% sequence similarity) and was described by Mayilraj *et al.* (2006). It originates from a cold desert soil in the Indian Himalayas. The remaining species of this genus is *O. pekingense* (Liu *et al.*, 2008) and was isolated from activated sludge.

**Table 3.3: Phylogenetic affiliation of Leg 307 isolates.** Isolates are compared to their closest phylogenetic cultivated relatives according to NCBI Blast. Also provided are the affiliate's origin and its accession number.

Strain	Sequence Length [bases]	Closest Match in BLASTN; Origin; (Accession Number)	%	Closest Cultivated Affiliation; Origin; (Accession Number) %	
<i>Arcobacter</i> sp.	F17IX	1001	Unc. Bact. Clone: crustal fluids; (AY704399)	98	<i>Arcobacter nitrofigilis</i> strain F2173; Mussels, Spain; (EU106661) 98
<i>Desulfovibrio</i> sp.	F16I	822	Unc. Bact. Clone: marine sediment, lugworm; (FJ716992)	99	<i>D. oceani</i> subsp. <i>galataeae</i> ; oxygen minimum waters off Peru; (FJ655908) 99
<i>Ornithinimicrobium</i> sp.	F18IV	974	<i>Ornithinimicrobium kibberense</i> , soil from Lahaul-Spiti valley in the Indian Himalayas (AY636111)	97	See closest match
<i>Photobacterium</i> sp.	F18I	958	<i>Photobacterium profundum</i> strain 3TCK; sediment sample within San Diego Bay (DQ027054)	99	See closest match
<i>Pseudomonas</i> sp.	F18II	841	Unc. <i>Pseudomonas</i> sp. clone; Pangong Lake; (HM072375)	99	<i>Pseudomonas</i> sp. BSw21399B; Kongsfjorden seawater, (FJ966156) 99
<i>Pseudomonas</i> sp.	F18V	873	<i>Pseudomonas</i> sp. BSw21399B; Kongsfjorden seawater, (FJ966156)	99	See closest match
<i>Raoultella</i> sp.	F17II	692	<i>Bacterium</i> sp.; groundwater in borehole (450m depth); FJ037707	88	See closest match
<i>Raoultella</i> sp.	F17VIII	895	<i>Bacterium</i> sp.; groundwater in borehole (450m depth); FJ037707	99	See closest match
<i>Shewanella</i> sp.	F16III	839	<i>Shewanella</i> sp. 204Z-25; water sample; GU584178	99	See closest match
<i>Shewanella</i> sp.	F16IV	907	<i>Shewanella</i> sp. 204Z-25; water sample; GU584178	99	See closest match
<i>Shewanella</i> sp.	F16V	926	<i>Shewanella</i> sp. 204Z-25; water sample; GU584178	100	See closest match
<i>Shewanella</i> sp.	F17III	896	<i>Shewanella</i> sp. LaSQ3; subantarctic ecosystem of Beagle Channel; EU075116	99	See closest match
<i>Shewanella</i> sp.	F17V	514	<i>Shewanella</i> sp. LaSQ3; subantarctic ecosystem of Beagle Channel; EU075116	89	See closest match
<i>Shewanella</i> sp.	F17VI	921	<i>Shewanella</i> sp. 204Z-25; water sample; GU584178	99	See closest match
<i>Shewanella</i> sp.	F17VII	955	<i>Shewanella</i> sp. 204Z-25; water sample; GU584178	99	See closest match
<i>Shewanella</i> sp.	F18III	809	<i>Shewanella livingstonensis</i> strain 171; oyster; AY870677	98	See closest match
<i>Vibrio</i> sp.	F16II	924	<i>Vibrio tasmaniensis</i> strain 373.11; associated with oysters and clams; AY620966	99	See closest match
<i>Vibrio</i> sp.	F17I	847	<i>Vibrio splendidus</i> LGP32; FM954972	98	See closest match
<i>Vibrio</i> sp.	F17IV	844	<i>Vibrio</i> sp. 57S-9; Shenzen coastal surface waters; GU371698	99	See closest match



**Fig. 3.2: Phylogenetic tree for *Ornithinimicrobium* sp. F181V.** The tree was constructed using the Nearest-Neighbour Joining method (Jukes-Cantor) in the phylogenetic software Mega4 (Tamura *et al.*, 2007). The tree is based on 1257 bases of aligned 16S rRNA gene sequences. Bootstrap support values over 50% (after 1000 replicates) are shown at nodes; first value derived by Jukes-Cantor algorithm, second value by Maximum Parsimony algorithm. The NCBI Accession number for each strain is given in brackets. The scale bar indicates substitutions per site. The 16S rDNA sequence of *Arthrobacter globiformis*<sup>T</sup> (X8073) was used as outgroup.

The presented phylogenetic tree (Fig. 3.2) is based on the Nearest-Neighbour Joining method (Jukes-Cantor) using aligned sequences, 1257 bases in length. It shows that strain F18IV forms a distinct operational taxonomic unit (OTU) within the genus. Therefore, additional phylogenetic computational algorithms were used to test the reliability and reproducibility of the tree. The results of the Maximum Parsimony and Minimum Evolution (LogDet) methods (see Appendix) show a very similar clustering of the phylogenetic groups, thus confirming the results of the Nearest-Neighbour Joining method.

Due to its position in the phylogenetic tree, strain F18IV most likely represents a new species within the genus *Ornithinimicrobium* and would be the first representative of the genus isolated from marine sediments. It was obtained from 22.9 mbsf at Reference site. Other affiliates such as the closely related *Serinicoccus* genus have recently been isolated from marine habitats. They originated from the water column (*S. marinus*; Yi *et al.*, 2004) and sediments (*S. profundus*; Xiao *et al.*, 2011).

### 2.1.2 Isolates belonging to the phylum *Proteobacteria*

*Proteobacteria* constitute the most abundant cultivated phylum isolated from Leg 307 samples and were obtained from all sites including representatives of the class *Gammaproteobacteria*, which dominated the culture collection.

#### a) Isolates belonging to the class *Gammaproteobacteria*

*Gammaproteobacteria* have been frequently isolated from numerous marine habitats and possibly constitute the most abundant seafloor bacterial group (Fry *et al.*, 2008). Therefore, it is not surprising that they also dominate the obtained culture collection with 16 representatives out of 19 total isolates. *Gammaproteobacteria* were found at all three sites but they are the only group in our culture collection obtained from the carbonate mound itself.

Most isolates were identified as members of the genus *Shewanella* (Fig. 3.3) with eight strains representing two distinct operational taxonomic units. Other

isolated *Gammaproteobacteria* belong to the genera *Vibrio* (3 strains, Fig 3.4), *Raoultella* (2 strains, Fig 3.5), *Pseudomonas* (2 strains, Fig 3.6), and *Photobacterium* (1 strain, Fig 3.7).

#### Isolates affiliated with the genus *Shewanella*

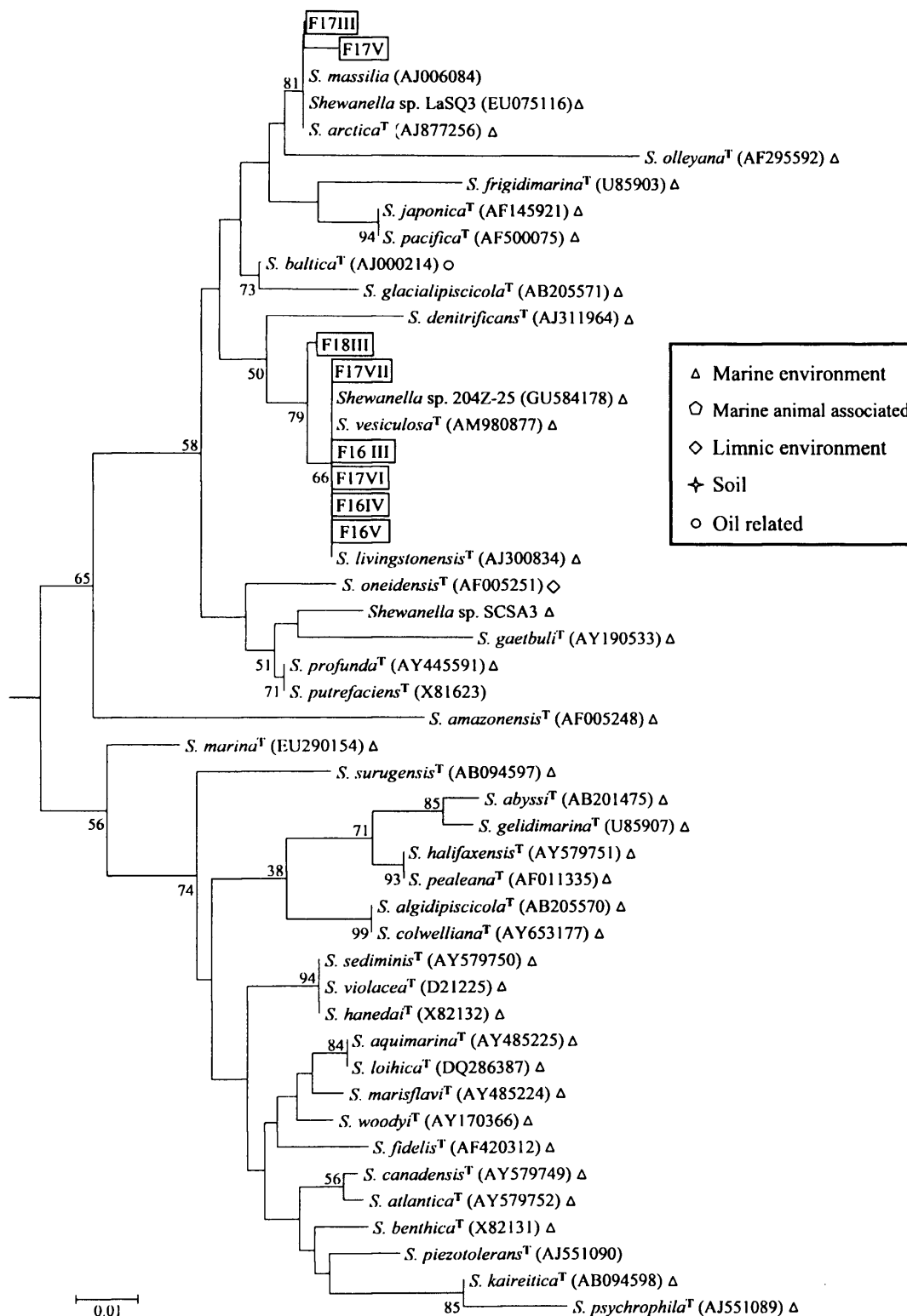
The genus *Shewanella* comprises more than 50 species (J. P. Euzéby [http://www.bacterio.cict.fr/] accessed Jan. 2011) inhabiting a variety of environments (from spoiled foods to deep-sea sediments, e.g. *S. profunda*, Toffin *et al.*, 2004a) most likely enabled by the wide variety of possible electron acceptors they can use including but not limited to oxygen, nitrate, thiosulphate, and manganese oxides, and their ability to ferment (Nealson and Scott, 2006).

Six of the *Shewanella* sp. strains, deriving from all three drilling sites and from within or below the carbonate mound, cluster together, close to *S. vesiculosa*, in the phylogenetic tree (Fig. 3.3). *S. vesiculosa* was isolated from marine coastal Antarctic sediments and was described as psychrotolerant by Bozal *et al.* (2009). In contrast, two isolates (F17III and F17V) obtained from within the carbonate mound are more closely affiliated to *S. arctica*, which was isolated from Spitzbergen (Quora *et al.*, unpubl.). The sequence similarities of the isolated strains to one another are based on the full sequence length obtained (Table 3.4). The constructed tree, however, is based on aligned sequences of 517 bases in lengths (Fig. 3.3).

**Table 3.4: 16S rDNA sequence similarities of isolated *Shewanella* strains.** Note that the sequence for strain F17V is of poor quality.

	F16III	F16IV	F16V	F17III	F17V	F17VI	F17VII	F18III
<b>F16III</b>	100							
<b>F16IV</b>	99.5	100						
<b>F16V</b>	99.8	99.8	100					
<b>F17III</b>	95.7	96.0	96.2	100				
<b>F17V</b>	87.7	87.9	87.9	89.1	100			
<b>F17VI</b>	99.6	99.7	99.9	96.3	87.1	100		
<b>F17VII</b>	99.8	99.8	99.9	96.2	87.9	99.8	100	
<b>F18III</b>	97.0	97.4	97.3	95.8	86.8	97.3	97.3	100



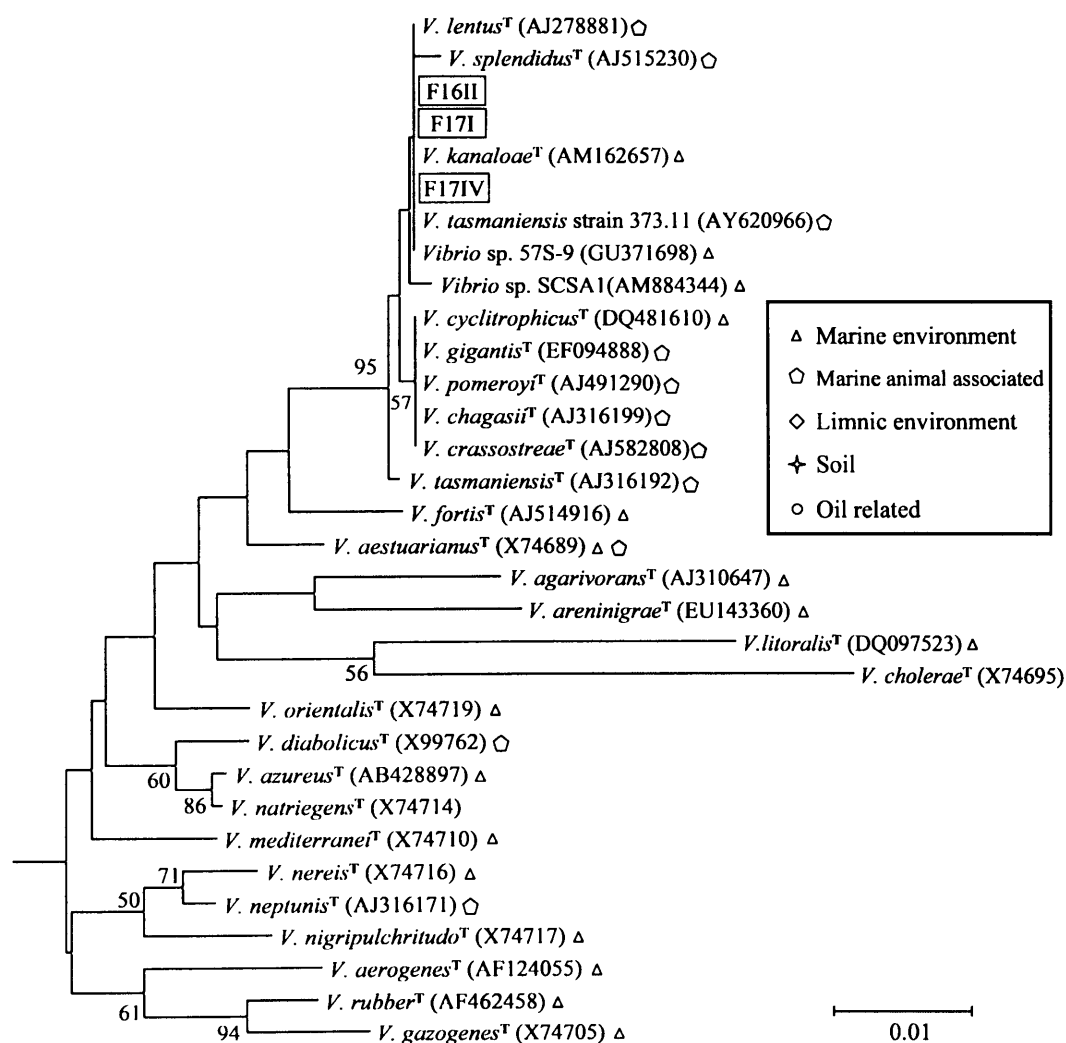


**Fig. 3.3: Phylogenetic tree for isolated *Shewanella* strains.** The tree was constructed using the Nearest-Neighbour Joining method (Jukes-Cantor) in the phylogenetic software Mega4 (Tamura *et al.*, 2007). The tree is based on 517 bases of aligned 16S rRNA gene sequences. Bootstrap support values over 50% (after 1000 replicates) are shown at nodes. The NCBI Accession number for each strain is given in brackets. The scale bar indicates substitutions per site. *Pseudoalteromonas haloplanktis*<sup>T</sup> (AF214730) was used as outgroup.

Isolates affiliated with the genus *Vibrio*

Members of the genus *Vibrio* represent the second largest phylogenetic group in the obtained culture collection. The type species of this genus, *Vibrio cholerae*, was described in 1854 (Pacini, 1854). Today the genus comprises 101 species (J. P. Euzéby [http://www.bacterio.cict.fr/] accessed April, 2011). Representatives of this genus are common in marine environments and some can cause severe illness to humans (e.g. *V. cholera*) and animals (Farmer and Hickman-Brenner, 2006).

The three *Vibrio* isolates from IODP Leg 307 are quite closely related to each other ( $\geq 96.9\%$ ) hence they fall in the same OTU in the phylogenetic tree (Fig 3.4).



**Fig. 3.4: Phylogenetic tree for *Vibrio* sp. strains.** The tree was constructed using the Nearest-Neighbour Joining method (Jukes-Cantor) in the phylogenetic software Mega4 (Tamura *et al.*, 2007). The tree is based on 593 bases of aligned 16S rRNA gene sequences. Bootstrap support values over 50% (after 1000 replicates) are shown at nodes. The NCBI Accession number for each strain is given in brackets. The scale bar indicates substitutions per site. *Salinivibrio costicola*<sup>T</sup> (X95527) was used as outgroup.

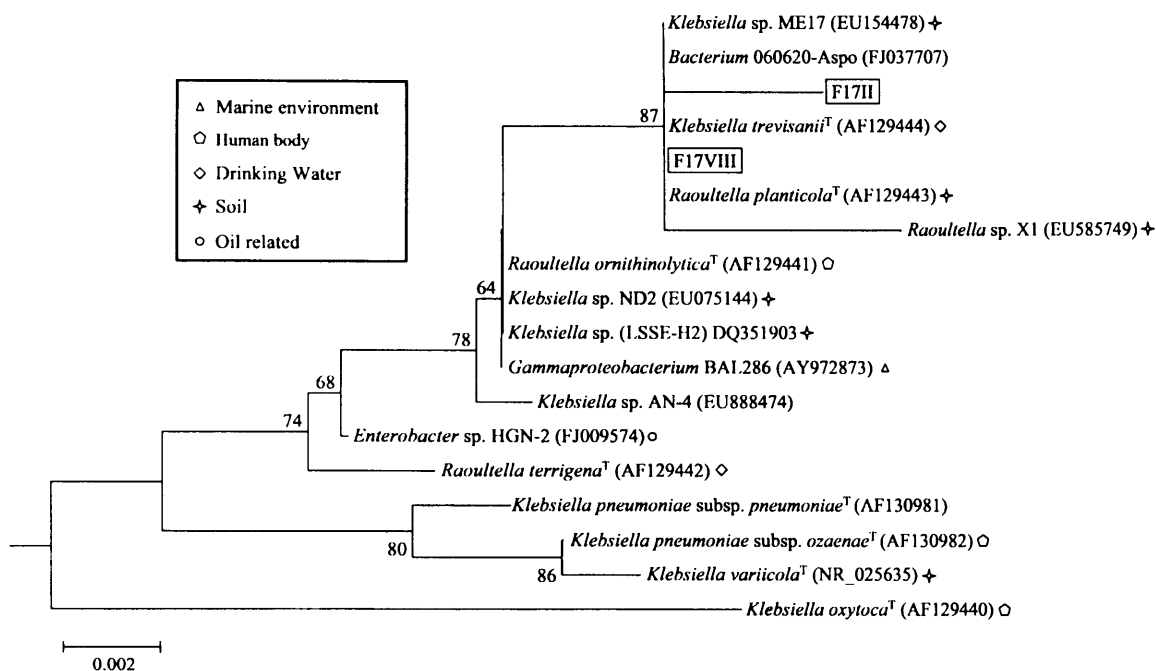
*V. splendidus*, *V. kanaloae*, *V. lentus*, and *V. tasmanensis* are closely related to these isolates and thus cluster together. These strains are of marine origin but not all are free-living organisms. Some of them are associated with marine animals such as *V. splendidus*, which can infect the pacific oyster (*Crassostrea gigas*). Two of the *Vibrio* strains (F17I and F17IV) were isolated from the carbonate mound itself, whereas strain F16II originates from sediments beneath the mound at the Flank site (Table 3.2).

#### Isolates affiliated with the genus *Raoultella*

The genus *Raoultella* was established recently by Drancourt *et al.* (2001) who compared sequences of the 16S rDNA and the beta subunit of RNA polymerase (*rpoB*) of nine *Klebsiella* strains and thus proposed the separation of the genera *Raoultella* and *Klebsiella*. This, however, remains debated in the literature (Janda, 2006; Brisse *et al.*, 2006). Due to this recent separation of the two genera and their close relationship the nomenclature in the literature is often ambiguous. Note in the presented tree, for example, that the strains *Klebsiella trevisanii* (AF129444) and *Raoultella planticola* (AF129443) share the same 16S rDNA sequence (Fig 3.5).

According to the German microbial culture collection (DSMZ) only three species of *Raoultella* are described. *R. planticola* is the closest affiliate to strain F17VIII with a 99.3% 16S rDNA sequence similarity and originates from a radish root. Other strains of this species were isolated from drinking water or air showing quite a diversity of habitats amongst the isolates, which is now extended to marine sediments by these novel strains. Representatives of *R. ornithinolytica* and *R. terrigena* were isolated from urine and also drinking water. Furthermore many strains of described *Klebsiella* species and the closely related *Enterobacter* species are of clinical relevance (Janda, 2006).

The two isolates appear only to be distantly related to each other (88.1%). This, however, is due to the low quality sequence obtained from one of the strains (F17II). Several efforts of retrieving a 16S rDNA sequence of better quality failed. Nonetheless, the two strains cluster together in the same OTU (Fig. 3.5). Their nearest affiliated sequence is an isolate obtained from a Swedish terrestrial borehole (450 m depth). Leg 307 strains originate from different depths at the Mound site; F17II from within the Mound, F17VIII from the underlying sediment (Table 3.2).

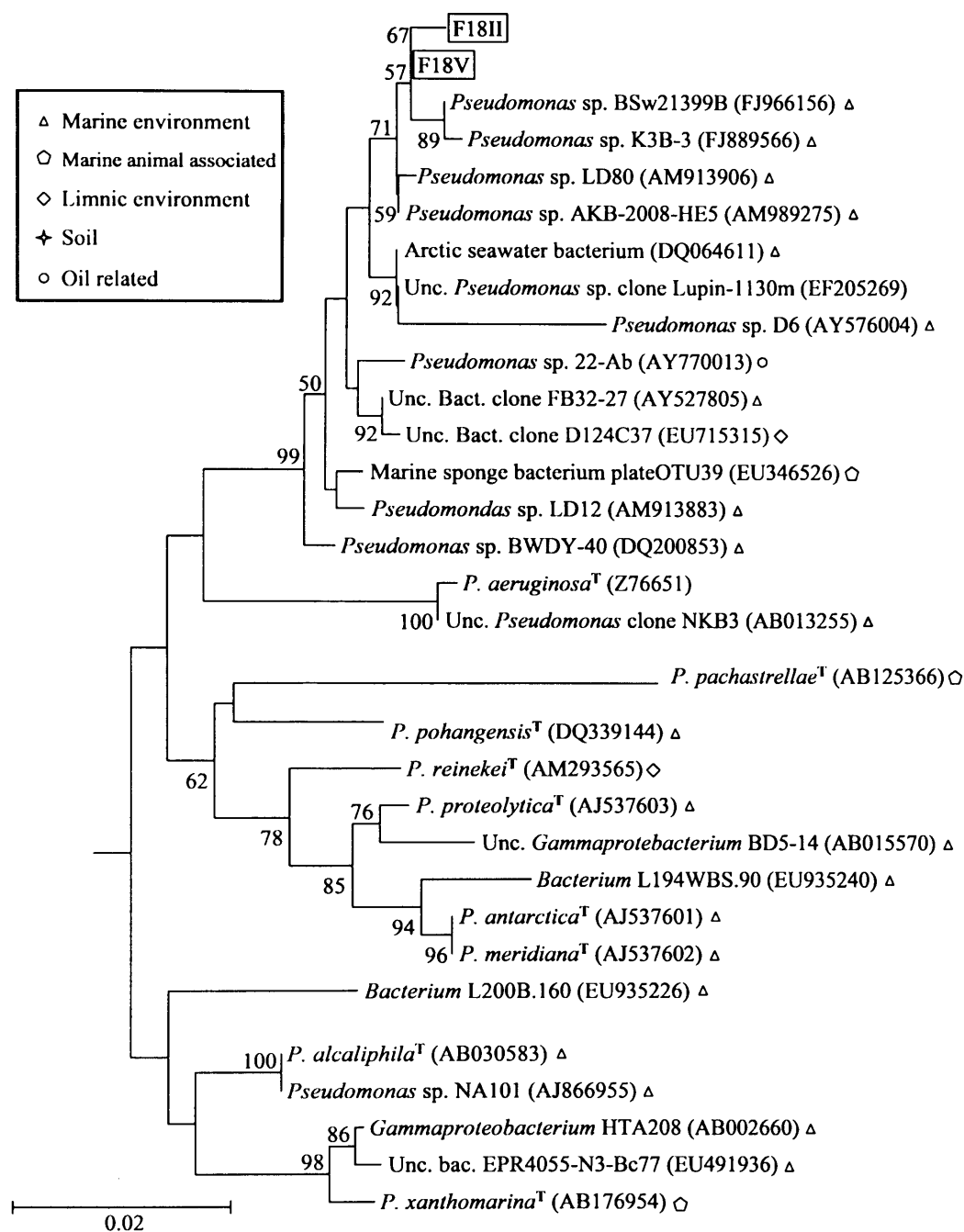


**Fig. 3.5: Phylogenetic tree for *Raoultella* sp. strains.** The tree was constructed using the Nearest-Neighbour Joining method (Jukes-Cantor) in the phylogenetic software Mega4 (Tamura *et al.*, 2007). The tree is based on 690 bases of aligned 16S rRNA gene sequences. Bootstrap support values over 50% (after 1000 replicates) are shown at nodes. The NCBI Accession number for each strain is given in brackets. The scale bar indicates substitutions per site. *Kluyvera chochleae*<sup>T</sup> was used as outgroup.

### Isolates affiliated with the genus *Pseudomonas*

The genus *Pseudomonas* was established by Migula in 1894 and contains a vast number of species including many clinical and environmental strains. At the time of writing it comprised 191 species and 18 subspecies (J. P. Euzéby [<http://www.bacterio.cict.fr/>] accessed Jan. 2011) but there is some debate about the legitimacy of some of these species within the genus (Anzai *et al.*, 2000). The type species is *P. aeruginosa* (Schroeter, 1872). Generally, *Pseudomonas* species utilise a wide range of organic and inorganic compounds and inhabit many environments, especially soils but are not uncommon in marine and freshwater environments (Moore *et al.*, 2006) and even in deep-sea sediments (Fry *et al.*, 2008).

The two novel isolates originate from different depths at the Reference site and are closely related to each other (99.5%). The closest affiliate to strain F18II is a clone from a hypersaline lake (India). Its closest cultured relative is a seawater isolate (*Pseudomonas* sp. BSw21399B), which in turn is the closest match for strain F18V (Table 3.3). They all cluster closely together in the phylogenetic tree (Fig. 3.6).

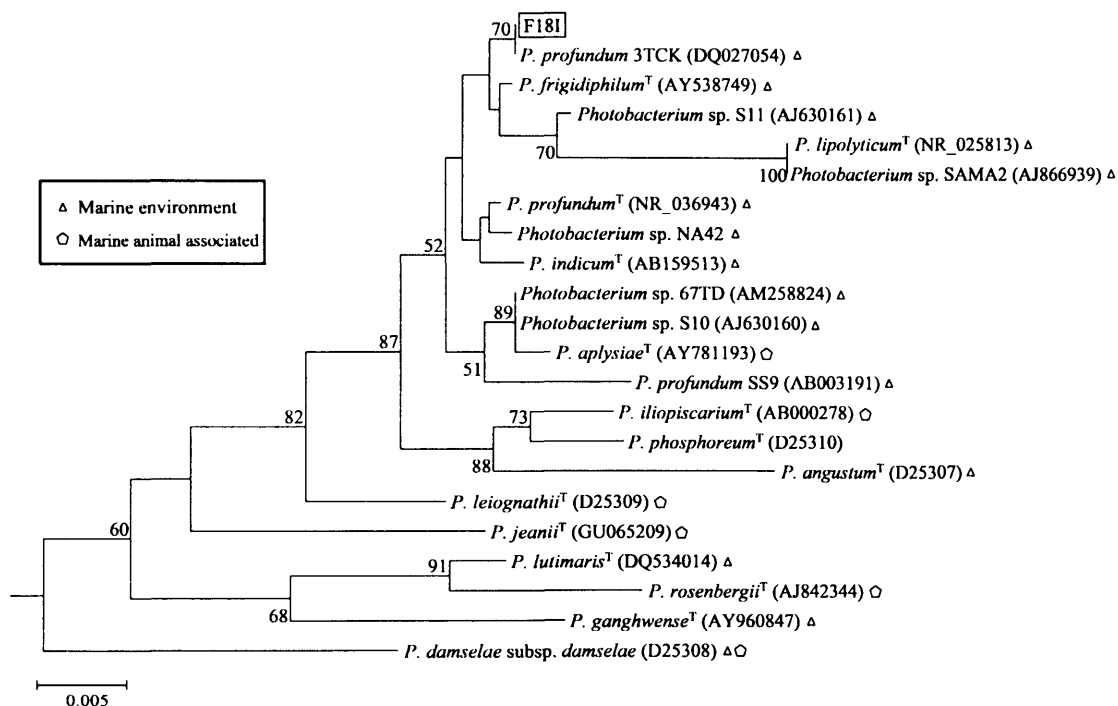


**Fig. 3.6: Phylogenetic tree for *Pseudomonas* sp. strains.** The tree was constructed using the Nearest-Neighbour Joining method (Jukes-Cantor) in the phylogenetic software Mega4 (Tamura *et al.*, 2007). The tree is based on 653 bases of aligned 16S rRNA gene sequences. Bootstrap support values over 50% (after 1000 replicates) are shown at nodes. The NCBI Accession number for each strain is given in brackets. The scale bar indicates substitutions per site. *Rhodanobacter lindanoelasticus*<sup>T</sup> (AF039167) was used as outgroup.

### Isolates affiliated with the genus *Photobacteria*

The remaining isolate of the *Gammaproteobacteria* belongs to the genus *Photobacterium*. This genus was established by Beijerinck (1889) and is named after the ability of some species to emit light (Hendrie *et al.*, 1970), both as a free-living

organism and as a symbiont in the light organs of marine animals (Farmer and Hickman-Brenner, 2006). The type strain of the genus is *P. phosphoreum*, which was first mentioned by Cohn in a letter in 1878. The genus contains 22 described species and two subspecies and is quite closely related to the genus *Vibrio*. According to Ruimy *et al.* (1994) these form a robust monophyletic unit within the family *Vibrionaceae*. It is therefore not surprising that there is some confusion about the taxonomy of some species and a certain degree of misclassification may still be present in the literature (Hendrie *et al.*, 1970; Urakawa *et al.*, 1999).



**Fig. 3.7: Phylogenetic tree for *Photobacterium* sp. F181.** The tree was constructed using the Nearest-Neighbour Joining method (Jukes-Cantor) in the phylogenetic software Mega4 (Tamura *et al.*, 2007). The tree is based on 544 bases of aligned 16S rRNA gene sequences. Bootstrap support values over 50% (after 1000 replicates) are shown at nodes. The NCBI Accession number for each strain is given in brackets. The scale bar indicates substitutions per site. *Salinivibrio costicola*<sup>T</sup> (X95527) was used as outgroup.

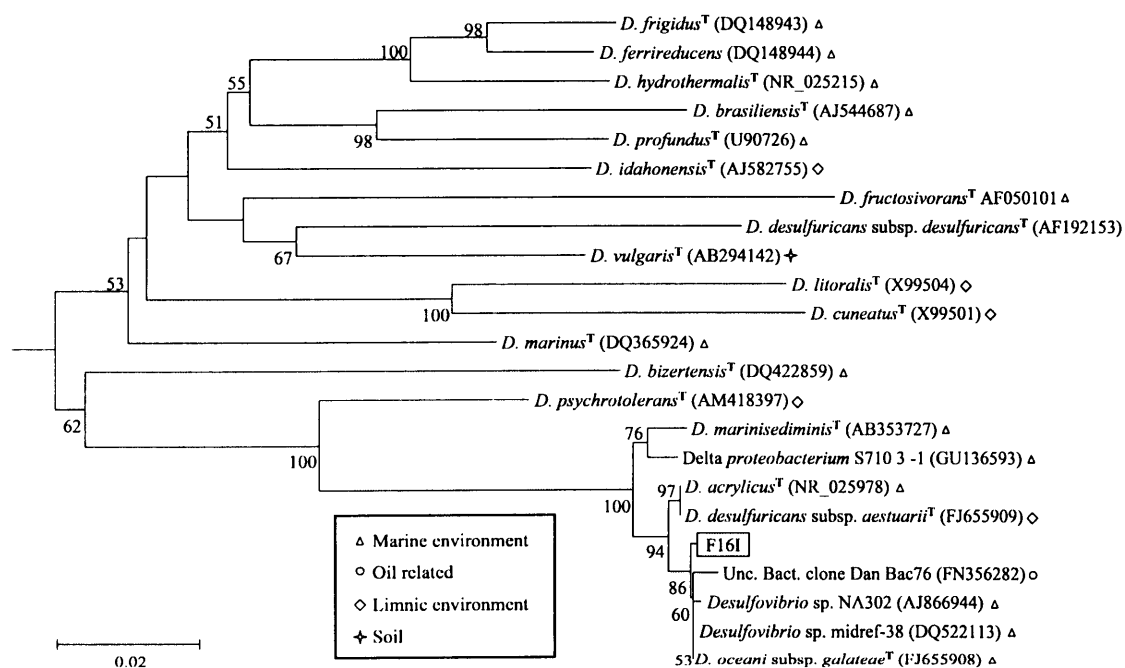
**Table 3.5: 16S rDNA sequence similarities of *Photobacterium* strains.**

		Leg 307	Mediterranean Cluster			<i>P. profundum</i>	Tidal Flat Strains		
		F181	I 67TD	I S10	II S11	SS9	3TCK	SAMA2	NA42
Leg 307	F181	100							
Mediterranean Cluster	I 67TD	97.4	100						
	I S10	97.2	97.8	100					
	II S11	98.2	97.5	96.4	100				
<i>P. profundum</i>	SS9	95.3	95.7	94.6	96.7	100			
	3TCK	99.6	96.8	96.0	97.6	96.9	100		
Tidal Flat Strains	SAMA2	97.6	97.3	96.8	98.3	97.3	97.6	100	
	NA42	98.0	96.6	96.2	96.7	97.2	98.0	95.8	100

*Photobacteria* are commonly found in the marine environment (pelagic, benthic, and associated with animals). Strain F18I was isolated from 4.25 mbsf at the Reference site and is closely related to *P. profundum* 3TCK (99.6%; Table 3.2; Fig 3.7) and other strains of the genus (Table 3.5). The type strain (SS9) of this species was isolated from deep sea sediments (Nogi *et al.*, 1998b).

#### b) Isolates belonging to the subclass *Deltaproteobacteria*

*Deltaproteobacteria* are commonly found in marine sediments but are less abundant in clone libraries than *Gammaproteobacteria* (Fry *et al.*, 2008). However, a large number of members of this subphylum are driving one of the main biogeochemical processes in marine sediments: sulphate reduction (Parkes and Sass, 2007). Although this process is of minor importance in deep-sea compared with coastal sediments, a number of isolates have been obtained from subsurface sediments, including strains affiliated with the genus *Desulfofrigus* (Süß *et al.*, 2004), *Desulfomicrobium* (Batzke *et al.*, 2007; Dias *et al.*, 2008) and *Desulfovibrio* (Bale *et al.*, 1997; Barnes *et al.*, 1998).



**Fig. 3.8: Phylogenetic tree for *Desulfovibrio* sp. F16I.** The tree was constructed using the Nearest-Neighbour Joining method (Jukes-Cantor) in the phylogenetic software Mega4 (Tamura *et al.*, 2007). The tree is based on 902 bases of aligned 16S rRNA gene sequences. Bootstrap support values over 50% (after 1000 replicates) are shown at nodes. The NCBI Accession number for each strain is given in brackets. The scale bar indicates substitutions per site. *Desulfohalobium retbaense*<sup>T</sup> (U48244) was used as outgroup.

Strain F16I belongs to the genus *Desulfovibrio*, which was established by Kluyver and van Niel (1936) and comprised 60 species and six subspecies at the time of writing (J. P. Euzéby [<http://www.bacterio.cict.fr/>] accessed Jan. 2011). The first pure culture, *Spirillum desulfuricans*, was described by Beijerinck (1895) and was later lost but is thought to be a member of the species *Desulfovibrio desulfuricans*.

Strain F16I was isolated from the sediment above the carbonate mound at the Flank site (50.8 mamb), which was within the sulphate reduction zone at this site (Fig. A3.1a). The closest relative of strain F16I is an uncultured bacterial clone from marine sediments (UK, 99%; Table 3.2). The closest described species with 99.2% sequence similarity is *D. oceani* subsp. *galatae* isolated from coastal waters off Peru (Finster and Kjeldsen, 2010). Furthermore, strain F16I is also closely related to *D. acrylicus* (98.9%), which was isolated from intertidal sediment of the Dutch Wadden Sea (van der Maarel, 1996; Fig. 3.8).

c) Isolates belonging to the subclass *Epsilonproteobacteria*

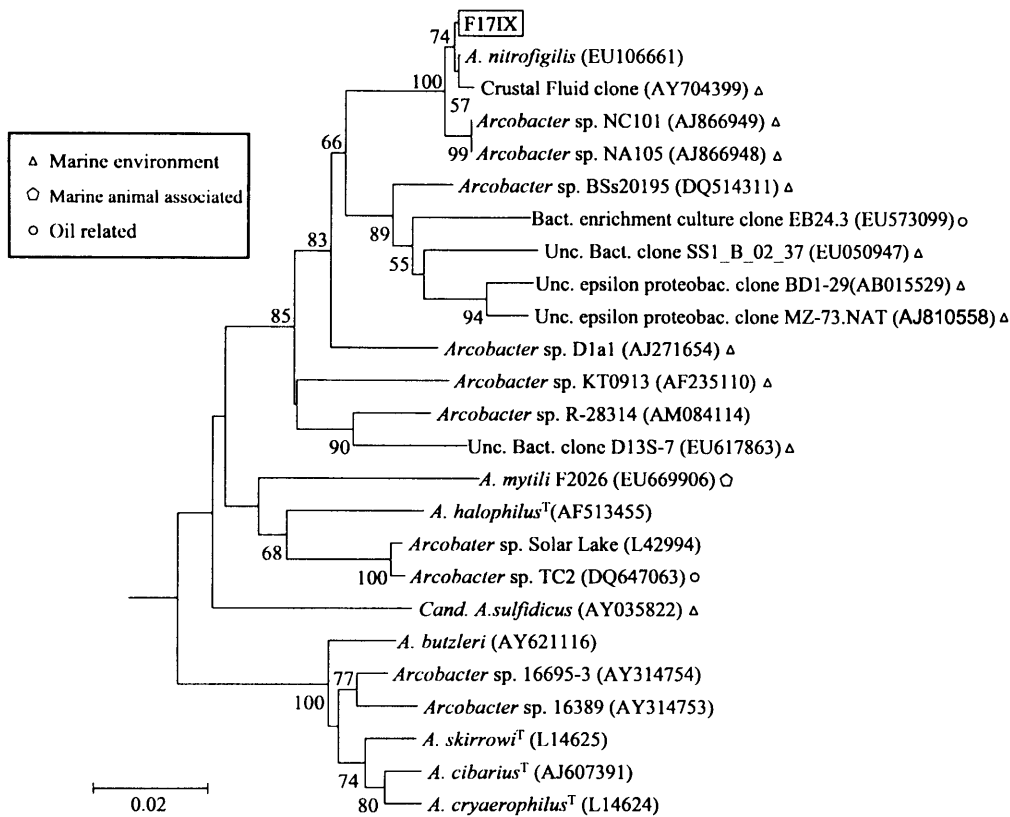
The remaining isolate belongs to genus *Arcobacter* within the subclass *Epsilonproteobacteria*. This genus was introduced by Vandamme *et al.* (1991) and comprised nine described species at the time of writing (J. P. Euzéby [<http://www.bacterio.cict.fr/>] accessed Jan. 2011). The type species is *A. nitrofigilis* (McClung *et al.*, 1983) but was initially misclassified as *Campylobacter nitrofigilis*. This nitrogen-fixing species originates from a plant root of a Canadian salt marsh.

A few representatives of the genus *Arcobacter* have been isolated from the marine environment (Thamdrup *et al.*, 2000; Wirsen *et al.*, 2002; Freese *et al.*, 2008b). Other species of this genus originate from animal or human tissue (Neill *et al.*, 1985; Vandamme *et al.*, 1992; Houf *et al.*, 2005 and 2009). The most infamous species, however, is probably *A. butzleri*, which was isolated from human and animal faeces (Kiehlbauch *et al.*, 1991) and poses a threat to public health (Skovgard, 2007).

Strain F17IX was isolated from the Mound site (-108 mamb; ca. 250 mbsf) and is closely related to a bacterial clone from crustal fluids (98%, Juan de Fuca Ridge; Huber *et al.* 2006). The closest cultivated relative is *A. nitrofigilis* strain F2173, which was isolated from mussels (Spain; Figuears *et al.*, 2008; Table 3.3). In the phylogenetic tree (Fig. 3.9) these sequences cluster closely together alongside



two isolates from intertidal sediments of the German Wadden Sea (strains NA105 and NC101; Freese *et al.*, 2008b).



**Fig. 3.9: Phylogenetic tree for *Arcobacter* sp. F17IX.** The tree was constructed using the Nearest-Neighbour Joining method (Jukes-Cantor) in the phylogenetic software Mega4 (Tamura *et al.*, 2007). The tree is based on 744 bases of aligned 16S rRNA gene sequences. Bootstrap support values over 50% (after 1000 replicates) are shown at nodes. The NCBI Accession number for each strain is given in brackets. The scale bar indicates substitutions per site. *Campylobacter fetus*<sup>T</sup> (DQ174127) was used as outgroup.

## 2.2 Phenotypical characterisation of isolates

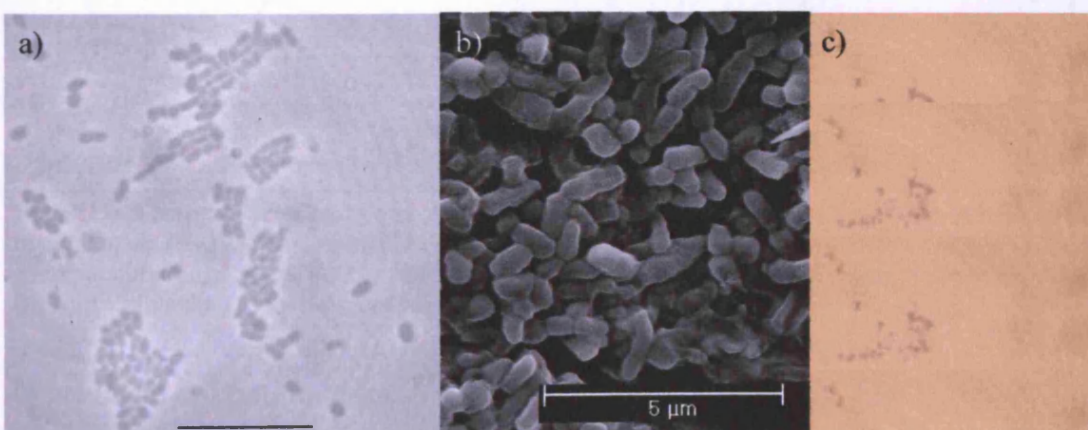
In addition to the phylogenetic analyses, all isolates were subjected to a general phenotypical characterisation. This is important in assessing the capacity of the organisms to be active in their marine deep-subsurface habitat. Furthermore, it allows comparisons of the isolates with each other, their close phylogenetic relatives, and with strains used in starvation experiments (Chapter IV). The tests included physiological experiments (determination of temperature, pH, and salinity ranges for growth, use of electron donors and acceptors, and for production of extracellular enzymes), morphological studies (shape, size, sporulation, motility, and presence of flagella, Gram staining) and additional tests such as antibiotic resistance.

### 2.2.1 Phenotypic properties of *Ornithinimicrobium* sp. F18IV

A summary of physiological properties of strain F18IV compared to its close phylogenetic relatives is given (Table A4.1) in addition to brief descriptions in the text (see below).

Strain F18IV was isolated from ~23 mbsf depth at site U1318. It forms circular, convex colonies with an entire margin. Colonies were white at early growth stages changing to yellow/orange after prolonged incubation. A similar colour change occurred for growth in liquid medium where strain F18IV formed homogenous cell suspensions. Cells were short rods, approximately twice as long as wide. The average length and width were  $1.3 \mu\text{m}$  ( $\pm 0.3 \mu\text{m}$ ) and  $0.7 \mu\text{m}$  ( $\pm 0.1 \mu\text{m}$ ), respectively ( $n=106$ ; Fig. 3.10a, b). Cells were Gram-positive. Motile cells or flagella were not observed.

*O. kibberense* (Mayilraj *et al.*, 2006), *O. humiphilum* (Groth *et al.*, 2001), and *O. pekingense* (Liu *et al.*, 2008) were chosen as reference strains to isolate F17IV as they represent the thus far only described species of the genus. Additionally, *Serinicoccus marinus* (Yi *et al.*, 2004), and *S. profundus* (Xiao *et al.*, 2011) are presented as they originate from the marine environment and are relatively closely related to the genus *Ornithinimicrobium* (Fig. 3.2).

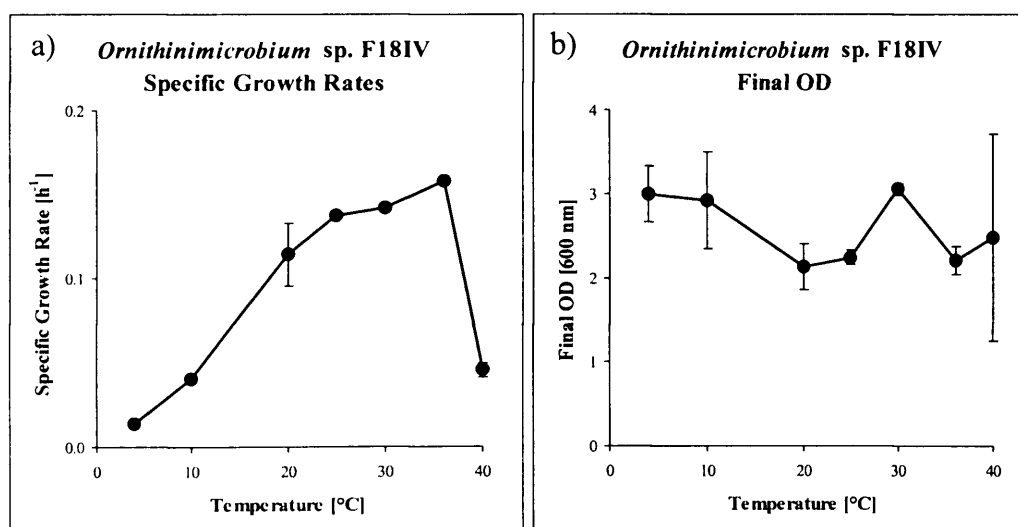


**Fig. 3.10: Micrographs of *Ornithinimicrobium* sp. F18IV.** Pictures were taken after 3 days of incubation in YPGL medium. a) Phase contrast image of strain F18IV, scale bar equals  $10 \mu\text{m}$ ; b) ESEM image of strain F18IV, scale bar equals  $5 \mu\text{m}$ ; c) Gram stain of F18IV

a) Temperature range for growth of *Ornithinimicrobium* F18IV

Strain F18IV grew over a wide range of temperatures (4°C to 39°C; Table A4.1) with an optimum at 36°C (Fig. 3.11). The specific growth rate at this temperature was 0.158 h<sup>-1</sup> (Fig. 3.11a), which equals a doubling time of 6.3 hours. No clear maximum for growth yield was observed and determined values had relatively high standard errors (Fig. 3.11b). However, the greatest cell density was obtained at 30°C where the final OD<sub>600</sub> was 3.06, only slightly higher than at 4°C and 10°C (final OD<sub>600</sub> 2.99 and 2.92, respectively; Fig. 3.11b).

In comparison to its relatives within the genus *Ornithinimicrobium*, strain F18IV shows a wider temperature range. It can grow at temperatures below 20°C and up to 39°C, which was only reported for *O. humiphilum*. Representatives of the genus *Serinicoccus*, which originate from the marine environment, show a temperature range for growth that is more similar to that of strain F18IV (Table A4.1).



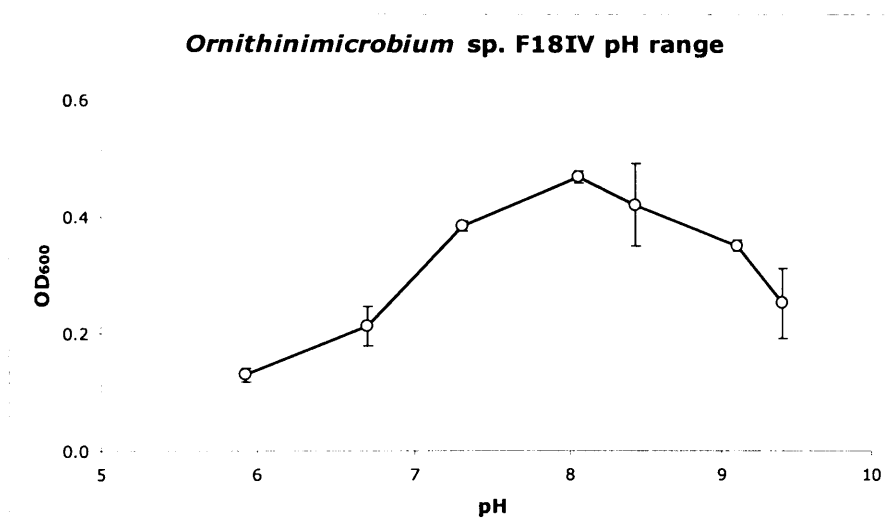
**Fig. 3.11: Temperature dependent growth of *Ornithinimicrobium* sp. F18IV.** a) Specific growth rate of isolate F18IV over a temperature range (shaken at 150 rpm). b) Final OD of isolate F18IV over a temperature range. Experiments were conducted in triplicate and averaged data is presented. If error bars not visible they fall within the size of the data points.

b) pH range and optimum for growth of *Ornithinimicrobium* sp. F18IV

For *Ornithinimicrobium* sp. F18IV the optimum initial pH for growth was pH 8.06 (Fig. 3.12). However, despite the media being buffered, in many cases the metabolic activity of the strains altered the final pH of the media. In most cases the pH was

lower after the incubation than at the beginning of the experiment due to volatile fatty acid production by the strains during growth, which was determined in separate experiments. Strain F18IV metabolised glucose to acetate, lactate, and formate (see Chapter IV). Nonetheless, the initial pH concentrations are compared with the final OD<sub>600</sub> (Fig. 3.12).

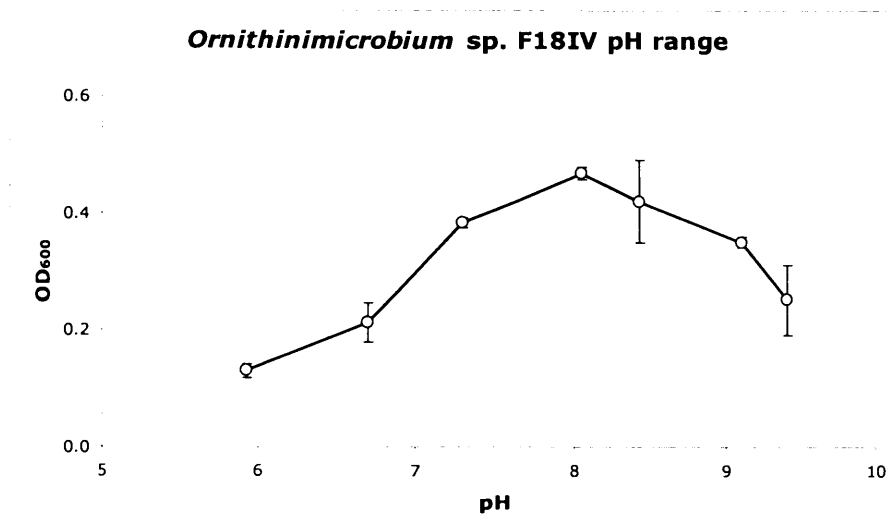
Strain F18IV grew over a similar pH range (pH 6 to pH 9) as other members of the genus *Ornithinimicrobium* (Table A4.1). In comparison, *Serinicoccus* spp. tolerate higher pH levels up to pH 11. The optimum pH for isolate F18IV is at around pH 8, which is similar to *O. pekingense* and *S. marinus* but not *S. profundus*, which has an optimum of pH 7.



**Fig. 3.12: Growth range and optimum of pH for strain F18IV.** Cells were incubated for three days at room temperature and OD<sub>600nm</sub> was determined using a submersible probe attached to the spectrophotometer. Each data point represents the average pH value of duplicates incubations, which themselves were measured in triplicate.

### c) Salinity range and optimum for growth of *Ornithinimicrobium* sp. F18IV

*Ornithinimicrobium* sp. F18IV grew at salinities between 3.5% and 7.5% (w/v), with an optimum of 3.5% NaCl (w/v; Fig. 3.13). *Ornithinimicrobium* sp. F18IV did not grow below 3.5% or above 7.5% NaCl (w/v). Other members of the genus are able to grow at 2% NaCl (w/v) and up to around 7%. Members of the genus *Serinicoccus* in comparison have a much larger salinity range. They grow from 0% to 14% NaCl (w/v) and their optima lie between 3% and 5% NaCl (w/v).



**Fig. 3.13: Growth range and optimum of salinity for strain F18IV.** OD<sub>600nm</sub> was determined after three days of growth. Each data point represents the average of three OD measurements.

d) Acid production of *Ornithinimicrobium* sp. F18IV

Acid production in strain F18IV was observed only for fructose. For this particular trait the isolate is comparable to *O. kibberense* as no acid was produced from fructose by *O. humiphilum* or *O. pekingense* (Table A4.1). Otherwise strain F18IV differs from these strains as they are positive for acid production from a variety of substrates, which F18IV was not. Hence, in regard to acid production F18IV is more similar to *O. pekingense* than it is to the other two species.

e) Substrates supporting growth of *Ornithinimicrobium* sp. F18IV

All *Ornithinimicrobium* and *Serinicoccus* strains are able to use D-glucose as an electron donor. In addition, strain F18IV is able to utilise glycerol, which is also metabolised by representatives of the *Serinicocci*. Reproducibility of growth tests with strain F18IV was low. In some tests it failed to grow on any and sometimes only on a few substrates. Variable growth occurred on acetate, which was also reported for *O. kibberense*, but positive in *S. marinus*. The same is true for cellobiose with the addition that it can also be metabolised by *S. profundus*. Results for sucrose consumption varied in strain F18IV but *O. kibberense*, *S. marinus*, and *S. profundus* grow with this substrate.

In addition to the listed substrates (Table A4.1), strain F18IV also grew aerobically with casamino acids, chitin, glycolate, laminarin, peptone, L-tryptophane,

xylan (variable), and yeast extract and showed weak growth when caproate, caprylate were used as substrates. F18IV also grew on Blood Agar (not haemolytic) and Muller-Hinton Agar but not on McConkey Agar.

No aerobic growth occurred with DL-alanine, L-arabinose, L-arginine, betaine, benzoate, *n*-butanol, butyrate, cellulose, crotonate, L-cysteine, ethanol, formate, fumarate, D-glucosamine, L-glutamine, glycol, glyconate, L-isoleucine, DL-lactate, lactose, malonate, methanol, methylamine, L-ornithine, phenylacetate, L-phenylalanine, *n*-Propanol, L-rhamnose, serine, starch, tween 80, or valerate.

#### f) Electron acceptors and fermentation ability of *Ornithinimicrobium* F18IV

The utilisation of electron acceptors other than oxygen was tested but found to be negative for nitrate (Table A4.1), thiosulphate, ferric citrate, ferrihydrite, manganese dioxide, trimethylamine N-oxide, DMSO and AQDS with DL-lactate. Weak growth occurred, however, on DMSO and AQDS when acetate was added. Of the six strains compared (Table A4.1), F18IV is the only one not using nitrate as an electron acceptor.

Growth under D-glucose fermentation was weak, no growth occurred with casamino acids under anaerobic conditions. Instead anaerobic growth occurred on lysine, L-arginine, and ornithine.

#### g) Vitamin requirements of *Ornithinimicrobium* sp. F18IV

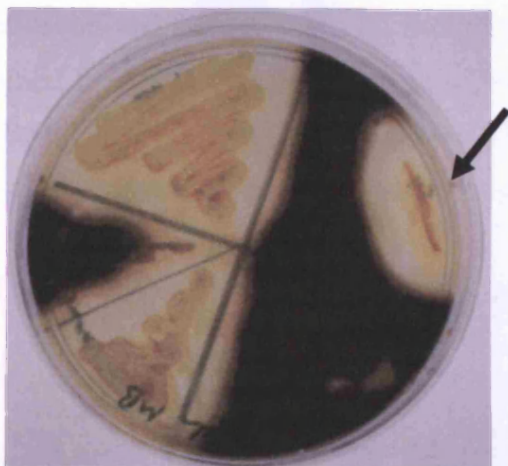
Vitamins essential for growth of strain F18IV were biotin, folate, lipoate, pantothenate, pyridoxine, and thiamine. Growth occurred without addition of nicotinate, *p*-aminobenzoate.

#### h) Hydrolytic properties and extracellular enzymes of *Ornithinimicrobium* F18IV

Strain F18IV showed  $\beta$ -galactosidase activity, which was also reported for *Serinicoccus* spp. Alkaline phosphatase was produced by strain F18IV as it is by *O. humiphilum* and *S. profundus*. Furthermore, strain F18IV hydrolysed chitin and starch (Fig. 3.14), the latter also being hydrolysed by most of the comparison strains (Table A4.1). Tween 80 was not hydrolysed by F18IV or the other



*Ornithinimicrobium* strains with the exception of *O. pekingense* but by the two *Serinicoccus* representatives. Aesculin hydrolysis did not occur in any *Ornithinimicrobium* strains but was reported for *Serinicoccus* spp. (Table A4.1).



**Fig. 3.14: Starch hydrolysis by strain F18IV.** Arrow indicates location of strain F18IV on the plate. A clear zone around the cell streak is the area where starch was hydrolysed (plate stained with Lugol's iodine to indicate presence of starch).

#### i) Antibiotic resistances of *Ornithinimicrobium* sp. F18IV

The isolate showed a remarkable resistance towards kanamycin and trimethoprim, still growing at the maximum concentration added to the medium ( $256 \mu\text{g} \times \text{ml}^{-1}$ ; Table A4.1). Strain F18IV was relatively sensitive towards erythromycin (MIC:  $0.016 \mu\text{g} \times \text{ml}^{-1}$ ) and penicillin G (MIC: 0.78 IU). Unfortunately, not much data are available for the comparison strains. Either they were not tested for antibiotic resistances or the antibiotics tested differ from the selection here. *O. humiphilum*, however, is somehow resistant to chloramphenicol, erythromycin, and penicillin but more sensitive to gentamycin, kanamycin, and streptomycin than strain F18IV.

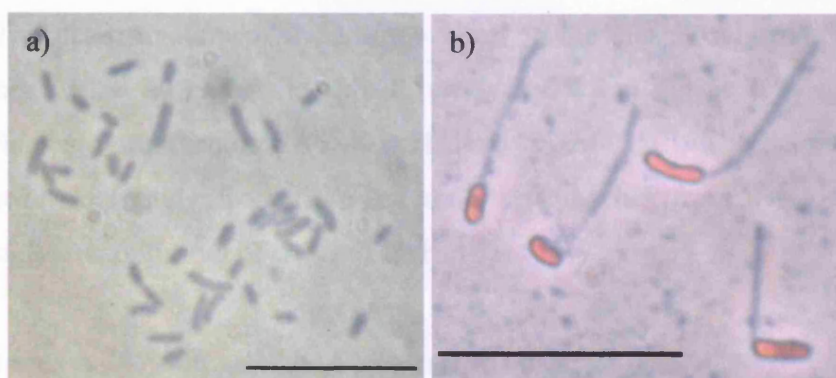
#### 2.2.2 Phenotypic properties of *Shewanella* isolates

All eight strains belonging to the genus *Shewanella* formed circular, convex, smooth (except F16IV) colonies with entire margins after a few days of growth on YPGL agar plates. For most strains the colonies exhibited an orange to brown colouration with the exception of strain F17V, which produced white colonies.

Cells of all strains were straight, sometimes slightly curved rods, Gram-negative and approximately 1.0 to 6.0  $\mu\text{m}$  long and 0.5 to 1.5  $\mu\text{m}$  wide. Motility was

observed in all but strain F17V, although all strains possessed a single, polar flagellum (Fig. 3.15). Sporulation was never observed.

Because of their marine origin and relatively close phylogenetic relationship to the *Shewanella* strains obtained from IODP Leg 307 samples (Fig. 3.3) *Shewanella* sp. SCSA3, *S. vesiculosa* (Bozal *et al.*, 2009), *S. frigidimarina* (Bowman *et al.*, 1997), and *S. profunda* (Toffin *et al.*, 2004a), were chosen as reference strains (Table A4.2).



**Fig. 3.15: Morphology of *Shewanella* sp. F17VI.** a) Phase contrast micrograph; b) Micrograph of stained flagella. Scale bar equals 10  $\mu$ m.

#### a) Temperature range for growth of *Shewanella* isolates

*Shewanella* strains from IODP Leg 307 grew over a wide temperature range from 2°C to a maximum of 39°C depending on the strain (Table A4.2). Their optimum temperatures, however, were at about room temperature at which after two to three days visible colonies formed. The temperature range for other *Shewanella* strains (Table A4.2) is similar to isolates from Leg 307.

#### b) pH range and optimum for growth of *Shewanella* isolates

The range of pH that supported growth in *Shewanella* isolates from IODP Leg 307 stretches from pH 6 to pH 9.1 or pH 9.4 with clear optima either at pH 7.3 or pH 8.4 (Table A4.2). Only strain F16III had similar OD values at both these pH values.

*S. vesiculosa* and *S. profunda* have a similar lower pH that supports growth compared to the Leg 307 isolates but have a lower maximum for growth at pH at 8.5 and 8.0, respectively (Table A4.2).





c) Salinity range and optimum for growth of *Shewanella* isolates

The salinity range for the different isolates from Leg 307 was almost identical and was between 3.5% up to either 7.5% or 10% (w/v; Table A4.2) with an optimum salinity of 3.5% for four out of eight strains (Strains F16III, F16IV, F17VII, and F18III). Strain F16V grew to similar optical densities in at 5% and 7.5% salinity whereas the remaining three strains had a clear optimum at 7.5% (Strains F17III, F17V, and F17VI).

The determination of the salinity range in the closely related strains is less comprehensive. *S. vesiculosa* is able to grow at 0% but not at 7% (w/v) salinity. *S. frigidimarina* was reported to grow below 9% of NaCl. The salinity range of *S. profunda* originating from deep-sea sediments reaches from 0% to 6% with a surprisingly low optimum of 0.5% (Table A4.2).

d) Substrates supporting growth of *Shewanella* isolates

All of the Leg 307 *Shewanella* strains grew aerobically on the following substrates: acetate, casamino acids, D-fructose, fumarate, D-glucose, L-glutamic acid, L-glutamine,  $\alpha$ -ketoglutarate, peptone, propionate, succinate, sucrose, and yeast extract. None of the strains utilised L-arabinose, cellulose, L-cysteine, formate, methylamine, L-rhamnose, salicylate, trehalose, L-tryptophan, and xylan (Table A4.2).

All *Shewanella* strains from the Flank site originated from the same sediment layer beneath the mound but were initially enriched in different media (Table 3.2). Their substrate spectra are very similar. They all utilised 24 substrates (e.g. DL-glucose, fructose, acetate) and none of them grew with 22 substrates (e.g. D-rhamnose, formate, L-cysteine) out of the 59 substrates tested. The remaining 14 substrates were generally used only by a subset of the strains (e.g. L-arginine, crotonate, starch). To highlight differences in substrate utilisation, the spectra of the strains were compared with each other (Table A4.2). Strains F16III and F16IV, for example, differed in their response to six substrates, strains F16III and F16V responded differently to seven substrates and strains F16IV and F16V showed opposite results for 13 substrates (Table A4.2 and 3.6).

These results show that although originating from the same sediment sample at the Flank site and despite clustering in the phylogenetic tree (Fig. 3.3) different strains of the genus *Shewanella* can differ with respect to their substrate spectrum and possibly represent different species. As these isolates were obtained from different MPN series/wells, their predecessor cells must have been present in the original sediment sample, indicating the presence of a physiologically diverse set of *Shewanella* strains in subsurface sediments.

The four *Shewanella* isolates from the Mound site originated from three different depths. Strains F17III (47 mamb), F17V and F17VI (14 mamb) were isolated from the mound itself whereas strain F17VII was obtained from an underlying sediment layer at -0.86 mamb. All four strains shared a common 16 utilised and 11 non-utilised substrates (Table A3.6).

**Table 3.6: Different responses to substrates provided to *Shewanella* isolates.** Given are the numbers of differences in substrate utilisation as revealed by the substrate test (out of 59 substrates).

Different Substrate Usage	F16			F17			F18	
	III	IV	V	III <sup>M</sup>	V <sup>M</sup>	VI <sup>M</sup>	III	
F16 III		6	7	19	8	13	1	8
F16 IV			13	12	14	17	5	14
F16 V				24	13	5	8	11
F16 III <sup>M</sup>					19	26	18	23
F17 V <sup>M</sup>						21	9	14
F17 VI <sup>M</sup>							12	13
F17 VII								9
F18 III								

Legend: Strains origination from the carbonate mound <sup>M</sup>.

The substrate spectra do not reveal any correlations amongst isolates originating from the mound itself or amongst isolates from the surrounding sediments. It is remarkable, however, that strains F16III and F17VII only differed in utilising a single substrate (chitin). Both cluster together in the phylogenetic tree (Fig. 3.3) but this can not be taken as a tool to predict substrate spectra as F16III, for example, differs from F17VI in 13 substrates tested despite the latter also being in the same OTU as F16III and F17VII.

In comparison with their close relatives results of substrate utilisation of Leg 307 strains do agree well in cases of acetate, butyrate, caprylate, cellobiose, fumarate, D-glucose, L-glutamic acid, DL-lactate, DL-malate, maltose, D-mannitol, peptone, L-

rhamnose, succinate, sucrose, valerate, and yeast extract. The *Shewanella* strains obtained from sediment samples of IODP Leg 307 differed from their reference strains by for the following substrates L-alanine, L-arabinose, citrate, D-galactose, glycerol, lactose, D-mannose, D-sorbitol, and trehalose.

e) Electron acceptors and fermentation ability of *Shewanella* isolates

Representatives of the genus *Shewanella* are known for their versatile range of electron acceptors (Nealson and Scott, 2006). It is therefore not surprising that all but one strain (F16V) reduced manganese oxides and nitrate. Five strains also utilised ferrihydrite but strains F16V, F17V, and F17VII did not. Weak growth with thiosulphate occurred in strains F17III and F18III and varied in strain F16III (Table A4.2). None of the strains reduced ferric citrate.

These findings match well the data reported for the reference strains. The three species (*S. frigidimarina*, *S. profunda*, *S. vesiculosa*) are also capable of using nitrate as an electron acceptor, two of them also utilise ferrihydrite. Strain SCSA3 is the only representative in this comparison not reducing nitrate but ferrihydrite, manganese oxides, and thiosulphate instead (Table A4.2).

Glucose was utilised by six of the eight strains with acetate, lactate, and formate being the fermentation products. Fermentation of casamino acids allowed growth to turbid cultures in strain F17V, whereas in strains F16III and F18III growth was reproducible but yielded hardly turbid cultures. In the remaining strains no growth by casamino acid fermentation occurred. The reference strain SCSA3 and *S. frigidimarina* also ferment glucose. No data were available for casamino acid fermentation.

f) Vitamin requirements of *Shewanella* isolates

Strain F17V was the only one amongst the *Shewanella* isolates that required certain vitamins for growth. It did not grow without biotin, folate, pantothenate, *p*-aminobenzoate, pyridoxine, and thiamine but did not require lipoate and nicotinate for growth.

g) Hydrolytic and extracellular enzymes of *Shewanella* isolates

All *Shewanella* isolates produced caseinase, DNase (except strain F16V), gelatinase, and alkaline phosphatase (Table A4.2). None of the strains produced arginine dihydrolase (except strain F17V), esterase (C4), urease, and lysine decarboxylase. Only two isolates produced extracellular amylase (strains F17III and F17V). Most strains hydrolysed either Tween 20 or Tween 80 or both, only strain F17V lacked this ability.

h) Antibiotic resistances of *Shewanella* isolates

All *Shewanella* strains were quite susceptible to chloramphenicol, erythromycin, gentamycin and kanamycin (Table A4.2). Some strains were sensitive towards penicillin G at a MIC of  $3.1 \text{ IU} \times \text{ml}^{-1}$  (e.g. F16III and F17V). Strain F17III was resistant even to the highest concentrations of penicillin G, streptomycin, tetracycline, and trimethoprim tested. Strains F16III and F18III also grew in the presence of any concentration of trimethoprim. For strain F17V the highest concentration of trimethoprim proved to be the MIC as was the highest concentration of penicillin G for strain F17VII. The remaining *Shewanella* strains showed a strong sensitivity towards these antibiotics.

2.2.3 Phenotypic properties of *Vibrio* sp. isolates

Three strains belonging to the genus *Vibrio* were isolated from sediment samples of IODP Leg 307. Strain F16II originated from a sediment horizon beneath the carbonate mound at the Flank site. The other two strains, F17I and F17IV were isolated from different depths at the Mound site (Table 3.2). Cells formed white, circular, convex colonies with an entire margin after three days of incubation on YPGL agar plates. Cells were Gram-negative, straight to curved rods and between  $1 \mu\text{m}$  to  $4 \mu\text{m}$  in length and approximately  $1 \mu\text{m}$  in width (Fig. 3.16). Sporulation and motility were not observed although cells of strain F17I had single polar flagella.

*Vibrio* sp. SCSA1, *V. lentus* (Macian *et al.*, 2001), and *V. kanaloae* (Thompson *et al.*, 2003), were chosen as reference strains (Table A4.3) to the *Vibrio* related isolates from IODP Leg 307 because of their very close phylogenetic affiliation relation (Fig. 3.4) and marine origin.



**Fig. 3.16: Morphology of *Vibrio* sp. F17IV.** Phase contrast micrograph scale bar equals 10  $\mu\text{m}$ .

a) Temperature range for growth of *Vibrio* sp. isolates

The temperature range of the three isolated *Vibrio* strains reached from below 2°C to below 27°C. This range was also observed in their close relatives strain SCSA1 and *V. lentus* (Table A4.3).

b) pH range and optimum for growth of *Vibrio* sp. isolates

The three *Vibrio* isolates grew over the entire pH range tested (pH 5.9 to pH 9.4). They differed, however, in their pH optimum. For strain F16II and F17I the optimum was at around pH 7.3 while F17IV showed the highest OD at pH 9.1. Unfortunately, the pH ranges for the reference strains are not published.

c) Salinity range and optimum for growth of *Vibrio* sp. isolates

Strains F16II and F17IV grew at salinities ranging from 3.5% to 10% yet differed in their optimum NaCl concentration for growth, which was 5% and 3.5%, respectively. The salinity range of strain F17I was shifted slightly downwards compared to the other two isolates (2.5% - 7.5% salinity) and the optimum salinity for strain F17I was 3.5% (Table A4.3).

d) Substrates supporting growth of *Vibrio* sp. isolates

All isolated *Vibrio* strains utilised acetate, DL-alanine, L-arginine, caproate, casamino acids, cellobiose, D-fructose, fumarate, D-gluconate, D-glucose, glutamate, L-glutamine, glycerol,  $\alpha$ -Ketoglutarate, maltose, mannitol, D-mannose, peptone, L-serine, starch, tween 80, xylan, and yeast extract for growth (Table A4.3).

They did not grow on L-arabinose, benzoate, betaine, *n*-butanol, butyrate, caprylate, cellulose, chitin, crotonate, L-cysteine, ethanol, formate, glycol, glycolate, L-isoleucine, laminarin, malonate, methanol, methylamine, L-phenylalanine, *n*-propanol, L-rhamnose, salicylate, D-sorbitol, sucrose, L-tryptophan, and xylose (Table A4.3).

The substrate spectra for the isolates are very similar with only a few in their substrate utilisation. Strain F16II did not use the uneven fatty acids propionate and valerate in the growth experiments whereas strains F17I and F17IV did. In total, *Vibrio* sp. F16II differed from strain F17I in its response to two substrates (propionate and valerate) and differed from strain F17IV by additional eight substrates (citrate, D-galactose, D-glucosamine, L-isoleucine, DL-lactate, lactose, DL-malate, and succinate). The different usage of these eight substrates also distinguishes F17I and F17IV (Table 3.7).

**Table 3.7: Different response to substrates provided to *Vibrio* isolates.** Given are the numbers of differences in substrate utilisation as revealed by the substrate test (out of 59 substrates).

Different Substrate Usage	F16II	F17I <sup>M</sup>	F17IV <sup>M</sup>	SCSA1
F16II		2	10	20
F17I <sup>M</sup>			8	22
F17IV <sup>M</sup>				20
SCSA1				

Legend: M, Strains origination from the carbonate mound.

Unfortunately, the two type strains were not tested for all the substrates that were used during the course of this project thus only limited conclusion can be drawn. For the substrate tested, Leg 307 strains, however, agree in most parts with those of their close phylogenetic relatives (Table A4.3). There are, naturally, a few exceptions as, for example, acetate, L-arginine and D-gluconate, which are not consumed by *V. lentus* while *V. kanaloae* does not grow on cellobiose but uses formate and

sucrose. However, the isolates differ in their substrate spectra from strain SCSA1 by 20 out of the 59 substrates tested (Table A4.3).

e) Electron acceptors and fermentation ability *Vibrio* sp. isolates

Members of the genus *Vibrio* are known to thrive in the absence of oxygen and are able to ferment glucose (Farmer, 2006). Leg 307 isolates are no exception and additionally grew by fermenting casamino acids. The use of electron acceptors of the isolates was, however, limited. F17IV showed varying results for nitrate as did F16II and F17I for thiosulphate and nitrate (Table A4.3). Ferric citrate, ferrihydrite and manganese oxides were not reduced. Strains SCSA1 and *V. lentus* in comparison are able to use nitrate (Table A4.3).

f) Vitamin requirements of *Vibrio* sp. isolates

Strain F17IV did not require any vitamins for growth. Strain F17I, however, required all eight vitamins tested (biotin, folate, lipoate, nicotinate, pantothenate, *p*-aminobenzoate, pyridoxine, thiamine) while strain F16II required all but lipoate.

g) Hydrolytic and extracellular enzymes of *Vibrio* sp. isolates

All isolated *Vibrio* strains produced L-arginine dihydrolase and alkaline phosphatase and hydrolyse starch. Strains F17I and F17IV liquefied gelatine whereas acid phosphatase activity was found in strains F16II and F17IV. None of the strains produced DNase, esterase, lipase (Tween 20 and 80), lysine decarboxylase, ornithine decarboxylase or urease (Table A4.3).

Some of these traits were reported for the reference strains. Strain SCSA1 also exhibited gelatinase activity (Fig. 3.17) as did *V. kanaloae* (Thompson *et al.*, 2003) and *V. lentus* (Macian *et al.*, 2001). These two type strains also produced L-arginine dihydrolase and showed a negative reaction for lysine and ornithine decarboxylase like Leg 307 isolates. There are some differences, however, between Leg 307 isolates and *V. lentus*, which produces caseinase, DNase, and lipase (Tween 80).



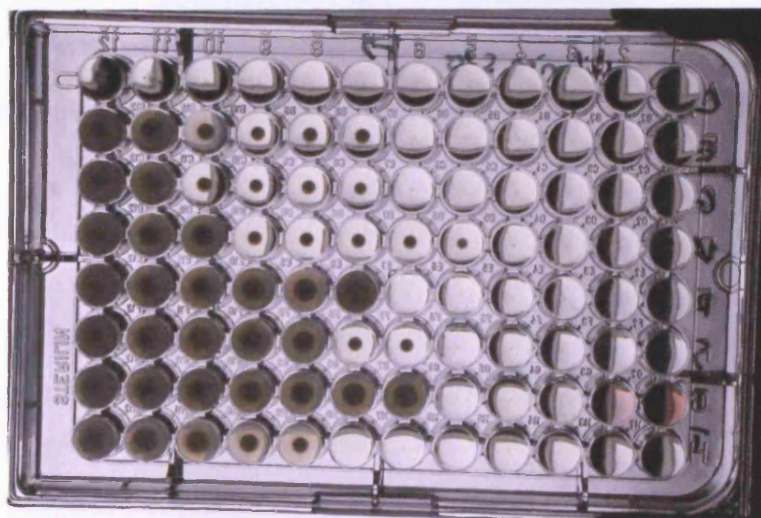


**Fig. 3.17: Gelatinase activity of *Vibrio* sp. SCSA1. *Shewanella* sp. SCSA3 and *Arcobacter* sp. NA105 are gelatinase negative.**

h) Antibiotic resistances of *Vibrio* sp. isolates

Only two of the Leg 307 *Vibrio* strains were tested for antibiotic resistance. Strain F16II was not included, instead reference strain SCSA1 was tested. *Vibrio* strains showed a higher sensitivity towards the antibiotics than, for example, some of the *Shewanella* isolates. Chloramphenicol was the most effective antibiotic with a MIC of  $\leq 0.5 \mu\text{g ml}^{-1}$ . Strain F17IV was relatively resistant towards gentamycin (MIC of  $64 \mu\text{g ml}^{-1}$ ) and penicillin G (MIC of  $25 \text{ IU ml}^{-1}$ , Table A4.3) whereas strain F17I was more resistant to kanamycin than its relatives.

*V. kanaloae* is classed sensitive for chloramphenicol and tetracycline depending on the radius of the inhibition zone around the disc (Table A4.3).



**Fig. 3.18: Antibiotic resistance tests of *Vibrio* sp. F17I.** Eight antibiotics were tested on each 96 multi-well plate with twelve, increasing concentrations (from left to right). Wells containing the Minimum Inhibitory Concentrations (MIC) for each antibiotic are the first ones in line (left to right) lacking growth.



#### 2.2.4 Phenotypic properties of *Raoultella* sp. isolates

Two strains of the genus *Raoultella* were isolated from the Mound site of IODP Leg 307, one from within the mound, the other from an underlying sediment layer. Both strains formed circular, convex colonies with an entire margin. Colonies of strain F17II were colourless whereas F17VIII formed white colonies. Cells were Gram-negative and generally short rods (Fig. 3.19) but sometimes ovoid in shape and also oblong. Cell lengths ranged from 1.0  $\mu\text{m}$  to 6.0  $\mu\text{m}$  with widths between 0.6  $\mu\text{m}$  and 1.8  $\mu\text{m}$ . Strain F17II was motile and had a single polar flagellum, both these characteristics were not observed for strain F17VIII.

*R. planticola* (Bagley *et al.*, 1981), *R. terrigena* (Izard *et al.*, 1981), and *R. ornithinolytica* (Sakazaki *et al.*, 1989), previously all assigned to the genus *Klebsiella* (Drancourt *et al.*, 2001) were chosen as reference strains (Table A4.4) to the *Raoultella* related isolates from IODP Leg 307 because they are the only validly described species of the genus and therefore closely related (Fig. 3.5).

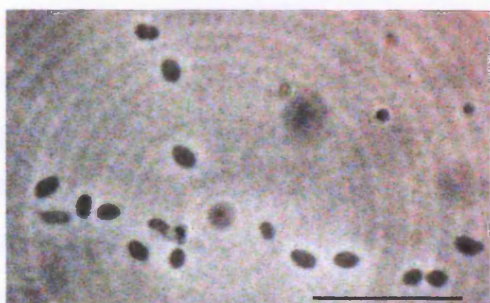


Fig. 3.19: Morphology of *Raoultella* sp. F17VIII. Phase contrast micrograph. Scale bar equals 10  $\mu\text{m}$ .

##### a) Temperature range for growth of *Raoultella* sp. isolates

Like most of the other isolates obtained from IODP Leg 307 samples, the two *Raoultella* strains grew at temperatures below 2°C. The two strains however differed from each other in their upper temperature limit for growth. Strain F17II grew up to 36°C, which was substantially exceeded by F17VIII still growing at 48°C. No data about the temperature range of the described species of *Raoultella* species could be retrieved from the literature.

b) pH range and optimum for growth of *Raoultella* sp. isolates

Both *Raoultella* isolates grew at the lower end of the pH range tested (pH 5.9). Strain F17II grew up to pH 9.1 and strain F17VIII even at the highest pH tested (pH 9.4). The optimum pH values for both strains were similar with pH 8.1 for strain F17II and pH 8.4 for strain F17VIII. No data were found in the literature for the pH range of the described species of *Raoultella* species.

c) Salinity range and optimum for Growth of *Raoultella* sp. isolates

Strains F17II and F17VIII did not grow at salinities below 3.5% (w/v), which was also the optimum NaCl concentration for growth. The upper limits were 7.5% for F17II and 10% for F17VIII. No data were available for the salinity range of the described *Raoultella* species.

d) Substrates supporting growth of *Raoultella* sp. isolates

Both *Raoultella* strains grew on acetate, DL-alanine, casamino acids, cellobiose, D-fructose, D-gluconate, D-glucosamine, D-glucose, glutamate, L-glutamine, DL-lactate, lactose, DL-malate, maltose, mannitol, peptone, L-serine, D-sorbitol, sucrose, and yeast extract (Table A4.4). Neither isolate utilised betaine, *n*-butanol, caprylate, L-cysteine, ethanol, formate, glycerol, L-isoleucine, propanol, salicylate, starch, and L-tryptophane. However, the two isolates differed in the usage of 20 out the 59 substrates tested and some substrates produced varying results for the individual strains during the substrate tests (e.g. xylan, Table A4.4).

For those substrates where data are available for the comparison strains the results are comparable to the Leg 307 isolates. The most variable results for all strains, however, can be found with benzoate, caproate, and trehalose (Table A4.4).

e) Electron acceptors and fermentation ability *Raoultella* sp. isolates

Both *Raoultella* strains used manganese oxides and nitrate as electron acceptors. Strain F17II also reduced ferrihydrite but neither used ferric citrate or thiosulphate.

f) Vitamin requirements of *Raoultella* sp. isolates

Tests for vitamin requirements showed that the two *Raoultella* strains were prototrophic. After five consecutive transfers into vitamin-free medium in each case strains developed turbid cultures upon a few days of incubation thus proving their ability to grow without added vitamins.

g) Hydrolytic properties and extracellular enzymes of *Raoultella* sp. isolates

The two *Raoultella* isolates produced caseinase and phosphatase (acid and alkaline) whereas neither produced arginine dihydrolase or esterase (C4). For the remaining tests of exo-enzymes the isolates differed in their response. Strain F17II, for example, was positive for DNase, gelatinase and lipase (Tween 80) whereas strain F17VIII as well as the reference strains are negative for these characteristics (Table A4.4). In contrast, F17II was negative for lysine decarboxylase and urease whereas strain F17VIII and all described *Raoultella* species were positive (Table A4.4). Generally, strain F17VII shares significantly more similarities with respect to physiological traits to other *Raoultella* species than strain F17II.

h) Antibiotic resistances of *Raoultella* sp. isolates

Both *Raoultella* strains were sensitive towards chloramphenicol but showed great resistance towards penicillin G exceeding the maximum concentration tested in the experiments for antibiotic resistance. This was also observed for strain F17VIII when exposed to tetracycline and trimethoprim. The two strains differed in their response to erythrocine, gentamycin, kanamycin, tetracycline, and trimethoprim with strain F17VIII exhibiting MICs two to 32 times higher than strain F17II.

Antibiotic resistance tests for *R. planticola* and *R. ornithinolytica* showed sensitivity to many of the antibiotics tested for Leg 307 isolates (e.g. chloramphenicol, Table A4.4). *R. ornithinolytica* is penicillin G resistant like the Leg 307 isolates but is sensitive towards tetracycline unlike strain F17VIII.

### 2.2.5 Phenotypic properties of *Pseudomonas* sp. isolates

Two strains isolated from Reference site of IODP Leg 307 belong to the genus *Pseudomonas* and originate from 4 and 80 mbsf (Table 3.2). Cells formed white, circular, convex colonies with an entire margin after two to three days of growth on YPGL agar plates. Cells were Gram-negative straight rods (Fig. 3.20) with a single polar flagellum. Motility was observed in strain F18V but not in strain F18II. The lack of motility is rare in *Pseudomonas* spp. (Moore *et al.*, 2006). Cell lengths ranged from 1  $\mu\text{m}$  to 6  $\mu\text{m}$ , the widths from 0.5  $\mu\text{m}$  to 1.5  $\mu\text{m}$ .

*Pseudomonas* sp. NA101 (Freese *et al.*, 2008b), *P. pohangensis* (Weon *et al.*, 2006), *P. antarctica*, and *P. proteolytica* (Reddy *et al.*, 2004) were chosen for comparison (Table A4.5) because of their origin of isolation (marine, cold habitats) and their close phylogenetic affiliation (Fig. 3.6).



**Fig. 3.20: Morphology of *Pseudomonas* sp. F18V.** Phase contrast micrograph. Scale bar equals 10  $\mu\text{m}$ .

#### a) Temperature range for growth of *Pseudomonas* sp. isolates

The two *Pseudomonas* strains F18II and F18V grew at temperatures from less than 2°C to 36°C and 39°C, respectively. This is a similar range as reported for the reference strains (Table A4.5) although Leg 307 strains showed potentially a slightly higher temperature tolerance for growth. Reddy *et al.* (2004) described *P. antarctica* and *P. proteolytica* as psychrophilic, strains F18II and F18V, however, are most likely psychrotroph.

b) pH range and optimum for Growth of *Pseudomonas* sp. isolates

Strain F18II grew over the entire pH range tested (pH 5.9 – pH 9.4) with an optimum of pH 8.4. Strain F18V did not grow at pH levels above 9.1 and its optimum pH for growth was 8.1. These results are similar to comparison strain *P. pohangensis*, which grows between pH 5 and pH 9 with an optimum pH between pH 7 and pH 8. The pH ranges for *P. antarctica* and *P. proteolytica* are not given in their species descriptions (Reddy *et al.*, 2004) but their pH optima were determined to be at pH 7 (Table A4.5).

c) Salinity range and optimum for growth of *Pseudomonas* sp. isolates

The two *Pseudomonas* isolates had an identical salinity range (3.5% to 7.0%) and optimum for growth of 3.5%. The three comparison species only tolerate NaCl concentrations up to 3%, which in the case for *P. pohangensis* appears to be quite low, as it was isolated from sea shore sands (Table A4.5).

d) Substrates supporting growth of *Pseudomonas* sp. isolates

The substrate spectra for the two *Pseudomonas* isolates are almost identical (Table A4.5). They only differed in their response to five electron donors. Strain F18V used L-isoleucine, which was not degraded by strain F18II. Formate, D-gluconate, D-glucosamine, xylan were degraded by strain F18V but with varying results, scored positive and negative in different parallels of the substrate test.

The substrate spectra of strains F18II and F18V and their close phylogenetic relatives are very similar and agree for 16 substrates (Table A4.5). However, F18II and F18V do not utilise citrate, D-fructose, D-galactose, D-glucose, mannitol, D-mannose, L-serine, D-sorbitol, and trehalose whereas *P. antarctica* and *P. proteolytica* degrade these compounds. The two Leg 307 *Pseudomonas* strains utilised succinate, which was not observed for *P. antarctica* and *P. proteolytica*.

e) Electron acceptors and fermentation ability *Pseudomonas* sp. isolates

The two *Pseudomonas* strains did not utilise any electron acceptors tested other than oxygen, although thiosulphate concentrations were reduced in one parallel

incubation by strain F18V (Table A4.5). Despite apparently not metabolising glucose under aerobic conditions, the two Leg 307 isolates produced acetate, lactate, and formate from glucose in one of the two anaerobically incubated parallels. In the same parallel 96-well plate strain F18V also grew by fermentation casamino acids. Neither of the strains reduced nitrate, which was confirmed using Ion-Chromatography whereas the comparison strains reduce nitrate.

f) Vitamin requirements of *Pseudomonas* sp. isolates

Strains F18II and F18V are prototrophic and did not require any added vitamins for growth.

g) Hydrolytic properties and extracellular enzymes of *Pseudomonas* sp. isolates

Both *Pseudomonas* isolates were positive for L-arginine dihydrolase, gelatinase, and lipase (Tween 20). In addition, F18V also produced ornithine decarboxylase and phosphatase (acid and alkaline). Neither of the two strains produced esterase (C4) which was reported for *P. pohangensis* (Weon *et al.*, 2006).

h) Antibiotic resistances of *Pseudomonas* sp. isolates

For almost all antibiotics investigated strain F18V showed much higher MICs than F18II (two to 32 times). The only exception was streptomycin, which was tolerated by F18II in higher concentrations than by F18V (Table A4.5). Strain F18V tolerated relatively high amounts of chloramphenicol, erythromycin, penicillin G, and tetracycline and grew even at the maximum concentration of trimethoprim.

In comparison, *P. antarctica* is sensitive towards most antibiotics and the results are comparable to strain F18II, whereas *P. proteolytica* shows similar responses towards many of the antibiotics as F18V.

## 2.2.6 Phenotypic properties of *Photobacterium* sp. F18I

Strain F18I was isolated from a sediment layer (4.3 mbsf) at the Reference site. Cells



formed white to yellowish, circular, convex colonies with an undulate margin after two to three days of growth on YPGL agar plates. Cells were Gram-negative, oval and between 1.0  $\mu\text{m}$  and 9.1  $\mu\text{m}$  long and between 0.7  $\mu\text{m}$  and 2.1  $\mu\text{m}$  wide (Fig. 3.21). Flagella and motility were not observed.

*Photobacterium* sp. SAMA2, NA42, S10, S11, 67TD (Süß *et al.*, 2008; and unpublished) and *P. profundum* (Nogi *et al.*, 1998b) were chosen for comparison (Table A4.6) to strain F18I because of their origin (marine habitats, including deep subsurface sediments) and their close phylogenetic affiliation (Fig. 3.7).



**Fig. 3.21: Morphology of *Photobacterium* sp. F18I.** Phase contrast micrograph. Scale bar equals 10  $\mu\text{m}$ . Most likely PHB storage is visible within the cells, which sometimes causes pleomorphism.

a) Temperature range for growth of *Photobacterium* sp. F18I

Isolate F18I grew from below 2°C to 33°C. This is comparable to other isolates from the German Wadden Sea and the Mediterranean Sea. *P. profundum*, however, is a true psychrophile and shows a lower temperature maximum for growth of only 18°C (Nogi *et al.*, 1998b).

b) pH range and optimum for growth of *Photobacterium* sp. F18I

Strain F18I grew over the entire pH range tested (pH 5.9 – pH 9.4) with its optimum being at pH 8.4. The Mediterranean comparison strains showed an almost identical pH spectrum (Süß *et al.*, 2008).

c) Salinity range and optimum for growth of *Photobacterium* sp. F18I

Strain F18I required salinities between 3.5% and 7.5% (w/v) for growth with an optimum of 5%. The three Mediterranean strains are able to grow at salinities below

3.5% (Table A4.6), which was not observed for F18I. Strain NA42 grew at salinities up to 10% and had an extended optimum between 3.5% and 7%.

d) Substrates supporting growth of *Photobacterium* sp. F18I

Strain F18I utilised 26 of the 59 substrates tested. Unfortunately, *P. profundum* was not subjected to a substrate test as thorough as conducted for strain F18I and the other comparison strains hence it was omitted for the following observations. Additionally, results of the substrates tests for strain S11 have to be considered with caution as this strain tested negative for D-glucose, which is very uncommon for a representative of this genus and it only used four electron donors.

Apart from strain S11 all *Photobacterium* strains used D-glucose (including *P. profundum*), DL-malate, maltose (including *P. profundum*), and yeast extract. None of the strains utilised benzoate, butyrate, cellulose, glycolate, L-rhamnose (including *P. profundum*), and salicylate.

Strain F18I showed an almost identical substrate spectrum to strain S10 (Table 3.8 and A4.6). These two strains only differ in their response to six (caproate, caprylate, cellobiose, chitin, propionate, and valerate) out of 52 substrates, all of which are consumed by strain S10. *P. profundum* and F18I share nine out of 14 characteristics. It appears that strain SAMA2 can use most of the substrates tested (41) whereas strain S11 only utilises four.

**Table 3.8: Different substrate responses of *Photobacterium* isolates.** Given are the numbers of substrates that a pair of strains responds differently to either being able to grow or not able to grow.

Different Substrate Usage		Tidal Flat Strains			Mediterranean Strains		
		F18I	SAMA2	NA42	S10	S11	67TD
Tidal Flat Strains	F18I		22	15	6	22	12
	SAMA2			26	17	36	31
	NA42				18	17	16
Mediterranean Strains	S10					31	15
	S11						17
	67TD						

e) Electron acceptors and fermentation ability *Photobacterium* sp. F18I

Strain F18I did not use any electron acceptors provided. Other *Photobacterium* strains utilised nitrate and potentially thiosulphate (Table A4.6). None of the strains used ferrihydrite or manganese oxides.



All *Photobacterium* strains are able to ferment glucose. Casamino acids were not used by F18I under anaerobic conditions but by three of the reference strains (strains S10, S11, 67TD; Süß *et al.*, 2008).

f) Vitamin requirements of *Photobacterium* sp. F18I

Strain F18I required biotin, folate, lipoate, nicotinate, and pyridoxine for growth. Strain NA42 did not grow without biotin, folate, pyridoxine and thiamine while strain SAMA2 is prototrophic. The remaining *Photobacterium* strains were not tested for their vitamin dependence.

g) Hydrolytic properties and extracellular enzymes of *Photobacterium* sp. F18I

*Photobacterium* sp. F18I was positive for L-arginine dihydrolase, DNase, gelatinase, phosphatase (alkaline), and starch hydrolysis. It did not produce caseinase, esterase (C4), lipase (tween 20 and 80), lysine decarboxylase, ornithine decarboxylase, phosphatase (acid) and urease.

*P. profundum* shows the same results for L-arginine dihydrolase, lysine decarboxylase, and ornithine decarboxylase (Nogi *et al.*, 1998b).

h) Antibiotic resistances of *Photobacterium* sp. F18I

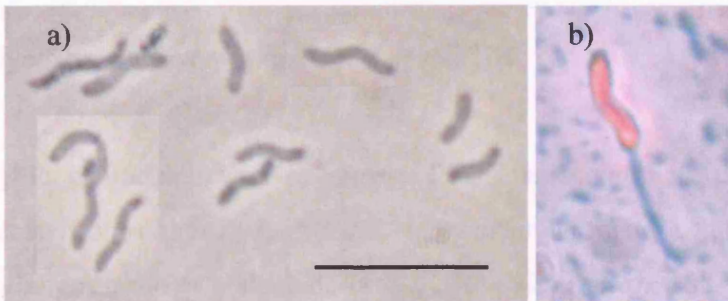
Antibiotic susceptibility was not tested for strain F18I but for SAMA2, S10, and S11. Strain SAMA2 was sensitive to most antibiotics tested and showed no growth at lower concentrations of e.g. chloramphenicol and erythromycin (Table A4.6). It grew, however, at trimethoprim concentrations higher than 256 µg ml<sup>-1</sup>, which was also observed for strain S10. This strain exhibited higher MICs to most antibiotics compared to the other two strains (e.g. erythromycin, gentamycin; Table A4.6).

### 2.2.7 Phenotypic properties of *Desulfovibrio* sp. F16I

Strain F16I was isolated from sediments (54.1 mamb/4.3 mbsf) overlying the carbonate mound structure at the Flank site. The strain belongs to the genus

*Desulfovibrio* and is most closely related to *D. oceani* subsp. *galatea* (Fig. 3.8). Cells were Gram-negative and vibrio-shaped (Fig. 3.22). Cells were 1.4  $\mu\text{m}$  to 14.3  $\mu\text{m}$  in length and 0.5  $\mu\text{m}$  to 1.1  $\mu\text{m}$  in width.

*Desulfovibrio oceani* subsp. *galatea* (Finster and Kjeldsen, 2010), *D. acrylicus* (van der Maarel *et al.*, 1996), *D. profundus* (Bale *et al.*, 1997), and *D. marinisediminis* (Takii *et al.*, 2008), were chosen as reference strains (Table A4.7) to strain F16I because of their origin of isolation (marine habitats, including deep subsurface sediments) and their close phylogenetic affiliation (Fig. 3.8). *D. desulfuricans* subsp. *desulfuricans* (Butlin *et al.*, 1949) was chosen as it represents the type species of the genus.



**Fig. 3.22: Morphology of *Desulfovibrio* sp. F16I.** a) Phase contrast micrograph. Scale bar equals 10  $\mu\text{m}$ ; b) Flagella staining of strain F16I.

#### a) Temperature range for growth of *Desulfovibrio* sp. F16I

Isolate F16I grew over a temperature range from 4°C to 30°C, which is identical with *D. oceani* subsp. *galatea*, the closest phylogenetic relative (Table A4.7). The biggest temperature range of the reference strains is that of *D. profundus*, which was isolated from deep sediments in the Japan Sea (Bale *et al.*, 1997).

#### b) Substrates supporting growth of *Desulfovibrio* sp. F16I

Strain F16I and its closest phylogenetic relative, *D. oceani* subsp. *galatea*, both degraded formate, fumarate, glycerol, hydrogen (heterotrophic), DL-lactate, and pyruvate. Neither of them used acetate, L-alanine, D-glucose, methanol, and *n*-propanol. They differ in their response to the other substrates tested. The substrate spectra for the other reference strains are very diverse and only agree in three positive (H<sub>2</sub>, DL-lactate, and pyruvate) but no common negative traits.

c) Electron acceptors and fermentation ability *Desulfovibrio* sp. F16I

Both, strain F16I and *D. oceani* subsp. *galatea*, used thiosulphate, sulphite, and sulphate as electron acceptors. Strain F16I was also weakly positive for DMSO and nitrate consumption.

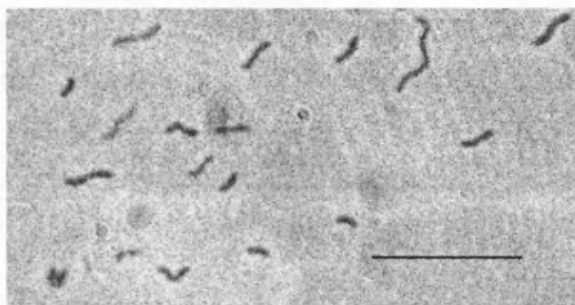
The comparison strains are quite diverse in their spectra (Table A4.7) but all use thiosulphate and sulphate. At least some of them differ from F16I in their response to DMSO and nitrate. None of the strains uses elemental sulphur. In the absence of an electron acceptor strain F16I is able to ferment pyruvate as are the comparison strains.

2.2.8 Phenotypic properties of *Arcobacter* sp. F17IX

The phenotypic characterisation of isolate F17IX is incomplete due to its weak growth under aerobic conditions. Instead *Arcobacter* strains are known to prefer a microaerophilic environment (Collado *et al.*, 2009; Neill *et al.*, 1885). Some strains, such as *A. nitrofigilis* are even obligate microaerophilic (McClung *et al.*, 1983). For this reason strain F17IX was not included in many of the tests conducted on other isolates of IODP Leg 307.

*Arcobacter* sp. F17IX was isolated from a sediment depth of -108 mamb (~254 mbsf) at the Mound site. It formed white to yellow, circular, convex, colonies with an entire margin within two to three days on YPGL agar plate under microaerophilic conditions. Cells were non-sporulating, Gram-negative, curved to S-shaped rods, on average 1.7  $\mu\text{m}$  ( $\pm$  0.7  $\mu\text{m}$ ) in length and 0.5  $\mu\text{m}$  ( $\pm$  0.1  $\mu\text{m}$ ) in width (n=66; Fig. 3.23). Motility and flagella were not observed, which differs to results from all comparison strains (Table A4.8).

*Arcobacter* sp. NA105, *A. nitrofigilis* (McClung *et al.*, 1983, described as *Campylobacter nitrofigilis*), *A. mytili* (Collado *et al.*, 2009), *A. halophilus* (Donachie *et al.*, 2005), and *A. cryaerophilus* (Neill *et al.*, 1985, described as *Campylobacter cryaerophilus*) were chosen as reference strains because of either their marine origin or their close affiliation to isolate F17IX (Fig. 3.9).



**Fig. 3.23: Micrograph of *Arcobacter* sp. F17IX.** Phase contrast image was taken after 3 days of incubation on YPGL Medium, scale bar equals 10  $\mu$ m.

a) Temperature range for growth of *Arcobacter* sp. F17IX

The temperature range for growth of strain F17IX is 4°C to 30°C, which is comparable to strain NA105 from an intertidal flat sediment (Table A4.8). All other strains are able to grow up to at least 37°C if not 42°C.

b) Substrates supporting growth of *Arcobacter* sp. F17IX

Strain F17IX was not subjected to thorough substrate tests as the other strains isolated from Leg 307. It grew well on YPGL agar plates under microaerophilic conditions, most likely using the peptone, yeast extract or vitamin components of the medium (Chapter II 3.2.1). Most of the comparison strains have been described to utilise organic or amino acids for aerobic growth but have not been observed to metabolise carbohydrates (McClung *et al.*, 1983; Neill *et al.*, 1985).

c) Electron acceptors and fermentation ability

The common electron acceptor used by members of the *Arcobacter* genus other than oxygen is nitrate. *A. nitrofigilis* can also use aspartate and fumarate as electron acceptors, *A. mytili* also uses aspartate (fumarate was not tested). Strain F17IX was not tested for the above but showed growth on ferric citrate, DMSO, and TMAO supplemented with acetate or lactate.

d) Vitamin requirements for *Arcobacter* sp. F17IX

Strain F17IX was not investigated in regard to vitamin requirements. The YPGL medium contains 2 ml l<sup>-1</sup> of a 10x vitamin solution (Chapter II 3.3.8). Strain NA105

was tested for growth without vitamins and found to be prototrophic. There was no information about the vitamin requirements in the publications of the *Arcobacter* type strains as they generally require complex media containing yeast extract or other complex additions it is likely they require some vitamins.

### 3. Discussion

During IODP Leg 307 for the first time an entire cold-water coral carbonate mound was investigated. Webster *et al.* (2009) reported the presence of a diverse microbial community and low consistent activities based on total cell counts (AODC, CARD-FISH), activity measurements, and culturability (MPN). The principal objectives of this part of the project were to

- Isolate sedimentary microorganisms, initially enriched by the aforementioned MPN dilution series or direct enrichments.
- Characterise the isolates using environmentally relevant tests.
- Compare and contrast the results from different isolates to one another and to close phylogenetic relatives.

#### 3.1 Cold-water coral carbonate mounds – a significant seafloor habitat?

Prior to IODP Leg 307 and the report of Webster *et al.* (2009) about microbial communities associated with the Challenger Mound other marine environments containing carbonates have been studied (Pimenov *et al.*, 1997; Aloisi *et al.*, 2002; Lein *et al.*, 2002; Michaelis *et al.*, 2002; Heijs *et al.*, 2006). Although carbonate precipitates can form chemically in the presence of metal ions ( $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ ) and  $\text{HCO}_3^-$  (Burton, 1993) many of these studies showed that microorganisms mediate and possibly accelerate this process aerobically as well as anaerobically by producing  $\text{CO}_2$ . Carbon dioxide dissolved in water forms carbonic acid, which dissociates and actually leads to the dissolution of carbonates unless protons are removed. This

removal is possibly mediated by sulphate reduction, which causes increased alkalinity, thus  $\text{HCO}_3^-$  remains. This molecule then precipitates as, for example,  $\text{CaCO}_3$ . At some sites anaerobic oxidation of methane (AOM) was observed and this process is thus assumed to be one of the major driving forces in carbonate precipitation (Heijs, *et al.*, 2006) providing the necessary high concentrations of bicarbonate concomitant with proton removal. Besides AOM, other microbial metabolisms also produce  $\text{CO}_2$  (e.g. metal ion reduction) and therefore would contribute not only to carbonate production but also towards its precipitation.

Thriving communities of  $\gamma$ - and  $\delta$ -*Proteobacteria* have been found at carbonate crusts associated with mud volcanoes in the Mediterranean (Heijs *et al.*, 2006). These crusts are seen as sinks for  $\text{CO}_2$  and may thus be relevant with respect to climate change (Petit *et al.*, 1999) but they are of particular interest to this project as the mud volcanoes at which they occur are constantly seeping methane into bottom waters. Molecular investigations revealed the presence of ANME 1 and ANME 2 sequences and the presence of *Desulfosarcina*-related sulphate-reducing bacteria using 16S rDNA and lipid biomarker analysis. These organisms are typically associated with AOM (Boetius *et al.*, 2000) what is supported by an isotopic analysis of the carbonates within these crusts suggesting they originated from AOM (Heijs *et al.*, 2006).

Although hydrocarbon (methane) seepage was also present at the Challenger Mound (Hovland *et al.*, 1994) no sequences of ANME 1 or ANME 2 were detected by Webster *et al.* (2009). However, the authors inferred from the geochemical gradients that at least some AOM should be present. In addition to methane, ethane and propane were also detected below the mound base (Mangelsdorf *et al.*, 2009) representing potential substrates for sulphate reduction and possibly contributing to carbonate formation.

Although Ferdelman *et al.* (2006) stated that the formation of the Challenger Mound was not due to microbially produced carbonate, they considered that microbial activity may have contributed to secondary cementation reactions. The prokaryotic activities were certainly present at the Challenger Mound (Webster *et al.*, 2009). This together with the isolation of several strains, which are capable of metal reduction (e.g. *Shewanella* spp.) and are thus able to produce alkalinity, may indicate the possible involvement of microorganisms in the build-up of these geological features.

### 3.2 Isolation of *Bacteria* from sediment layers with elevated microbial activities

In the summary of the results from Webster *et al.* (2009) (see Appendix) it was shown that at all sites (within and below the mound) local maxima for cell counts (qPCR, CARD-FISH, MPN) and/or elevated activities (radiotracer experiments) were present. It is therefore not surprising that isolates were predominantly obtained from these layers.

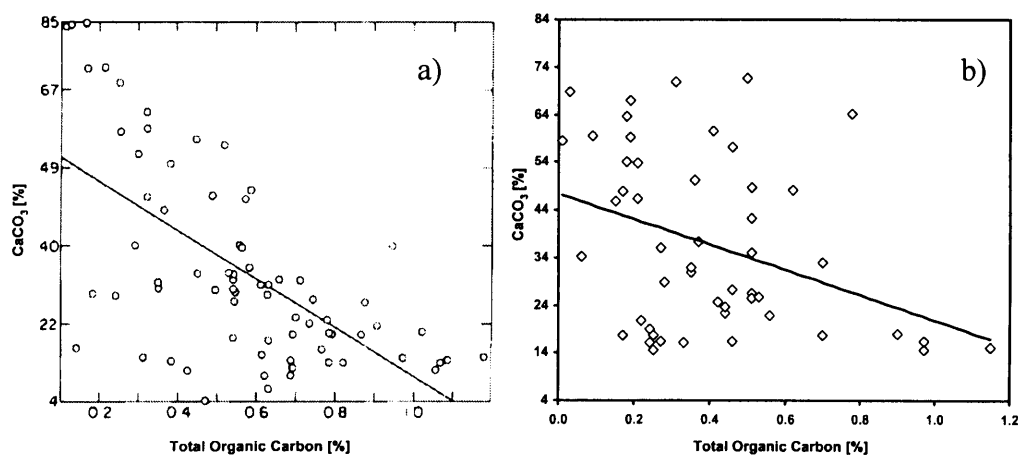
At the Flank site, for example, three *Shewanella* strains (F16III-V) were isolated from -47.9 mamb. This sediment horizon showed local maxima for cell concentrations determined by qPCR ( $2 \times 10^7$  cells  $\text{cm}^3$ ; Fig. A3.1), culturability (MPN for heterotrophs  $7 \times 10^4$  cells  $\text{cm}^3$ ; Fig. A3.1), and a maximum of thymidine incorporation rates of the entire sediment column at this site concomitant with a local maximum for acetate oxidation (Fig. A3.4). In the performed substrate tests these isolates were able to utilise acetate as an electron donor (Table A4.2) and hence could have been at least partly responsible for acetate oxidation in radiotracer experiments. *Arcobacter* sp. F17IX presents the deepest isolate obtained from sediments collected during IODP Leg 307. It originates from a sediment layer below the mound base at the Mound site (-108 mamb), which showed a local maximum for CARD-FISH cell counts and thymidine incorporation. And finally, at the Reference site *Ornithinimicrobium* sp. F18IV was obtained from a sediment horizon (22.9 mbsf) showing elevated CARD-FISH based cell counts and a local maximum for thymidine incorporation.

There are, of course, also sediment layers that showed no elevated microbial activities and yet isolates could be obtained. *Vibrio* sp. F17I, for example, was isolated from 71.9 mamb where the thymidine incorporation rate was lowest for all mound samples and no growth occurred in MPNs from this horizon.

Overall 10 of the 19 Leg 307 isolates (including a putatively new species) originate from sediment layers that, in one form or another, showed local activity maxima. Geological interfaces (Parkes *et al.*, 2005) or sediment layers with high total organic carbon such as the Mediterranean sapropels (Coolen *et al.*, 2002) are usually “hot spots” for microbial activities. As far as the geological interfaces between the

Challenger Mound and over- or underlying sediments are concerned no elevated microbial activities were reported for these (Webster *et al.*, 2009; Soffientino *et al.*, 2009). Likewise, most enrichments (and MPN series) inoculated with these sediments were negative and showed, if at all, only weak and unsustainable growth.

Organic matter availability, in turn, may be more important for the physiological state of microorganisms in sediments, their capacity to initiate growth in microbiological media and eventually being obtained in pure culture. For example, Süß *et al.* (2004) reported elevated MPNs from organic matter-rich sapropel layers compared to intermediate layers in Mediterranean sediments and the successful isolation of a relatively large number of pure cultures. It is therefore not surprising that only six isolates from Leg 307 originate from the nutrient-poor carbonate mound itself whereas the remaining strains come from surrounding sediments, which contain up to three times more total organic carbon and only half the amount of carbonate (Table 3.1). An additional problem is the “great plate count anomaly” (Staley and Konopka, 1985), which is the phenomenon that not all microorganisms in a given sample can be brought into laboratory culture. A possible explanation for this is the viable but nonculturable state (Roszak and Colwell, 1987) that prokaryotes might enter upon changing environmental condition. These phenomena, however, will be discussed in more detail in Chapter IV and VI.



**Fig. 3.24: Sedimentary total organic carbon concentrations versus  $\text{CaCO}_3$  concentrations.** a) Marine surface sediments worldwide, modified from Premuzic *et al.*, 1982; b) select depths of IODP Leg 307 sediments from all sites, data from Ferdelman *et al.*, 2009, Mangelsdorf *et al.* 2009, Leonide *et al.*, 2009.

An inverse correlation between carbonate content and TOC has been reported for surface sediments worldwide (Premuzic *et al.*, 1982;  $R^2 = 0.41$ ; Fig. 3.24a).



However, a similar analysis for subsurface sediments from sites of Leg 307 only showed a very weak correlation between these two important factors ( $R^2 = 0.13$ ; Fig 3.24b). Nonetheless these relationships might explain why fewer cultures were obtained from carbonate-rich and thus organic matter-poor sediment layers.

To summarise, tools that assess the *in situ* microbial activity in deep marine sediments such as radiotracer experiments and cell number estimates based on ribosomes (CARD-FISH) or culturability (MPN) might aid in the endeavour of obtaining novel isolates from this barren environment.

### 3.3 Molecular versus cultivation-based diversity

The predominant phylogenetic group isolated from sediment samples of IODP Leg 307 was the phylum *Proteobacteria*. Within this phylum members of the *Gammaproteobacteria* were most abundant with strains belonging to *Shewanella* being the most frequently isolated genus. As Fry *et al.* (2008) reported *Gammaproteobacteria* are among the most abundant bacterial group in marine sediments. It is therefore not surprising that members of this class also dominate the obtained culture collection. Other isolates belong to the *Delta*-, *Epsilonproteobacteria*, and *Actinobacteria*. Molecular analyses by Webster *et al.* (2009) revealed the *in situ* presence of these phyla in sediments from the Challenger Mound sites and the Reference site although none of the obtained sequences matched any of the cultivated isolates.

The molecular investigation showed that a more diverse microbial community is present in the sediments than is reflected by the culture collection obtained during the course of this project. The latter lacks, for example, members of the *Alpha*-, *Betaproteobacteria*, *Firmicutes*, and *Chloroflexi*, all of which were present in at least some layers (e.g. *Alphaproteobacteria*) or dominated certain horizons (e.g. *Betaproteobacteria*; see A3.4).

*Epsilonproteobacteria* were not detected using molecular biological techniques yet *Arcobacter* sp. F17IX was isolated from -108 mamb at the Mound site.

This once more highlights the discrepancy between results of molecular and of cultivation-based surveys as previously reported for marine and other environments (Santegoeds *et al.*, 1996; Suzuki *et al.*, 1997; Eilers *et al.*, 2000a, b; Lanoil *et al.*, 2000; Ranchou-Peyruse *et al.*, 2006). Furthermore, Parkes and Sass (2009) stated that the microbial community composition of deeply buried sediments assessed with molecular tools differs from surface sediments whereas cultivation-based methods tend to detect similar genera. This is certainly true for the culture collection obtained here. All isolates are closely related to previously isolated strains or described species (> 97% sequence similarity; Table 3.3). When analysed phylogenetically, all isolates affiliated with known genera and only one isolate possibly constitutes a new species. There are, however, also studies where molecular biologically detected strains could be brought into laboratory culture (e.g. Sass, *et al.*, 2001; Bruns *et al.*, 2002; Süß *et al.*, 2006). Biases introduced when assessing the microbial diversity, for example, by the low resolution of the commonly applied DGGE (Muyzer *et al.*, 1993) combined with PCR biased amplification of 16S rDNA genes (Speksnijder *et al.*, 2001), might lead to an underestimation of the actual diversity. Thus, many microorganisms might evade molecular biological detection (Wintzingerode *et al.*, 1997) whilst being readily cultivated.

### 3.4 Ecological significance and potential *in situ* activity of isolates

All isolates obtained from IODP Leg 307 were subjected to a polyphasic taxonomic study including phylogenetic (see 2.1) and physiological analyses (see 2.2). Certain strains were selected for polar lipid analysis and sent to the German Research Centre for Geosciences. Unfortunately, to date the results have not yet been received. In this section, however, the ecological role and potential *in situ* activity of the novel isolates are discussed.

All isolates grew over a wide range of temperatures, pH and salinities. As growth occurred at temperatures less than 20°C and most strains exhibited the ability of growth at less than 2°C they can be classed as psychrotroph. This, together with the fact that most strains required salinities of 3.5% and above, indicates that the isolates were indigenous to the marine environment and capable of growth under *in situ*

conditions. Two further findings support the potential *in situ* activity of the isolates. Firstly, the widespread ability of isolates to produce extracellular enzymes suggests that they potentially hydrolyse extraneous organic matter and thus provide themselves and possibly others with substrate (Kobayashi *et al.*, 2008). Secondly, a number of isolates were obtained from sediment layers showing elevated microbial activities suggesting their potential *in situ* activity.

#### 3.4.1 Ecological significance of *Ornithinimicrobium* sp. F18IV

Prior to this study strains belonging to the genus *Ornithinimicrobium* have been isolated from the L'Atalante Basin sediment (Mediterranean Sea) but these were closely affiliated to already described species ( $\geq 99\%$ ; Dr. Andrea Sass, personal communication). Isolate F18IV, however, is the first marine strain of the genus *Ornithinimicrobium* with a distinct phylogenetic relationship and a different physiology compared with other species of the genus (Table A4.1). The three validly described species of the genus were isolated from soil (Mayilraj *et al.*, 2006; Groth *et al.*, 2001) or activated sludge (Liu *et al.*, 2008).

The presence of *Actinomycetales* in marine sediments has been known as early as 1969 when Weyland (1969) investigated sediments from North Sea and Atlantic Ocean. Despite this report it was widely accepted that isolated *Actinomycetales* were not indigenous to the marine environment but merely revived cells of terrestrial origin (Cross, 1981). Mincer *et al.* (2002), however, reported the widespread occurrence of a major taxon of *Actinomycetales* (designated MAR1) within marine sediments from three distinct locations. These isolates were classified as obligate marine due to their  $\text{Na}^+$  requirement for growth and later assigned to the genus *Salinispora* (Family: *Micromonosporaceae*; Mincer *et al.*, 2005).

Since then a number of *Actinomycetales* have been isolated from the marine environment (Colquoun *et al.*, 1998a, b; Maldonado *et al.*, 2005; Cho *et al.*, 2006; Lee, 2007; Stevens *et al.*, 2007). Recently, two members of the genus *Serinicoccus* (family *Intrasporangiaceae*) were obtained from the marine habitat. *S. marinus* (Yi *et al.*, 2004) originated from the waters of the East Sea (Korea) whereas *S. profundus* (Xiao *et al.*, 2011) was isolated from deep marine sediments in the Indian Ocean.

Promising novel cultivation techniques using sediment gradient cultures, however, have led to the increased enrichment of *Actinomycetales* (e.g. *Micrococcus* spp.) from Mediterranean Sea sediments indicating that members of this phylum may prefer complex compounds for growth (Jacqueline Süß, PhD Thesis, 2006, Carl von Ossietzky Universität, Oldenburg, Germany). Furthermore, *Actinomycetales* isolates are often categorised as strict aerobes. During starvation-survival experiments (Chapter IV), however, anaerobic metabolic activity was detected in both *S. marinus* and *Ornithinimicrobium* sp. F18IV. Under anaerobic conditions no pellet formation occurred, indicating low growth yields. Hence anaerobic growth might often be overlooked as cultures do not produce enough biomass to cause turbidity in the media.

So far though, the ecological role and significance of *Actinomycetales* in marine sediments remains unclear, also hindered by the fact that these microorganisms evade detection with molecular biological techniques (Mincer *et al.*, 2005; Parkes and Sass, 2009).

### 3.4.2 Ecological *in situ* significance of isolated *Proteobacteria*

#### a) *Shewanella*

Isolates belonging to the genus *Shewanella* dominated the culture collection obtained from IODP Leg 307. Members of this genus have been isolated from a variety of habitats including the marine environment (DeLong *et al.*, 1997; Nogi *et al.*, 1998a, Ivanova *et al.*, 2003) and are not uncommon in marine sediments (Venkateswaran *et al.*, 1998; Toffin *et al.*, 2004a; Köpke *et al.*, 2005). They can grow at a range of temperatures (Ghosh *et al.*, 2003; Bozal *et al.*, 2009) and use a variety of electron acceptors (Nealson and Scott, 2006). Furthermore a number of species have been described as psychrophilic as well as piezotolerant (Nogi *et al.*, 1998a; Toffin *et al.*, 2004a). The adaptation of *Shewanella* strains to life in marine sediments has been confirmed during this study. Their widespread occurrence in this harsh environment has been extended by the isolation of four strains from sediment samples of the carbonate Challenger Mound.

b) *Vibrio* and *Photobacterium*

Similarly to *Shewanella*, members of the genera *Vibrio* and *Photobacterium* are typical inhabitants of the marine environment (Farmer and Hickman-Brenner, 2006). Hence their isolation from sediment samples is not surprising. Members of these genera have frequently been obtained from anoxic sediments around the globe (Nogi *et al.*, 1998b; Biddle *et al.*, 2005; Köpke *et al.*, 2005; Batzke *et al.*, 2007; Süß *et al.*, 2008; Freese *et al.*, 2009). It was shown that isolates are adapted to their habitat by their ability to grow at low temperatures (Nogi *et al.*, 1998b; Biddle *et al.*, 2005; Süß *et al.*, 2008) and at elevated pressures (Nogi *et al.*, 1998b). It is further assumed that these genera thrive in their environment as they are nutritional versatile and facultatively anaerobic (Süß *et al.*, 2008) and produce extracellular enzymes (Biddle *et al.*, 2005), which degrade organic matter and aid survival. The here isolated strains are no exception to these characteristics and thus represent further examples of these genera being active and viable members of marine sediment microbial communities. Furthermore, with *Vibrio* sp. F17IV a representative of this genus was now isolated from a sediment layer within a cold-water coral carbonate mound.

c) *Raoultella*

Assessing the ecological role of *Raoultella* species in the environment is difficult as the genus is relatively young (Drancourt *et al.*, 2001) and species were previously classified in the genus *Klebsiella*. Strains of this genus have been found in a variety of habitats (for review see Brisse *et al.*, 2006) including the marine environment where they have been associated with nitrogen fixation (Werner *et al.*, 1974; Jones and Rhodes-Roberts, 1980). One particular strain was isolated from soil and is potentially methanotrophic (Zhao *et al.*, 2009). Strain BAL286 (Bostrom *et al.*, 2007; Fig 3.5), probably belonging to the species *R. ornithinolytica*, was isolated from coastal waters in the Baltic Sea and linked to acetylene reduction in the presence of nitrogen.

The physiological data of strain F17II and F17VIII indicate their adaptation to the marine environment. Especially their ability to grow at low temperatures, their wide range of substrates (mostly sugars and organic acids), their versatile production of hydrolytic enzymes and high growth yields in the absence of oxygen makes them

well adapted to the marine environment and seafloor sediments. To the best of my knowledge strains F17II and F17VIII represent the first isolates from deep subsurface sediments with strain F17IV being the first representative of this genus to be obtained from a cold-water coral carbonate mound

d) *Pseudomonas* and the presence of strict aerobes in anoxic marine sediments

Moore *et al.* (2006) described the genus *Pseudomonas* as “truly ubiquitous” as a result of its genetic and metabolic adaptability and thus it inhabits soil and water environments as well as animal and plant tissue. The only limitation, however, appears to be the need for oxygen, which is why they are not prominent in anoxic environments although sometimes nitrate can be used for anaerobic growth and arginine and pyruvate fermentation have been observed in some species (Moore *et al.*, 2006). Despite this, *Pseudomonas* strains have been detected in anoxic sediments by molecular (Fry *et al.*, 2008) and anaerobic cultivation methods (Parkes *et al.*, 2009).

The presence of strictly aerobes in oxygen-depleted marine sediments raises legitimate concerns about their environmental role. In the case of strictly aerobic isolates from IODP Leg 307 no spore formation was observed, which indicates that the strains must be either be surviving in a vegetative state in the sediment or fulfilling microbial niches that remain elusive for the moment. The two *Pseudomonas* isolates did not grow under anaerobic conditions during the phenotypical characterisation. The reference strains of these isolates (Table A4.5) are, however, able to utilise nitrate anaerobically, which was not observed for strains F18II and F18V.

Recently a few examples for anaerobic metabolisms in *Pseudomonas* were reported. Wolterink *et al.* (2002) isolated *P. chloritidismutans*, a species able to reduce chlorate, from a bioreactor. Nitrogen fixation has been reported for strains of *P. stutzeri* and *P. aeruginosa* (Moore *et al.*, 2006). Additionally, *Pseudomonas* related isolates have been obtained from deep sea sediments prior to this study (Takami *et al.*, 1997; Toffin *et al.*, 2004b; Parkes *et al.*, 2009). This shows that the “strictly aerobic” genus *Pseudomonas* is not necessarily confined to aerobic environments. Thus it is probably better to state that the aerobic isolates from

IODP Leg 307 were strictly aerobic only under the conditions tested but might have been able to utilise certain compounds anaerobically if these had been investigated.

e) *Desulfovibrio*

Strain F16I was isolated from sediments overlying the Challenger Mound at the Flank site where active sulphate reduction occurred (54.1 mamb; Fig. A3.1) and *dsrAB* copy numbers were relatively high ( $4.8 \times 10^4$  *dsrAB* copies  $\text{cm}^{-3}$  sediment, data from Webster *et al.*, 2009). This isolate represents the only obligate anaerobe obtained from sediments of IODP Leg 307. It belongs to the genus *Desulfovibrio* (Family *Desulfovibrionaceae*) within the delta subgroup of the *Proteobacteria*. Members of this genus carry out sulphate reduction, which is an important terminal oxidation process in anoxic marine sediments (Parkes *et al.*, 2000, D'Hondt *et al.*, 2004).

Sulphate reduction is carried out by a physiologically and phylogenetically diverse group of microorganisms (Devereux *et al.*, 1989) that are able to use more electron acceptors than “just” sulphate. The reduction of nitrate, nitrite (Widdel and Pfennig, 1982), Fe(III) (Coleman *et al.*, 1993), uranium(VI) (Lovley and Phillips, 1992), chromate (Lovley and Phillips, 1994), arsenate (Newman *et al.*, 1997) and Mn(IV) (Tebo and Obraztsova, 1994) by sulphate-reducing species have been observed. A study by Cypionka (2000) showed that some of these organisms not only tolerate but even use oxygen coupling it to ATP conservation. Aerobic growth, however, is low or absent and the consumption of oxygen appears to have protective functions (Cypionka, 2000). Nevertheless, these findings could explain the presence and activity of *D. oceanii* subsp. *galatae* in oxygen containing waters (Finster and Kjeldsen, 2010).

The presence of sulphate reducers in marine sediments has been known for some time due to pure cultures (van der Maarel *et al.*, 1996; Bale *et al.*, 1997; Takii, 2008), measurements of sulphate reduction rates (Elsgaard *et al.*, 1994; Parkes *et al.*, 2005) and molecular studies (Devereux and Mundfrom, 1994; Teske *et al.*, 1996). The detection of these microorganisms with 16S rDNA as the target is low (Parkes *et al.*, 2005; Schippers and Neretin, 2006) hence more specific primers are often needed. Targeting functional genes such as the *dsrAB* gene (Wagner *et al.*, 1998) proved to be a successful strategy and was applied to marine sediments (Webster *et*

al., 2006a, b; Wilms *et al.*, 2007). Sequences of the *dsrAB* gene were detected at all three sites of IODP Leg 307 and throughout the entire sediment column indicating the existence of sulphate reducing prokaryotes even in zones where no or only little sulphate reduction occurred (Webster *et al.*, 2009). Yet strain F16I is the only sulphate reducing isolate from sediments of IODP Leg 307, which indicates that other sulphate-reducers evaded enrichment and subsequent isolation most likely due to “inappropriate” cultivation media.

f) *Arcobacter* sp. F17IX

*Epsilonproteobacteria* have been frequently detected in marine sediments using clone libraries but are often less abundant than other members of the *Proteobacteria* (Fry *et al.*, 2008). A few studies also detected strains affiliated to the genus *Arcobacter* in other marine environments (Llobet-Brossa *et al.*, 1998, Eilers *et al.*, 2000b; Thamdrup *et al.*, 2000; Wirsen *et al.*, 2002; Bowman and McCuaig, 2003; Fera *et al.*, 2004; Omoregie *et al.*, 2008). Members of this genus have been connected to nitrogen (McClung *et al.*, 1983; Wirsen *et al.*, 2002; Donachie *et al.*, 2005) and sulphur cycling (Teske *et al.*, 1996; Wirsen *et al.*, 2002) thus representatives from deep marine sediments might be involved in the biogeochemical cycles of these elements.

The oxygen sensitivity of the genus *Arcobacter* implies their preference for dysaerobic environments and their occurrence in deep sea sediments is thus not surprising. They also show a diversity of metabolic properties, which has not been fully assessed for strain F17IX. For example, the ability of Candidatus *A. sulfidicus* to fix CO<sub>2</sub> and nitrogen and produce filamentous sulphur from H<sub>2</sub>S (Wirsen *et al.*, 2002) was not tested for strain F17IX. These processes might be ecologically important and could potentially contribute significantly to primary production (Wirsen *et al.*, 2002).

### 3.4.3 Frequent isolation of facultative anaerobes from anoxic sediments

Only one strictly anaerobic (*Desulfovibrio* sp. F16I) and one microaerophilic strain (*Arcobacter* sp. F17IX) were amongst the isolates from IODP Leg 307. The



remaining isolates are either facultative anaerobes (14 strains) or even strict aerobes (*Pseudomonas* spp. and *Ornithinimicrobium* sp. F18IV).

Facultative anaerobes have been frequently isolated from deep marine sediments (Ivanova *et al.*, 2003; Süß *et al.*, 2004; Köpke *et al.*, 2005). Although the final isolation steps and the maintenance of IODP Leg 307 strains occurred aerobically (except for *Desulfovibrio* sp. F16I) the initial enrichments and parts of the subsequent isolation were performed anaerobically. This and the ability of many strains to grow in the absence of oxygen suggest that their potential activity in deeply buried marine sediments. To support this, alternative electron acceptors were tested during the phenotypic characterisation of the isolates. It was shown that especially *Shewanella* and *Raoultella* strains were able to use manganese oxide and nitrate as alternative to oxygen (Table A4.2 and A4.4). *Vibrio* and *Photobacterium* isolates on the other hand did not use many of the electron acceptors provided. These genera are known for their ability to ferment glucose. An electron acceptor is thus not necessary if sedimentary humic acids serve as sinks for electrons (Lovley *et al.*, 1996; Benz *et al.*, 1998). In conclusion, facultative anaerobes potentially play an important role in anoxic deep subsurface sediments.

#### 3.4.4 Are IODP Leg 307 isolates involved with *in situ* hydrogen metabolism?

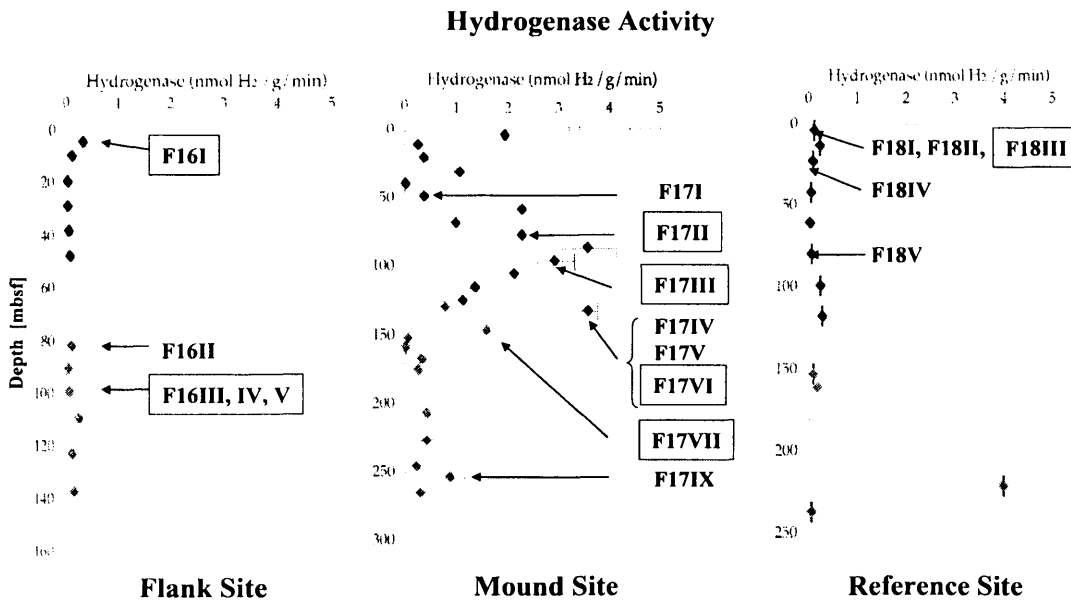
Soffientino *et al.* (2009) investigated hydrogenase activities in sediments of IODP Leg 307 and found it to be present in all samples tested (Fig. 3.25). They further reported that mean activities were greater at the Challenger Mound site compared with the Flank and Reference site. The latter two sites did not significantly differ from each other.

Hydrogenase is a membrane-bound or intracellular enzyme that enables the formation or lysis of molecular hydrogen. It is thus essential in microorganisms that either produce or metabolise this electron donor (Adams, 1990; Vignais *et al.*, 2001). Molecular hydrogen is an important intermediate in most anaerobic communities (Wolin, 1982) and connects fermenting with terminal oxidation processes (Lovley and Klug, 1982). Hydrogen is an important substrate for methanogens (next to acetate) and also serves as electron donor for sulphate reducers who are suspected to outcompete methanogens for this substrate under *in situ* conditions (Lovley and

Klug, 1983). It is possibly involved in AOM as intermediate between the sulphate reducing and the methanogenic members of AOM consortia (Boetius *et al.*, 2000),

A number of Leg 307 isolates (especially the genera *Shewanella* [Table A4.2] and *Raoultella* [Table A4.4]) produced hydrogen under anaerobic conditions most likely during glucose fermentation and thus must possess the responsible enzyme hydrogenase. *Desulfovibrio* sp. F16I also produced hydrogen in low amounts during growth on lactate and in the presence of peptone and yeast extract but more importantly was found to be able to use it as substrate in the presence of acetate

These data indicate that isolates from Leg 307 might have been partly responsible for hydrogenase activities detected by Soffientino *et al.* (2009) and may thus be contributing to the production of this important metabolite in anoxic sediment layers thereby possibly stimulating growth in methanogens and/or sulphate reducers. This underlines that the Challenger Mound is inhabited by an active microbial community



**Fig. 3.25: Correlation between Origin of Isolates and Hydrogenase Activities in Sediment layers of IODP Leg 307.** a) Flank Site U1316; b) Mound Site U1317; c) Reference Site U1318; strains that produced molecular Hydrogen under anaerobic conditions are highlighted by a surrounding box; data points with different shadings have been obtained from holes cores of the respective sites, modified from Soffientino *et al.* (2009).

#### 4. Summary and conclusions

Cold-water coral carbonate mounds are widespread along the European continental margin. Despite low organic matter concentrations these geological features harbour an active microbial population (Webster *et al.*, 2009). As part of this project the phylogeny and physiology of isolates obtained from this harsh environment were examined. Novel strains belonging to previously identified typical deep biosphere microorganisms such as the *Gammaproteobacteria* were obtained. Their potential *in situ* activity was confirmed by the phenotypical characterisation (e.g. temperature range for growth, spectra for substrate and electron acceptor utilisation, and production of extracellular enzymes). These analyses suggest that the isolates represent active and viable members of the *in situ* microbial community present in carbonate mounds.

The isolates were not subjected to experiments regarding their ability to grow at elevated pressures, which should be included in future studies with novel isolates to underpin their origin from and adaptation to the conditions in deep marine sediments.

Isolate F18IV might putatively constitute a novel species within the genus *Ornithinimicrobium* and would be the first representative of this genus isolated from the marine environment and deep subsurface sediments. Further studies, however are necessary to confirm its suggested status as a new species (e.g. GC content, cell wall peptidoglycan, pressure experiments).

## Chapter IV:

# Starvation-survival of deep biosphere prokaryotes compared with near-surface relatives

## 1. Introduction

Microbes inhabiting the natural environment face many challenges to survive and even multiply. These challenges are often thought to be extreme conditions, such as, high or low temperatures, pH, and salinities or even desiccation. One condition, however, that can be considered extreme in its own right is frequent and exists in many habitats: the lack of substrates and nutrients. The absence of substrates is ubiquitous in oligotrophic lakes and rivers, sea waters and marine sediments. Often several growth factors are limited at the same time (e.g. carbohydrates and phosphate) while others are abundant (e.g. electron acceptors like oxygen or sulphate). Substrate limitation in nature is not a rare event but commonplace (Kolter *et al.*, 1993). In fact, probably the only place it does not exist is in rich culture media in microbiological laboratories and even there limitation is rapidly reached and growth slows or stops.

If substrate deprivation is that widespread how then do microorganisms cope with starvation conditions? How do they survive extended periods of time without electron donors, and therefore, energy to maintain an intact, living cell?

In the marine environment prokaryotic abundance is high, especially in marine sediments (Parkes *et al.*, 1994) and although considerable organic matter is often present (1-10 kg carbon per m<sup>3</sup>; Hedges and Keil, 1995), it appears to be only very slowly utilised by microbes (Parkes *et al.*, 2000). Nonetheless, vast numbers of microbes have been found in deep, ancient marine sediments (1626 mbsf, 111 million years, Roussel *et al.*, 2008) possibly containing up to a third of Earth's total living biomass (Whitman *et al.*, 1998). The microbes, however, face constant starvation due to low organic matter input (1 g of carbon per m<sup>2</sup> and year; Jahnke and Jackson, 1992) and low energy fluxes (D'Hondt *et al.*, 2002) thus the phrase

“starving majority” (Jorgensen and D’Hondt, 2006 and title of a workshop in Aarhus, Denmark 2007) is a good description of the *in situ* conditions.

Despite the apparent lack of substrates a significant fraction of deep biosphere prokaryotes have been shown to be active (Coolen *et al.*, 2002; Parkes *et al.* 2005; Schippers *et al.*, 2005) rather than “just” surviving, being dormant or dying slowly. These microorganisms, however, exhibit extremely long division times of 0.2 to 100,000 years (Whitman *et al.*, 1998; Parkes *et al.*, 2000; Schippers *et al.*, 2005; Biddle *et al.*, 2006). Thus life in the deep biosphere is characterised by slow growth and very low energy availability (Coolen *et al.*, 2002), challenging our understanding of minimum energy requirements for prokaryotes to sustain cellular biopolymers and integrity and ultimately life (Jorgensen and Boetius, 2007). With increasing depth, however, cell numbers decrease in marine sediments (Parkes *et al.*, 1994), clearly indicating that there is a time and/or energy limit for withstanding starvation. Jorgensen and Boetius (2007) consider the transitions from live to dead cells a gradual development. Stages of this are i) actively growing, ii) metabolising but not growing, iii) intact cells without metabolic activity, and finally iv) dead cells. This pattern of growth, maintenance, survival was described by Price and Sowers (2004). According to their calculations, cells in marine sediments can repair macromolecular damage but are probably largely dormant.

The VBNC state is an often described example in the literature as a strategy to survive harsh environmental conditions (for recent reviews see Kell *et al.*, 1998; Colwell, 2000; Oliver, 2005, 2010) and has been attributed to changes in environmental conditions (e.g. temperature; Muela *et al.*, 2008; Vattakaven *et al.*, 2006) and/or can be caused by starvation (Amel *et al.*, 2008; Weichart and Kjelleberg, 1996; Vattakaven *et al.*, 2006). This phenomenon might be responsible for the great plate anomaly (Staley and Konopka, 1985), the difference between total and culturable cell counts in environmental samples. After all, and despite many efforts, cultivation efficiencies of environmental prokaryotes remain low (< 1%; Amann *et al.*, 1995; Kaeberlein *et al.*, 2002).

Although prokaryotes in the mainly anaerobic ocean sediments are adapted to the slow pace of life (Kerr, 2002) many studies, especially on the molecular basis of starvation, have been conducted on aerobic, “live fast – die young” bacteria such as *E. coli* (Kolter *et al.*, 1993; Siegele and Kolter, 1992). These studies revealed that cells in the stationary phase are not as inactive as might appear. Various survival

mechanisms such as stationary phase contact-dependent inhibition (SCDI; Lemonnier *et al.* 2008) or growth advantage in stationary phase (GASP; Zambrano and Kolter, 1996) have been identified. Additionally, cellular starvation adaptation mechanisms such as the formation of ribosomal dimers (Ortiz *et al.*, 2010) and production of starvation response genes (Groat and Matin, 1986; Lange and Hengge-Aronis, 1991; Jenkins *et al.*, 1991; Matin, 1991; Loewen and Hengge-Aronis, 1994; Williams *et al.*, 1994) were revealed.

A considerable number of publications on starvation-survival of marine pelagic bacteria are available (Novitsky and Morita, 1976, 1977; Amy and Morita 1983b; Baker *et al.*, 1983; Humphrey *et al.*, 1983; Kurath and Morita, 1983; Johnstone and Stone, 1988a, b; Moyer and Morita, 1989a,b; Albertson *et al.*, 1990; Nyström *et al.*, 1990; Sanchez-Amat and Torrella, 1990; Lebaron and Joux, 1994; Shiba *et al.*, 1995; Eguchi *et al.*, 1996; Joux *et al.*, 1997; Fouz *et al.*, 1998; Fegatella and Caicchioli, 2000; Abdallah *et al.*, 2009; Orchard *et al.*, 2009; Gomez-Consarnau *et al.*, 2010) but so far marine sedimentary prokaryotes who are specialists in surviving extended periods of starvation *in situ* have been largely overlooked (with a few exceptions, starvation period less than one month Marteinsson *et al.*, 1997). The longest period of starvation investigated that I am aware of is the survival of *V. vulnificus*, a marine pelagic strain, over a 20 year period (Doyle and Oliver; Poster at ISME-13, 2010, Abstract number: PS.13.005).

Therefore, as part of this project a variety of different, typical deep biosphere microorganisms were selected and subjected to long-term starvation. Furthermore, isolates from deep and shallow sediment layers were compared to investigate any potential differences in their response to substrate deprivation. The methods used to elucidate the physiology of starving bacteria are widely applied to study *in situ* populations (e.g. FISH and MPN) without really knowing how starved, pure cultures are detected by these measures of viability under laboratory conditions. Additionally, bacteria were studied under different starvation conditions. One treatment contained no substrate (“A”), another was supplemented with a minute amount of substrate (~15  $\mu$ M, “B”), and the third represents the continued incubation of the original culture. These treatments were chosen to investigate any potential effects the complete absence of substrate (“A”) might have compared to a minute amount (“B”) reflecting conditions in many deep sediment layers and which potentially provides some energy to maybe “prepare” for starvation. Treatment “C” is in some respect

similar to marine sediments as extracellular carbon is present but cannot be used by the organisms (metabolic endproducts such as acetate) plus the environment has been modified by the previous prokaryotic activity.

## 2. Results of starvation-survival

The procedure for the onset of starvation is described in Chapter II (3.1.1). As a reminder, cells were grown anaerobically (where possible) in liquid culture until turbid. For starvation treatments “A” and “B” cells were washed and resuspended in substrate and vitamin-free artificial seawater. No amendments were made to “A”. Treatment “B” was supplemented with 15  $\mu$ M of substrate (glucose or lactate; in case of *Arcobacter* sp. F17IX 0.0001% of yeast extract and peptone were added), whereas “C” is the continued incubation of the original culture.

### 2.1 Starvation-survival of *Actinobacteria*

*Ornithinimicrobium* sp. F18IV was isolated from sediments of IODP Leg 307 (see Chapter III). Currently there are no marine species described for this genus. Thus, *Serinicoccus marinus* was chosen as a comparison strain for starvation experiments because of its marine origin and the relatively close phylogenetic relationship between the two genera.

#### 2.1.1 *Serinicoccus marinus*

*Serinicoccus marinus* is a marine pelagic bacterium isolated from the East Sea, Korea (Yi *et al.*, 2004). During starvation no apparent loss of AODC total cell numbers occurred in any of the three treatments over the 270 days of the experiment (Fig. 4.1 a-c). Statistically, no significant change in cell numbers occurred in parallels “A” and “C” but in “B” ( $p < 0.05$ ). Total cell numbers estimated, using the LIVE/DEAD<sup>®</sup> BacLight<sup>™</sup> Bacterial Viability Kit (“live” and “dead” scored cells), resulted in total cell counts continuously below the AODC total count with a marked

decrease after 90 days of starvation. Already after ten days of incubation the percentages of dead cells increased in two (“A” and “B”) of the three cultures and continued to rise until 180 days where there was an average of 88% dead cells in the samples.

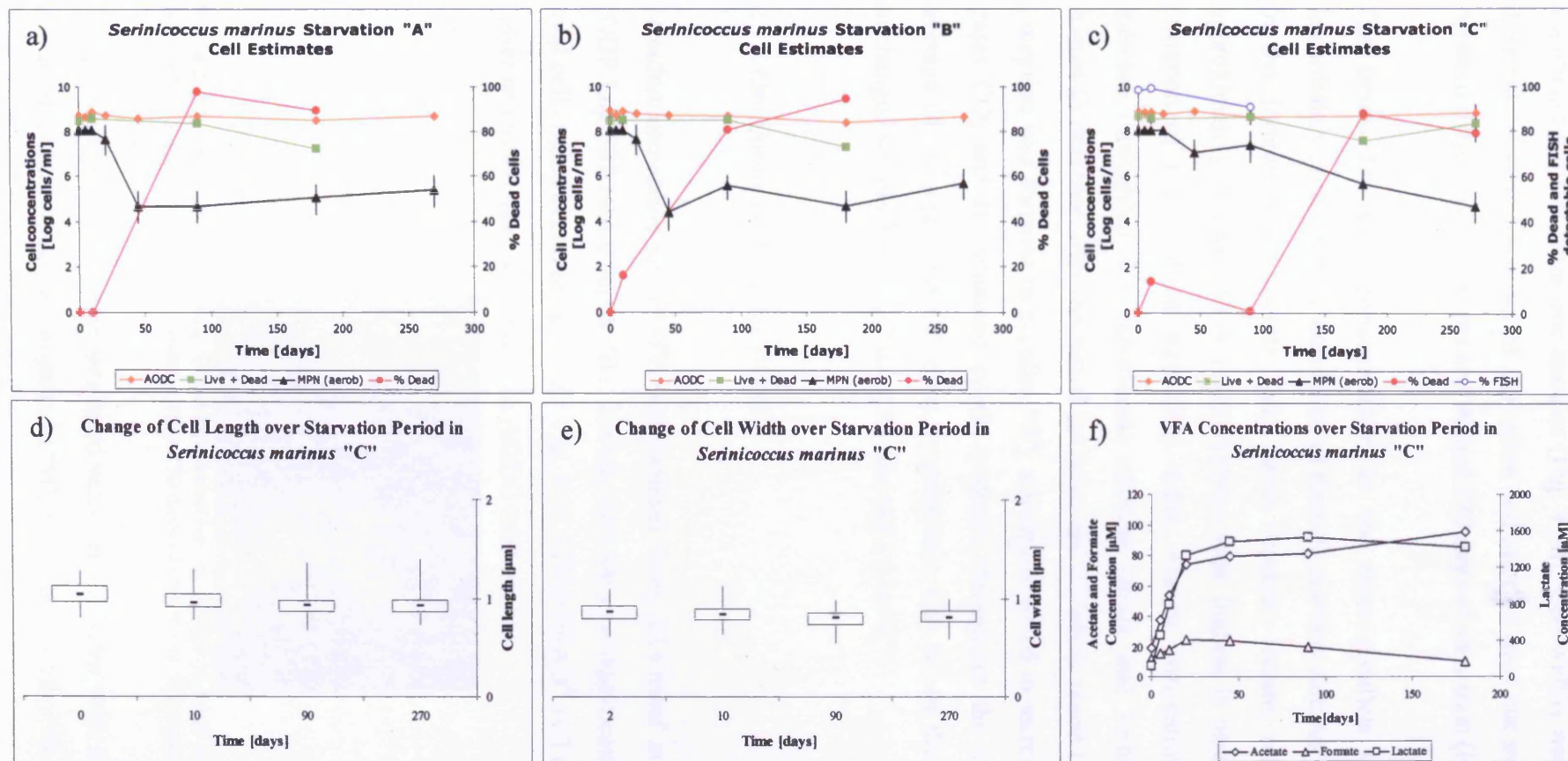
FISH detectability of cells in parallel “C” decreased slightly with continuing starvation (Fig. 4.1c). Unfortunately, for the last sampling point DAPI did not stain any of the cells hence a percentage of hybridised cells compared to the total DAPI count of the sample can not be given. The actual cell count using the probe, however, was higher than at previous sampling points, clearly indicating the presence of viable cells. This problem also occurred several times for other bacterial strains investigated and will be discussed further below.

Culturable cell numbers of *S. marinus* during the first 10 days of starvation exceeded the upper detection limit ( $> 10^8$  cells ml<sup>-1</sup>) as this strain grew in all dilution steps of the MPN dilution series (hence no confidence limits are shown in Fig. 4.1). After ten days culturable cells decreased in “A” and “B” (to  $10^4$  and  $10^5$  cells ml<sup>-1</sup>, respectively) where they remained relatively unchanged until the end of the experiment (Fig. 4.1 a-b). In “C” a less steep decrease over time occurred resulting in a similar number of culturable cells towards the end of the experiment compared to “A” and “B” (Fig. 4.1c). The loss of culturability in *S. marinus* reflects the increasing number of dead cells in the cultures. The time for this species to fully grow in the MPN wells increased from ca. 10 days at the beginning of the experiment to more than 20 days after prolonged starvation.

The lengths and widths of cells were measured for selected samplings (Fig. 4.1 d-e). Shown in the plots are the 25 and 75 percentile (box), the median (horizontal dash) and the data range (vertical dashes). The width of the box has no meaning. This particular type of plot was chosen to show all measured data, which enables the visualisation of changes in the data range. The drawback is that significant changes cannot easily be identified by looking at the graphs but will be described in the text.

Cell sizes decreased significantly during starvation. At the beginning, cells were significantly longer than after 10 days of starvation ( $p < 0.05$ ). Cells at





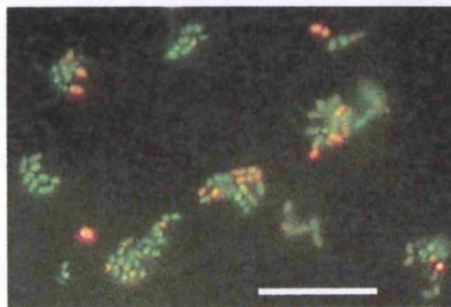
**Fig. 4.1: Physiological responses of *S. marinus* to starvation.** a-c) Cell number estimates for treatments "A", "B", and "C". Confidence limits are given for AODC, Live + Dead, and MPNs, if not visible they fall within the size of the data points. d-e) Change of size during starvation in "C". Shown are the 25 and 75 percentile (box), the median (horizontal dash) and the data range (vertical dashes). f) Changes in VFA concentrations during starvation in "C".

90 and 270 days of starvation were even shorter than after 10 days but did not significantly differ from one another (Fig. 4.1d). Cell widths were not significantly different between the onset of starvation and after 10 days but significantly differed between these two timepoints and 90 and 270 days of starvation (Fig. 4.1e).

The trends for VFA concentrations in the three parallels were very similar. Immediately at the start of starvation all three *S. marinus* cultures showed an increase in the formation of volatile fatty acids (acetate, lactate, and formate) up to approximately 20 days. VFA concentrations were highest in parallel “C” due to the omission of the washing step (Fig. 4.1f). Acetate concentrations continued to increase throughout the experiment whereas lactate and formate concentrations started to decrease after the initial increase and a plateau phase (90 days). The only exception was formate in parallel “B”, which continued to increase. The headspace gases CO<sub>2</sub> and H<sub>2</sub> remained nearly constant throughout the experiment with an average of 10<sup>4</sup> ppm and 10<sup>2</sup> ppm, respectively. The pH of the medium remained unchanged with pH 7.4 in “A” and “B” and pH 6.2 in “C”.

### 2.1.2 *Ornithinimicrobium* sp. F18IV

*Ornithinimicrobium* sp. F18IV was isolated from 22.9 mbsf at Reference site of IODP Leg 307 (see Chapter III). During starvation no significant change in AODC total cell counts occurred ( $p < 0.05$ ; Fig. 4.3). LIVE/DEAD<sup>®</sup> cell counts were always lower and much more variable than AODC total counts.



**Fig. 4.2: Micrograph of starving *Ornithinimicrobium* sp. F18IV stained with the LIVE/DEAD<sup>®</sup> Kit.** Cells from parallel “C” were stained after 270 days of starvation. Bar equals 10  $\mu$ m.

The percentages of dead cells increased with starvation but were relatively variable and are thus not considered reliable. In “B”, for example, after 90 days of starvation

75% of dead cells were detected, whereas after 180 days only 2% of the cells were scored as dead (Fig. 4.2 and 4.3 a-c).

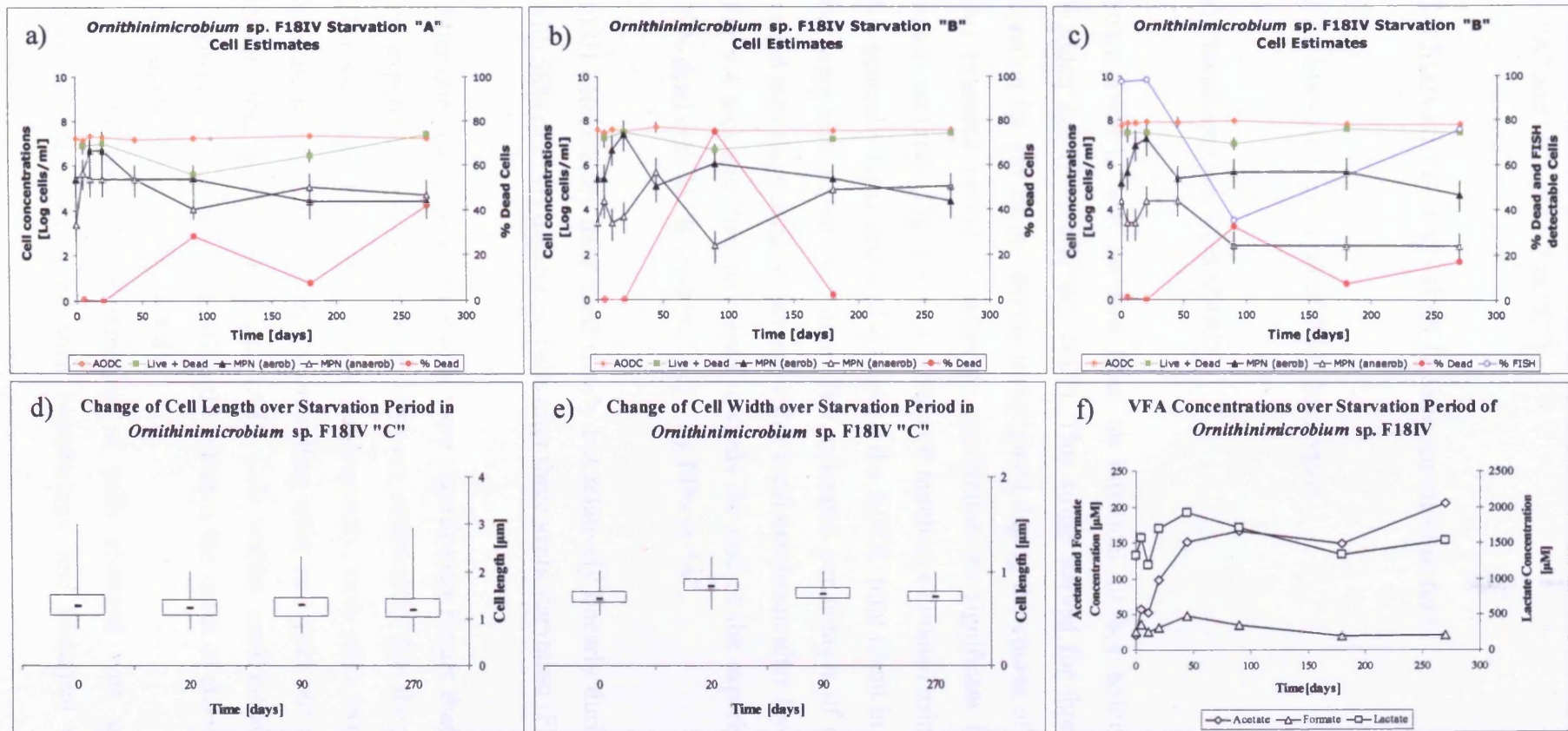
The number of FISH detectable cells in “C” decreased rapidly after 20 days of starvation showing a local minimum (35%) coinciding with a local maximum of “dead” cells (32%) at 90 days but was increased in the final sample. Overall there was a decrease in FISH detectable cells during the experiment from 98% to 76% (Fig. 4.3c).

In the beginning of starvation, culturability in aerobic MPN dilution series increased in all three parallels (Fig. 4.3 a-c). After this initial period (20 days) the number of culturable cells decreased and resulted in  $10^4$  cells ml<sup>-1</sup> after 270 days of incubation. For treatment “C” anaerobic MPNs were consistently lower than the aerobic MPNs (up to three orders of magnitude). Pellet formation, however, was rarely observed but with ongoing incubation the redox indicator changed colour. This suggests metabolic activity of this strain under anaerobic conditions although it was found to be strictly aerobic (Chapter III). With continued starvation strain F18IV required longer incubation times to fully grow in MPNs. Incubation times increased in aerobic MPNs from five days at the beginning to more than 10 days after 11 days of starvation. The time needed for activity to occur in anaerobic incubations increased from more than 10 days to more than 20 days.

The changes in cell sizes for strain F18IV are somewhat confusing. Cells were significantly shorter after 20 and 180 days of starvation than at the beginning but cells measured after 90 days were not. Cells were actually wider after 20 and 90 days than at the beginning of starvation. After 270 days cell widths showed the same mean as at the start of starvation.

Lactate and formate concentrations in the three cultures decreased during starvation whereas acetate showed different profiles in the different treatments. After 180 days acetate was completely depleted in “A” and declined in parallel “B”. In contrast, acetate consistently increased during starvation in “C”, especially in the first 50 days and then again after 180 days (Fig. 4.3f). Headspace gas concentrations (CO<sub>2</sub> and





**Fig. 4.3: Physiological responses of *Ornithinimicrobium* sp. F18IV to starvation.** a-c) Cell number estimates for treatments "A", "B", and "C". Confidence limits are given for AODC, Live + Dead, and MPNs, if not visible they fall within the size of the data points. d-e) Change of size during starvation in "C". Shown are the 25 and 75 percentile (box), the median (horizontal dash) and the data range (vertical dashes). f) Changes in VFA concentrations during starvation in "C".

H<sub>2</sub>) and the pH of the medium remained relatively constant during starvation (pH 7.3 in “A” and “B”; pH 6.2 in “C”).

## 2.2 Starvation-survival of *Gammaproteobacteria*

### 2.2.1 Starvation-survival of *Photobacterium*

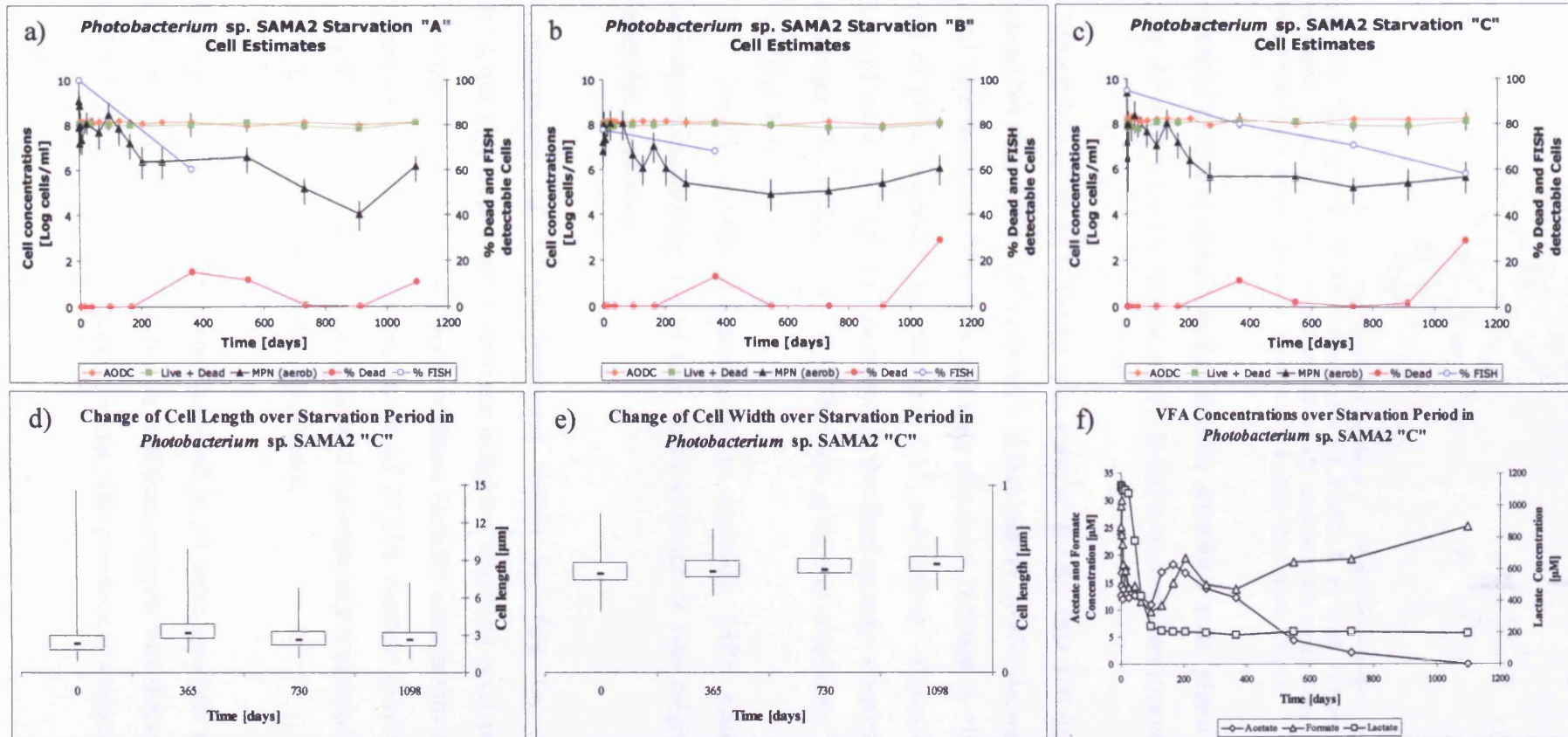
#### a) *Photobacterium* sp. SAMA2

Strain SAMA2 was isolated from an intertidal surface sediment of the German Wadden Sea (Freese *et al.*, 2009). This strain starved for three years, the longest duration for any of the strains investigated during the course of this project. During this extended period of substrate deprivation no significant loss of AODC total counts occurred (Fig. 4.4 a-c). Total cell numbers estimated using the LIVE/DEAD<sup>®</sup> Kit generally remained slightly below the AODC total count in all three treatments, but were also stable over time. The estimated percentages of dead cells, however, varied over the starvation period with a local maximum after 1 year (average of 13%; Fig. 4.4 a-c) and then increased towards the end of the experiment reaching up to 29% dead cells in “B” and “C” but only 11% in “A”.

FISH detectability decreased slowly but relatively linearly during starvation in “C” with 58% of FISH detectable cells after three years starvation (Fig. 4.4c).

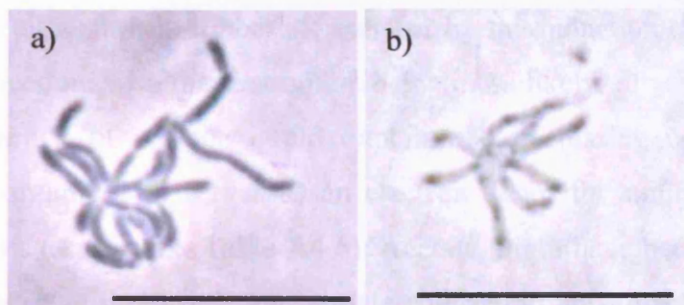
After one year of starvation cells were significantly longer than at the beginning of the experiment ( $p < 0.05$ ). It is, however, noteworthy that the range of cell lengths decreased with time (Fig. 4.4d). Subsequently, cells after two and three years of starvation were significantly shorter than after one year but did not significantly differ from each other (Fig. 4.4d). Cell widths continuously increased during starvation and differed significantly between the onset of starvation compared with two and three years (Fig. 4.4 d).

The intracellular structure of cells changed with continued starvation. Although cells preserved their morphology and remained relatively long they



**Fig. 4.4:** Physiological responses of *Photobacterium* sp. SAMA2 to starvation. a-c) Cell number estimates for treatments "A", "B", and "C". Confidence limits are given for AODC, Live + Dead, and MPNs, if not visible they fall within the size of the data points. d-e) Change of size during starvation in parallel "C". Shown are the 25 and 75 percentile (box), the median (horizontal dash) and the data range (vertical dashes). f) Changes in VFA concentrations during starvation period in parallel "C".





**Fig. 4.5: Micrographs of *Photobacterium* sp. SAMA2.** a) fresh culture; b) after three years of starvation. Although cells in b) are still relatively long they are much more translucent and appear to have areas of higher density often at poles of the cells. Bars equal 10  $\mu\text{m}$ .

appeared fainter or more translucent after starvation under phase contrast microscopy (Fig. 4.5 a-b), which indicates autolysis and a concomitant loss of cell density.

Culturability of strain SAMA2 was variable for the first 100 days of starvation and ranged between  $10^7$  to  $10^8$  cells  $\text{ml}^{-1}$ . Subsequently, MPNs decreased to  $10^5$  cells  $\text{ml}^{-1}$  until approximately 270 days and then remained constant in “B” and “C” until the end of the experiment. In treatment “A”, culturability decreased further until 912 days of starvation but was increased in the final sample comparable to the other two treatments. Thus variability of MPNs was greater in treatments “A” than in “B” and “C” (Fig. 4.4).

Strain SAMA2 did not grow in anaerobic MPN plates despite extended periods of incubation (up to one year) although it was originally isolated under anaerobic conditions.

Concentrations of VFAs decreased within the first 96 days of starvation. Subsequently, lactate concentrations remained relatively constant at around 200  $\mu\text{M}$  until the end of the experiment, whereas formate concentrations slightly increased during this time reaching a maximum of 25  $\mu\text{M}$ . Acetate showed a local maximum (18  $\mu\text{M}$ ) after 165 days of starvation and subsequently decreased below the detection limit (0.2  $\mu\text{M}$ ) at the end of the experiment.

Headspace  $\text{CO}_2$  concentrations increased in all three parallels (between  $2 \times 10^3$  and  $1.6 \times 10^4$  ppm) during starvation. In addition, oxygen was detected in parallels “B” and “C” from at least 1.5 years onwards. The presence of oxygen in the samples can

be explained by either brittle rubber stoppers or by the induction of oxygen during the sampling procedure with the first option being more likely.

The presence of oxygen could explain the decreasing concentrations of acetate, as this organic acid serves as an electron donor for strain SAMA2 under aerobic conditions (Appendix, Table A4.6). Acetate might have been consumed and formate and CO<sub>2</sub> were produced. The acetate concentrations, however, were minute (25 µM) and the possible energy obtained from the degradation would be minimal yet potentially sufficient to maintain cellular activity and integrity. Additionally, the slow disappearance indicates a very slow consumption possibly limited to the slow diffusion of oxygen into the vials.

In contrast, lactate was not completely degraded under these conditions (~200 µM remaining) although strain SAMA2 is able to grow on lactate under aerobic conditions (Appendix, Table A4.6). Why lactate was not used is uncertain but this finding could indicate a lack of activity contradicting the limited possible acetate metabolism.

The pH of the cultures slowly decreased during starvation from pH 7.1 to pH 6.5 in parallels “A” and “B” and from pH 6.8 to pH 6.4 in parallel “C” between one and three years of starvation.

#### b) *Photobacterium* sp. NA42

Strain NA42 was isolated from an intertidal surface sediment of the German Wadden Sea (Sass, unpublished). A significant change in AODC total counts occurred in parallels “A” and “B” during starvation ( $p < 0.05$ ; Fig. 4.6 a-c). In the final samples of these treatments the total cell counts were 21.5% and 29.6% of the initial total counts in “A” and “B”, respectively. No significant change of cell numbers occurred in “C”. Total cell numbers using the LIVE/DEAD<sup>®</sup> Kit were comparable to those of AODC for all cultures (Fig. 4.6 a-c). Dead cells were present in all samples examined and were always higher in “A” and “B” (6.7% to 25.5%) than in “C” (1.8% to 5.3%). The relative numbers of dead cells in the parallel cultures is consistent with the decrease in total cell numbers.



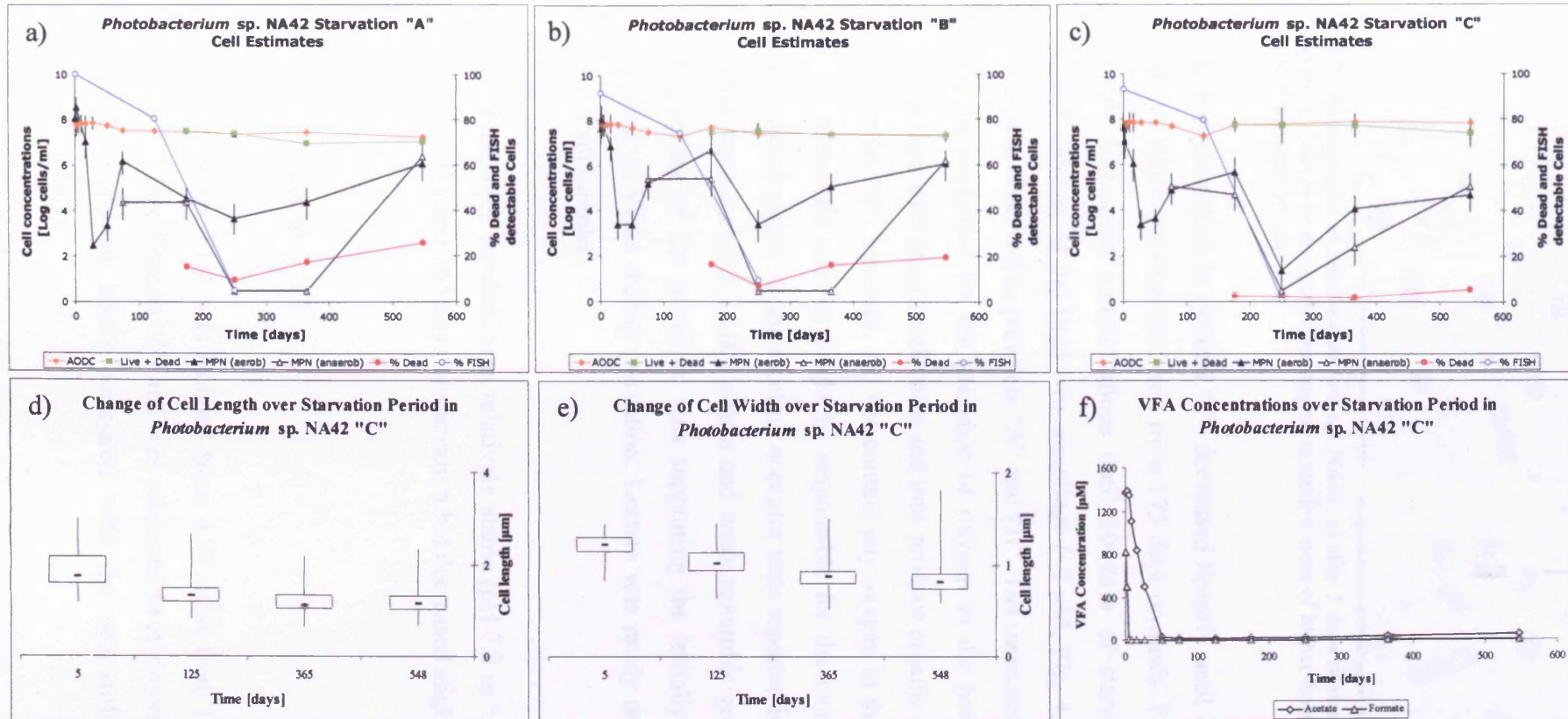
FISH detectable cells decreased rapidly within the first 250 days of starvation and trends were very similar in the three parallels (Fig. 4.6 a-c). The lowest percentage of cells detected by FISH were found in “C” after 250 days with only 0.7% of DAPI stained cells detected.

Cell lengths and widths of strain NA42 decreased significantly during starvation (Fig. 4.6 d-e). There was a significant difference for these two measures between five days of starvation versus 125, 365, and 548 days as well as 125 days versus 365 and 548 days. There was no significant difference in cell sizes between the last two samplings, which indicates that the minimum cell size of strain NA42 was reached after (less than) one year of starvation with an approximate loss of 30% in length and width.

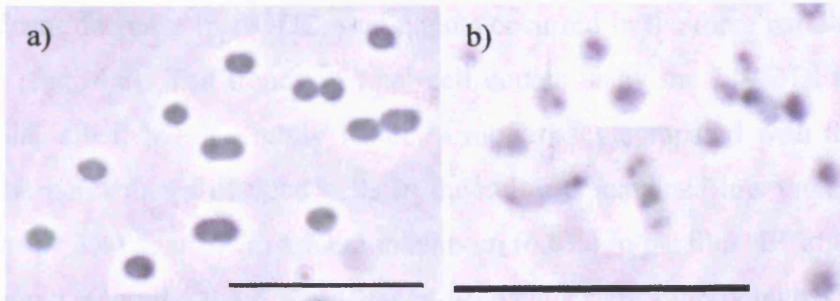
Similar to strain SAMA2, the appearance of starved cells of strain NA42 observed under the microscope changed from dense and healthy looking cells to faint, ghost-like cells (Fig. 4.7). Again denser areas appeared to be located near the poles of the cells.

Culturability in *Photobacterium* sp. NA42 was unreliable under normal laboratory maintenance conditions, which was enhanced during starvation. Nonetheless, aerobic culturability decreased rapidly from  $10^8$  cells ml<sup>-1</sup> at  $t_0$  to  $10^2 - 10^3$  cells ml<sup>-1</sup> after 27 days of starvation. Subsequently, an increase in MPNs occurred ( $10^4$ - $10^6$  cells ml<sup>-1</sup> between 50 and 175 days) but variability between consecutive samplings remained high (Fig. 4.6 a-c). At the final sampling, aerobic MPNs were  $10^4$  to  $10^6$  cells ml<sup>-1</sup> culturable cells in all three parallels, which was higher than for some of the previous time points.

Anaerobic MPNs were only conducted from 75 days of starvation onwards and for this timepoint these were similar to the aerobic incubations for parallels “B” and “C” ( $\sim 10^5$  cells ml<sup>-1</sup>) but two orders of magnitude lower in “A”. Subsequently, culturability remained constant for the following timepoint in all parallels, then no growth ( $< 3$  cells ml<sup>-1</sup>) was observed for two consecutive samplings in parallels “A” and “B” and for one time point in “C” (Fig. 4.6 a-c). For the final sampling, however, MPNs were higher than in any of the previous anaerobic incubations and even higher than the aerobic MPNs for this time point showing  $10^5$  cells ml<sup>-1</sup> in parallel “C” and  $10^6$  cells ml<sup>-1</sup> in “A” and “B”.



**Fig. 4.6:** Physiological responses of *Photobacterium* sp. NA42 to starvation. a-c) Cell number estimates for treatments "A", "B", and "C". Confidence limits are given for AODC, Live + Dead, and MPNs, if not visible they fall within the size of the data points. d-e) Change of size during starvation in "C". Shown are the 25 and 75 percentile (box), the median (horizontal dash) and the data range (vertical dashes). f) Changes in VFA concentrations during starvation in "C".



**Fig. 4.7: Micrographs of *Photobacterium* sp. NA42.** a) after 5 days of starvation; b) after 548 days of starvation. Cells in b) are fainter but appear to exhibit areas of higher density mostly at the poles of the cells. Bars equal 10  $\mu\text{m}$ .

Acetate concentrations in parallel “C” decreased linearly until 75 days of starvation (31  $\mu\text{M}$ ). A slight increase occurred from 175 days onwards. Formate had a similar trend with decreasing concentrations until 50 days of starvation (2  $\mu\text{M}$ ) and a subsequent increase at the final two samplings ( $\sim 8 \mu\text{M}$ , Fig. 4.6f). For formate this trend was also observed in parallels “A” and “B”. The increases in “A” and “B” can probably be explained by the presence of oxygen in the headspace. Cells might metabolise their own macromolecules and thus produce organic acids.

Parallel “C”, however, did not contain any oxygen in the headspace gas thus anaerobic metabolic activity might be responsible for the increases in acetate and formate concentrations. In the electron acceptor tests reported in Chapter III, acetate and lactate were included in the blanks and weak anaerobic growth of strain NA42 occurred in one of the parallels, thus supporting the initially observed anaerobic consumption of VFAs during starvation. Lactate was rarely detected in any of the parallels or subsamples.

The pH of the three parallels was relatively stable (pH 7.2 in “A” and “B”; pH 7 in “C”) between 175 and 365 days of starvation but decreased slightly towards 548 days (pH 6.3).

#### c) *Photobacterium* sp. F18I

*Photobacterium* sp. F18I was isolated from 4.25 mbsf from the Reference site of IODP Leg 307 (see Chapter III) and was subjected to starvation as a representative from deeper sediment layers compared with the near-surface isolates (strains SAMA2 and NA42).

No significant decrease in AODC total counts occurred in the three parallels during starvation (Fig. 4.8). The trends of total cell counts using the LIVE/DEAD<sup>®</sup> stain were similar albeit being slightly lower in most cases compared with the AODC counts. The percentages of dead cells in the cultures remained low throughout the experiment (< 3%) apart from a local maximum (6.6%) in parallel "B" after 50 days of starvation. Generally, the percentages of dead cells were slightly higher in parallel "A" and "B" (between 1% and 6%) than in "C" (< 1%, Fig. 4.8 a-c).

FISH detectability decreased during starvation of strain F181 from 87% at  $t_0$  via a local maximum of 77% after 181 days to 52% after one year (Fig. 4.8c). This latter value lies between values of FISH detectability of the near-surface strains. Strain SAMA2 showed 80% FISH detectable cells after one year of incubation whereas only 0.7% of cells of strain NA42 gave a FISH signal after 270 days of starvation.

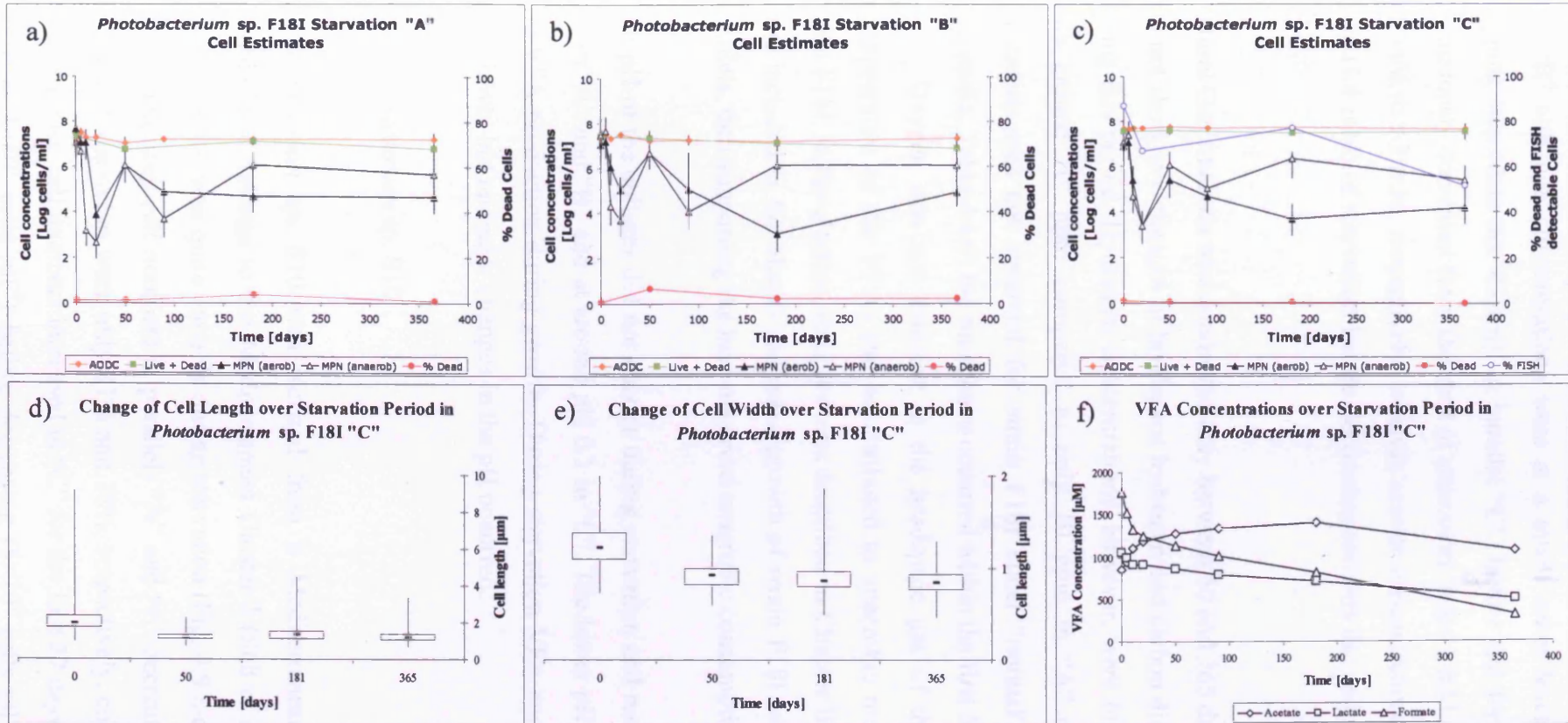
Cells of strain F181 were significantly longer at the beginning (2.2  $\mu\text{m}$ ) than after 50, 181, and 365 days of starvation (average 1.3  $\mu\text{m}$ ;  $p < 0.05$ ; Fig. 4.8e). However, cells after 50 days of starvation were significantly shorter than after 181 and 365 days. Cell widths also decreased during starvation and were significantly narrower after 50, 181, and 365 days (0.9  $\mu\text{m}$ ) of incubation compared with the initial width (1.2  $\mu\text{m}$ ;  $p < 0.05$ ; Fig. 4.8f).

With starvation, culturability decreased in all three parallels. At the beginning, approximately  $10^7$  culturable cells  $\text{ml}^{-1}$  were detected in the aerobic as well as in the anaerobic MPN dilution series. There was some variation in MPNs between treatments but a local minimum of aerobic and anaerobic MPNs occurred simultaneously after 20 days of starvation in all three cultures ( $10^2$ - $10^5$  cells  $\text{ml}^{-1}$ ; Fig. 4.8 a-c).

Subsequently, MPNs increased again, ranging between  $10^3$  and  $10^6$  cells  $\text{ml}^{-1}$  for the remainder of the experiment. In parallel "C" anaerobic MPN incubations resulted in higher culturable cell numbers than aerobic incubations from 50 days onwards. A similar trend occurred in parallels "A" and "B" but only for the two samplings at 181 and 365 days of starvation.

The necessary incubation periods of MPNs until no further growth occurred was usually five to eight days and did not increase with prolonged starvation.





**Fig. 4.8: Physiological responses of *Photobacterium* sp. F181 to starvation.** a-c) Cell number estimates for treatments "A", "B", and "C". Confidence limits are given for AODC, Live + Dead, and MPNs, if not visible they fall within the size of the data points. d-e) Change of size during starvation in "C". Shown are the 25 and 75 percentile (box), the median (horizontal dash) and the data range (vertical dashes). f) Changes in VFA concentrations during starvation in "C".

The trends of VFA concentrations for parallel “C” (Fig. 4.8f) were similar in “A” and “B” although concentrations were at a much lower levels due to the initial washing step (data not shown). In parallel “C”, lactate and formate concentrations continuously decreased from the start of starvation (from 1 mM to 0.5 mM and from 1.7 mM to 0.3 mM, respectively) whereas acetate concentrations increased until 181 days (1.4 mM) of starvation before decreasing towards the end of the experiment (1 mM).

Natural Gas Analysis was conducted only between 50 and 365 days of starvation and did not show any changes in headspace hydrogen and carbon dioxide concentrations during this period. Hydrogen concentrations, however, were higher in parallel “C” with around  $10^3$  ppm compared to only 10 ppm in “A” and “B”. Hydrogen production was not observed for strain F18I under “normal” growth conditions (Appendix, Table A4.6) but must have occurred within the first 50 days of starvation.

Oxygen was not detected in the headspace gas of the cultures thus the disappearance of the VFAs can be attributed to anaerobic metabolic activities of strain F18I. In the electron acceptor tests described in Chapter III, acetate and lactate were included in the blanks and weak growth of strain F18I occurred in one of the parallels, thus supporting the here observed anaerobic consumption of lactate.

The pH of the cultures did not change during starvation and remained at around pH 7.5 in “A” and “B” and at around pH 6.1 in “C”. The lower pH in parallel “C” is to the VFA production during growth. During starvation VFA concentrations changed (see above) but no major changes in the pH occurred.

#### d) *Photobacterium* sp. S10

*Photobacterium* sp. S10 was isolated from a Mediterranean sapropel layer at 2.6 mbsf and belongs to the Mediterranean Cluster I (Süß *et al.*, 2008). The total AODC counts were quite variable during starvation (Fig. 4.9 a-c). Immediately after the washing step cell numbers in parallels “A” and “B” decreased rapidly and after 50 days of starvation were only 37% and 38%, respectively, compared to AODC at  $t_0$ . In contrast, cell numbers increased in “C” for the first 27 days of incubation (from  $3 \times 10^7$  to  $7 \times 10^7$  cells  $\text{ml}^{-1}$ ) before decreasing ( $5 \times 10^6$  cells  $\text{ml}^{-1}$ ) and subsequently

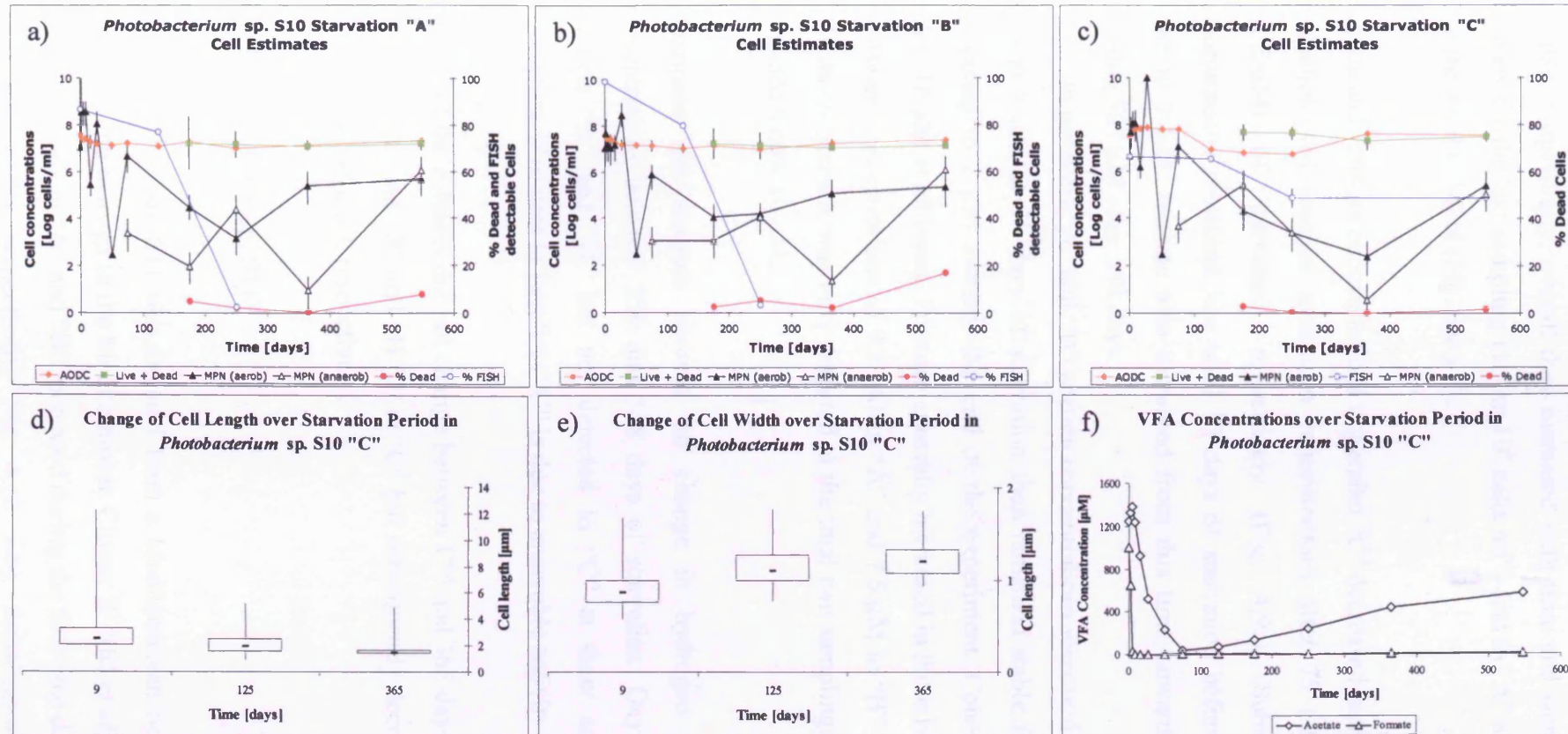
increasing ( $4 \times 10^7$  cells  $\text{ml}^{-1}$ ) again. Total cell counts using the LIVE/DEAD<sup>®</sup> Kit were comparable to AODC total counts in parallels “A” and “B”. In “C”, however, they were much higher than those obtained with AODC for two samplings (175 and 250 days; Fig. 4.9c). This and the curve of the AODC total count suggest that there might have been a procedural error rather than an actual change in cell numbers.

The percentages of dead cells remained relatively low throughout the experiment (maximum 5%) until the last sampling (548 days) where elevated numbers of dead cells were approximately 8% in “A” and 16% in “B”. The proportions of dead cells were always lower in “C” (final sample < 2%) than in “A” and “B”.

FISH detectability decreased slightly between  $t_0$  and 125 days of starvation followed by a rapid decline until 250 days in parallels “A” and “B”, down to only ~3% (Fig. 4.9 a-b). A similar change occurred for parallel “C” with only 0.4% of FISH detectable cells at 250 days of starvation. However, the total FISH counts are shown (Fig. 4.9c) rather than the percentages of FISH detectable cells compared with DAPI. This was necessary as for the final sampling the relationship between hybridised cells and the total DAPI count was 186%. This is once more caused by the effect that DAPI did not stain all the starved cells on the filter.

Cell sizes decreased significantly during starvation. Cells after nine days were significantly longer (2.8  $\mu\text{m}$ ) than those measured after 125 (2.1  $\mu\text{m}$ ) and 365 days (1.6  $\mu\text{m}$ ;  $p < 0.05$ ; Fig. 4.9d). Additionally, cells after 125 days were also significantly longer than after one year of starvation ( $p < 0.05$ ). Contrarily, cell widths increased with time and were significantly different between the three samplings (Fig. 4.9e)

Culturability of strain S10 was unreliable under “normal” laboratory culture conditions. This was also so during starvation, especially in the first 75 days, where aerobically incubated MPNs varied over several orders of magnitude in consequent samplings (for first 75 days; Fig. 4.9 a-c). Nonetheless, a decrease in culturability occurred with ongoing incubation from  $\sim 10^8$  cells  $\text{ml}^{-1}$  at the beginning of starvation to  $10^3$ - $10^4$  cells  $\text{ml}^{-1}$  after 250 days. Subsequently an increase of MPNs occurred towards the end of the experiment resulting in approximately  $\sim 10^5$  cells  $\text{ml}^{-1}$ .



**Fig. 4.9: Physiological responses of *Photobacterium* sp. S10 to starvation.** a-c) Cell number estimates for treatments "A", "B", and "C" including total count for FISH as DAPI staining was unreliable. Confidence limits are given for AODC, Live + Dead, FISH, and MPNs, if not visible they fall within the size of the data points. d-e) Change of size during starvation in "C". Shown are the 25 and 75 percentile (box), the median (horizontal dash) and the data range (vertical dashes). f) Changes in VFA concentrations during starvation in "C".



Anaerobic MPNs were first inoculated after 75 days of starvation and were also quite variable. Surprisingly, overall they increased with time and were highest in all the parallels for the last sampling ( $10^5$  to  $10^6$  cells  $\text{ml}^{-1}$ ) and in “A” and “B” even higher than the aerobic MPN (Fig. 4.9 a-b).

Acetate and formate concentrations in parallel “C” decreased rapidly after the start of incubation and reached minimum concentrations after 75 (39  $\mu\text{M}$ ) and 9 days ( $< 0.2 \mu\text{M}$ ) of starvation, respectively (Fig. 4.9c). Subsequently, formate concentrations remained low until 75 days of starvation before slowly increasing again to 9  $\mu\text{M}$ . Acetate also increased from this time onwards but more rapidly reaching 565  $\mu\text{M}$  after 548 days.

In parallels “A” and “B” acetate concentrations increased slightly from 5  $\mu\text{M}$  at  $t_0$  to 9  $\mu\text{M}$  at 75 days of starvation then remained stable for 100 days before decreasing to 2  $\mu\text{M}$  towards the end of the experiment. Concentrations remained below 10  $\mu\text{M}$  at all times. Formate generally increased in these two cultures reaching maximum concentrations of 9.3  $\mu\text{M}$  in “A” and 7.5  $\mu\text{M}$  in “B” in the final samples (548 days). Acetate was only detected at the final two samplings of the cultures and remained below 10  $\mu\text{M}$ .

Headspace gas analysis showed no change in hydrogen or carbon dioxide concentrations between 250 and 548 days of starvation. Oxygen was present in parallels “A” and “B” but not detected in “C” at these samplings. Thus the production of acetate in parallels “C” is due to anaerobic activity of the strain.

The pH of the cultures did not change between 175 and 365 days of starvation being pH 7.2 in “A” and “B” and pH 6.9 in “C” but subsequently decreased to pH 6.3 and pH 6.2 after 548 days, respectively.

#### e) *Photobacterium* sp. S11

*Photobacterium* sp. S11 was isolated from a Mediterranean Sea sediment layer at 2.35 mbsf and belongs to the Mediterranean Cluster II (Süß *et al.*, 2008). The AODC total cell counts in “A” and “B” increased during the first nine days of starvation (by 300% and 650% respectively; Fig. 4.10 a-b). Subsequently, they decreased

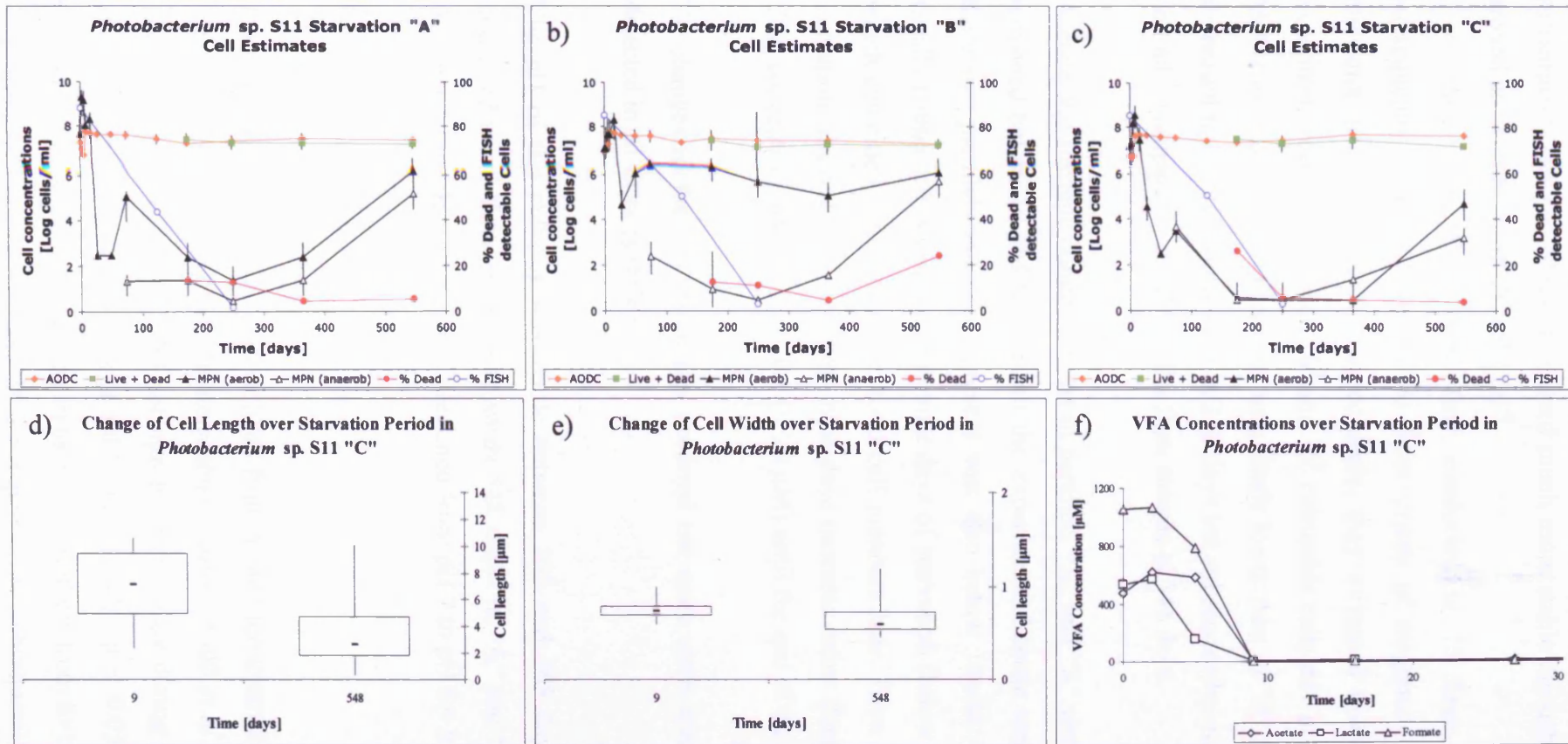
significantly ( $p < 0.05$ ) below 50% after 125 days of starvation compared to the total cell count at nine days. Afterwards they remained relatively stable until the end of the experiment. In “C” cell counts also increased initially (840% in the first nine days) but subsequently no significant changes in cell numbers occurred ( $p < 0.05$ ) until the end of the experiment.

Total cell counts estimated with the LIVE/DEAD<sup>®</sup> Kit remained just below the total AODC counts, but the trends were similar (Fig. 4.10 a-c). The percentages of dead cells in the cultures were generally higher than for any other strain of the genus *Photobacterium* but were quite variable over time and between treatments. The highest percentage of “dead” cells occurred in “C” after 125 days of starvation (26%). Subsequently, this proportion decreased to less than 6% in later samples and was lowest in the final sample (4%). The percentages of dead cells in “A” followed the trend in “C” with decreasing numbers of dead cells over time. In parallel “B” the percentages of dead cells increased towards the end of starvation reaching ~24% at 548 days from 12% at 175 days.

FISH detectability decreased almost linearly with time in the three treatments from an average of 91% at the beginning to 2% by 250 days. For parallel “C” the final sample (548 days) showed a higher number of hybridised cells compared to the previous sample but there was no signal for DAPI stained cells on the filter. Treatments “A” and “B” were not tested again.

Both cell lengths and widths decreased significantly between 9 and 548 days of starvation from 7.0  $\mu\text{m}$  to 3.7  $\mu\text{m}$  and from 0.75  $\mu\text{m}$  to 0.64  $\mu\text{m}$ , respectively ( $p < 0.05$ ; Fig. 4.10 d-e).

Culturability of strain S11 decreased rapidly in aerobic MPNs for “A” and “C” dropping six and four orders of magnitude (Fig. 4.10a and c) within the first 27 and 50 days of starvation, respectively. Subsequently, MPNs in “C” decreased further and almost no growth occurred for three subsequent samplings (175 to 365 days). Towards the end of the experiment, however, culturable cell numbers increased again to  $10^4$  cells  $\text{ml}^{-1}$ . The trend in “A” was similar to “C” but MPNs were usually two orders of magnitude above culturable cell numbers in “C”.



**Fig. 4.10: Physiological responses of *Photobacterium* sp. S11 to starvation.** a-c) Cell number estimates for treatments "A", "B", and "C". Confidence limits are given for AODC, Live + Dead, and MPNs, if not visible they fall within the size of the data points. d-e) Change of size during starvation in "C". Shown are the 25 and 75 percentile (box), the median (horizontal dash) and the data range (vertical dashes). f) Changes in VFA concentrations during starvation in "C".

The development of culturability in parallel “B” was similar for the first 27 days but subsequently aerobic MPNs remained much more stable throughout the experiment around an average of  $8 \times 10^5$  cells ml<sup>-1</sup>.

Anaerobic MPNs were first conducted at 75 days of starvation and subsequently decreased by two to three orders of magnitude until 250 days of starvation in “B” and “C”. Subsequently, they increased towards the end of the experiment reaching between  $10^3$  and  $10^5$  culturable cells ml<sup>-1</sup> in the cultures. In “A” MPNs were one to two orders of magnitude lower than in “B” and “C” at 75 days, decreased to  $< 3$  culturable cells at 250 days but subsequently increased reaching  $10^5$  cell ml<sup>-1</sup>, comparable to “B”, in the final sample at 548 days.

Acetate and formate concentrations in parallels “A” and “B” were quite variable but remained below 10  $\mu$ M throughout the experiment. Lactate was rarely detected in these two parallels but when present was also below 10  $\mu$ M. In “C” VFAs were rapidly consumed within the first nine days of starvation (below 10  $\mu$ M; Fig. 4.10f), which coincides with increase in total cell numbers here. Thus, actually the start of starvation survival in “C” is from nine days onwards rather than at  $t_0$ . Subsequently VFA concentrations remained low ( $< 20$   $\mu$ M) until the end of the experiment.

No changes in the headspace gas occurred but once more, traces of oxygen were detected in all three parallels.

The pH of the cultures was stable between 175 and 365 days of starvation but decreased in all three parallels towards 548 days. In “A” and “B” the pH dropped from pH 7.3 to pH 6.1 whereas it declined from pH 7 to pH 6.4 in “C”.

#### f) *Photobacterium* sp. 67TD

*Photobacterium* sp. 67TD was isolated from a Mediterranean Sea sediment layer at 0.2 mbsf and belongs to the Mediterranean Cluster I (Süß *et al.*, 2008). The AODC total counts in “A” and “B” did not significantly change during starvation remaining at around  $4 \times 10^6$  and  $6 \times 10^6$  cells ml<sup>-1</sup>, respectively ( $p < 0.05$ ; Fig. 4.11 a-b). In parallel “C” a seven fold increase in total cell numbers from  $8 \times 10^6$  to  $5 \times 10^7$  cells ml<sup>-1</sup> occurred in the first 16 days of the experiment. Subsequently, they decreased to

$3 \times 10^6$  cells  $\text{ml}^{-1}$  at 250 days before increasing again to  $10^7$  cells  $\text{ml}^{-1}$  in the final two samplings (365 and 548 days; Fig. 4.11c).

Total cell counts estimated using the LIVE/DEAD<sup>®</sup> Kit were in the range of the AODC total cell counts (Fig. 4.11 a-c). In “cultures “A” and “B” the percentages of dead cells increased towards the end of the experiment from < 10% at 175 days to 33% and 27% dead cells in the final samples (548 days), respectively. An opposite trend occurred for parallel “C” where the proportion of dead cells was highest after 175 days of starvation (10%) and subsequently decreased to 0.7% after 548 days. Neither FISH nor cell size analyses were performed for any of the samples of *Photobacterium* sp. 67TD.

Aerobic culturability in parallels “A” and “C” decreased with prolonged starvation from  $\sim 10^8$  cells  $\text{ml}^{-1}$  and reached a minimum of culturable cells at 250 days of 23 and < 3 cells  $\text{ml}^{-1}$ , respectively, before increasing towards the end of the experiment reaching  $10^5$  cells  $\text{ml}^{-1}$  at 548 days. In parallel “B” culturability remained relatively unchanged throughout starvation with an average of  $9 \times 10^5$  culturable cells  $\text{ml}^{-1}$ .

Anaerobic culturability was first investigated at 75 days of starvation and quickly decreased from  $10^2$  cells  $\text{ml}^{-1}$  in “A” and “B” and  $10^4$  cells  $\text{ml}^{-1}$  in “C” to < 3 culturable cells in either of the parallels from 250 days onwards (Fig. 4.11 a-c).

VFA concentrations in parallel “C” decreased rapidly at the beginning of the experiment (Fig. 4.11d). Lactate was depleted after just two days whereas acetate and formate reached minimum concentrations after 27 days (13  $\mu\text{M}$  and 2  $\mu\text{M}$ , respectively). This initial depletion coincided with the increase in total cell counts. Thus, the actual start of starvation survival in “C” lies somewhere between these sampling points rather than at  $t_0$ . Concentrations of both acetate and formate increased again from 75 days to maximum values of 260  $\mu\text{M}$  and 10  $\mu\text{M}$ , respectively, in the final sample.

In parallels “A” and “B” VFA concentrations were relatively variable yet remained low. Acetate generally remained below 10  $\mu\text{M}$  except for a single peak (260  $\mu\text{M}$ ) in “B” at 27 days of starvation but no trend was observed. Lactate was rarely detected in the cultures and if present was below 8  $\mu\text{M}$ . Formate concentrations were also variable but generally increased with time in parallels “A”

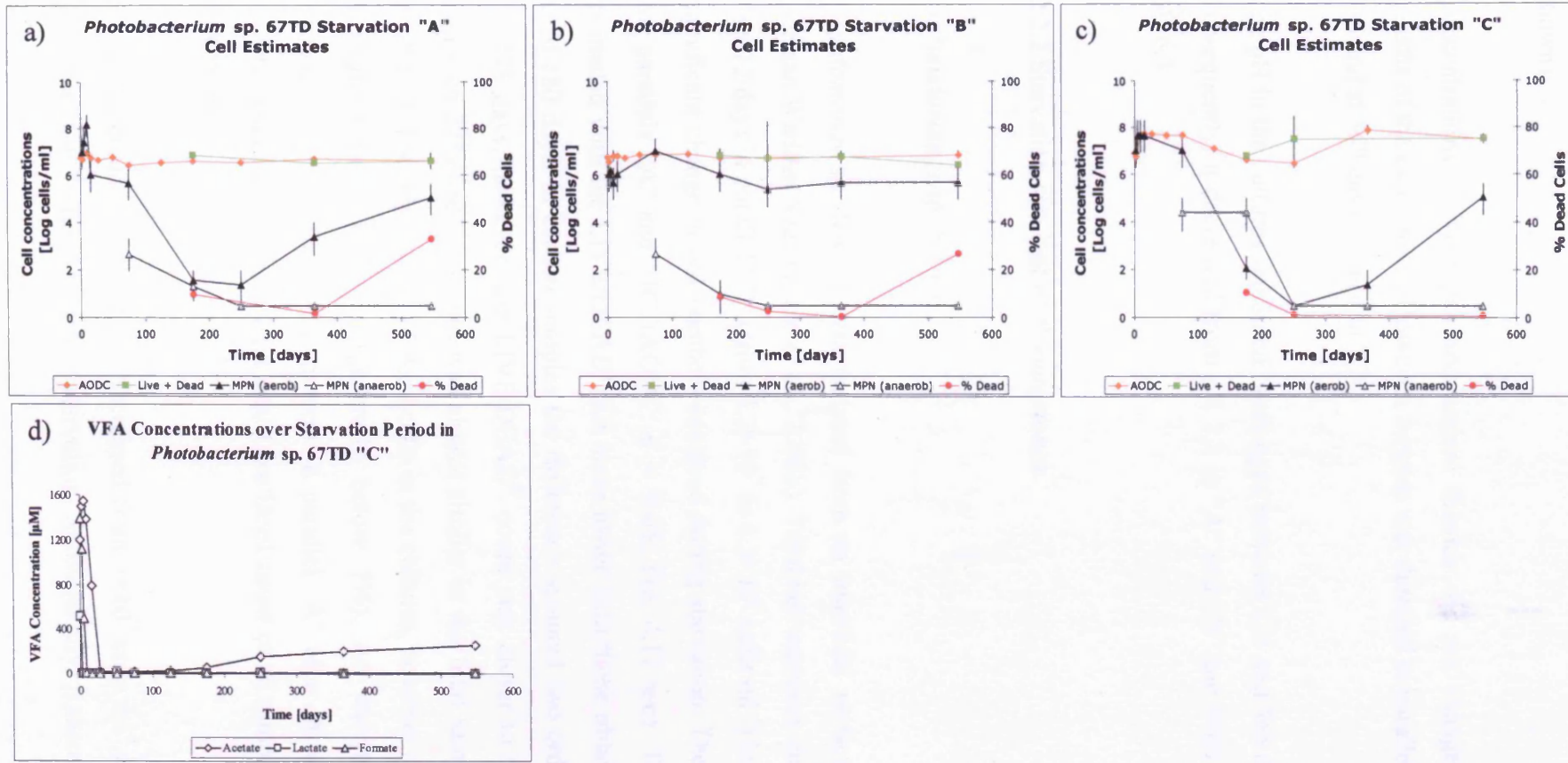


Fig. 4.11: Physiological responses of *Photobacterium* sp. 67TD to starvation. a-c) Cell number estimates for treatments "A", "B", and "C". Confidence limits are given for AODC, Live + Dead, and MPNs, if not visible they fall within the size of the data points. f) Changes in VFA concentrations during starvation in "C".

and “B”. In the final samples concentrations of  $\sim 10 \mu\text{M}$  were detected (data not shown).

Concentrations of hydrogen and carbon dioxide did not change in the final six months of the experiment. However, oxygen was detected in parallels “A” and “B” at 365 and at 549 days but not in “C”.

The pH in the cultures remained unchanged between 175 and 365 days of starvation. Subsequently, it decreased from pH 7.2 in “A” and “B” and from pH 6.9 in “C” to pH 6.3.

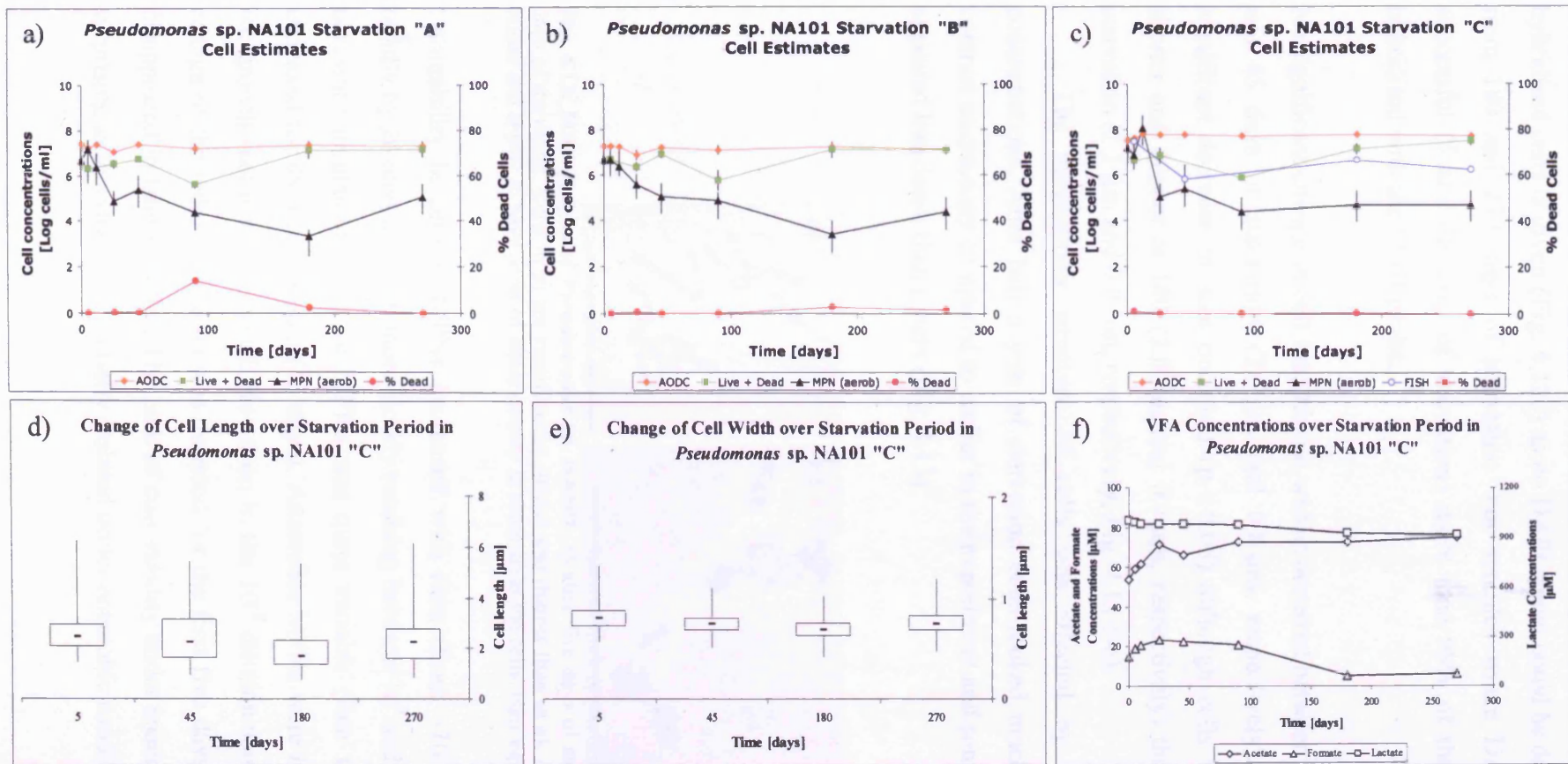
### 2.2.2 Starvation-survival of *Pseudomonas*

#### a) *Pseudomonas* sp. NA101

*Pseudomonas* sp. NA101 was isolated from an intertidal surface sediment of the German Wadden Sea (Freese *et al.*, 2008b). Total cell numbers increased during the first 12 days in parallel “C” (from  $3.2 \times 10^7$  to  $6.3 \times 10^7$  cells  $\text{ml}^{-1}$ ) but subsequently no significant change in cell numbers occurred during starvation. The latter is also true for parallels “A” and “B” (AODC;  $p < 0.05$ ; Fig. 4.12 a-c). Total cell numbers estimated with the LIVE/DEAD<sup>®</sup> Kit were lower than those obtained using AODC until 180 days. In certain samples the difference spanned two orders of magnitude. At 175 days, however, the LIVE/DEAD<sup>®</sup> count was closer to the AODC counts (between 22% and 78%) and was almost similar in the final sample (between 61% and 95%). The percentages of dead cells in the cultures, however, remained very low throughout the experiment (generally below 1%). An exception was a local maximum of 14% dead cells present in parallel “A” after 90 days of starvation, which coincides with the lowest total Live/Dead count of all samples and parallels of this strain.

The FISH count in parallel “C” decreased from  $3 \times 10^7$  cells  $\text{ml}^{-1}$  at the beginning to  $7 \times 10^5$  cells  $\text{ml}^{-1}$  after 45 days of starvation. Subsequently, it recovered to  $\sim 10^6$  cells





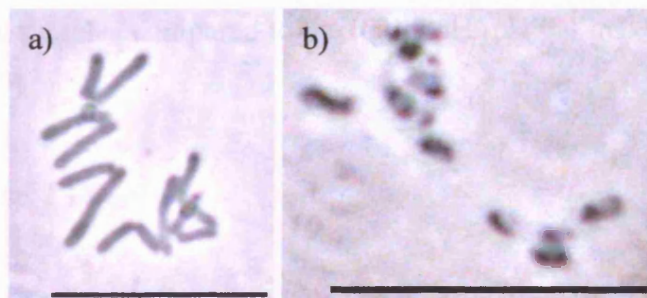
**Fig. 4.12:** Physiological responses of *Pseudomonas* sp. NA101 to starvation. a-c) Cell number estimates for treatments "A", "B", and "C". Confidence limits are given for AODC, Live + Dead, FISH, and MPNs, if not visible they fall within the size of the data points. d-e) Change of size during starvation in "C". Shown are the 25 and 75 percentile (box), the median (horizontal dash) and the data range (vertical dashes). f) Changes in VFA concentrations during starvation in "C".



ml<sup>-1</sup> where it remained until the end of the experiment. Note that the total count of hybridised cells is given (Fig. 4.12c) as no DAPI signal could be detected in samples from 180 and 270 days of starvation. For samples where DAPI staining was successful (5 and 45 days of starvation) more than 98% of the total cells were hybridised with the FISH probe.

No significant change in cell lengths and widths occurred between samples from five and 45 days of starvation (2.5  $\mu$ m and 0.8  $\mu$ m, respectively). Subsequently, a significant decrease in size occurred ( $p < 0.05$ ) although cells were significantly shorter and thinner at 180 (2.0  $\mu$ m and 0.7  $\mu$ m, respectively) than at 270 days of starvation (2.3  $\mu$ m and 0.8  $\mu$ m, respectively; Fig. 4.12 d-e).

The intracellular structure of cells was affected by the low nutrient concentrations. After half a year of starvation cells looked much fainter in phase contrast microscopy compared to earlier in the experiment and some areas of the cell appeared less dense than others (Fig. 4.13).



**Fig. 4.13: Micrographs of *Pseudomonas* sp. NA101.** a) after five days of starvation; b) after 180 days of starvation. Cells in b) are significantly shorter and thinner than in a). Additionally, cells are fainter and appear to have areas of lesser density in some areas the cells. Bars equal 10  $\mu$ m.

Culturability in aerobic MPNs decreased with time (from  $\sim 10^7$  cells ml<sup>-1</sup>) most rapidly by 26 days and then more slowly reaching between  $10^3$  and  $10^5$  cells ml<sup>-1</sup>. The necessary incubation time for MPNs was quite variable (four to nine days) and increased for the final sample (13 days). Anaerobic MPNs were initially inoculated but growth was never observed. However, in the  $10^{-1}$  dilution some activity (colour change of the redox indicator) was detected for the first five days of starvation but disappeared in later samples. The lack of culturability under anaerobic conditions is surprising as this strain was initially isolated under anaerobic conditions.

The fate of VFAs was very similar for the three starvation treatments although concentrations in “A” and “B” were much lower ( $< 25 \mu\text{M}$ ) than in “C” due to the washing step prior to starvation. Acetate increased within the first 26 days of starvation (from  $54 \mu\text{M}$  to  $71 \mu\text{M}$ ) and continued to increase slowly until the end of the experiment ( $75 \mu\text{M}$ ). Lactate slowly decreased over time (from  $1 \text{ mM}$  to  $0.9 \text{ mM}$ ) whilst formate initially increased (from  $15 \mu\text{M}$  at the beginning to  $23 \mu\text{M}$  after 26 days) before decreasing to  $6 \mu\text{M}$  at the end of the experiment (Fig. 4.12f). The initial production of VFAs is most likely linked to the increase in total cell counts that occurred in treatment “C”.

In all three cultures, headspace carbon dioxide concentrations approximately halved within the first 12 days (from  $1.2 \times 10^5 \text{ ppm}$  to  $7.5 \times 10^4 \text{ ppm}$ ) but subsequently remained constant throughout the experiment. No change in hydrogen concentration occurred in any of the treatments ( $\sim 12 \text{ ppm}$ ).

The pH in the cultures slowly increased during starvation and was  $\sim 0.3$  pH units higher in the final samples compared to the initial pH (pH 7.4 in “A”, pH 7.3 in “B”, and pH 6.4 in “C”).

#### b) *Pseudomonas* sp. F18V

*Pseudomonas* sp. F18V was isolated from 80 mbsf at Reference site of IODP Leg 307 (see Chapter III). During starvation no significant loss of cell numbers occurred in any of the parallels (AODC;  $p < 0.05$ ; Fig. 4.14 a-c). In “C”, however, an initial increase in cell counts occurred (from  $4.4 \times 10^7 \text{ cells ml}^{-1}$  at the beginning to  $1.0 \times 10^8 \text{ cells ml}^{-1}$  after days). Similar to strain NA101, the total cell counts using the LIVE/DEAD<sup>®</sup> Kit were considerably below the AODC total counts often differing by one order of magnitude but increased with continued incubation. This increase between the beginning and the final sample was significant ( $p < 0.05$ ) for treatments “B” and “C” but not “A”.

The percentages of dead cells increased with time but decreased again towards the end of the starvation period. Parallels “A” and “B” generally exhibited more dead cells than were present in parallel “C” with local maxima of up to 8% after 90 days of starvation in “A” and 7% after 180 days in “B”. Treatment “C” also

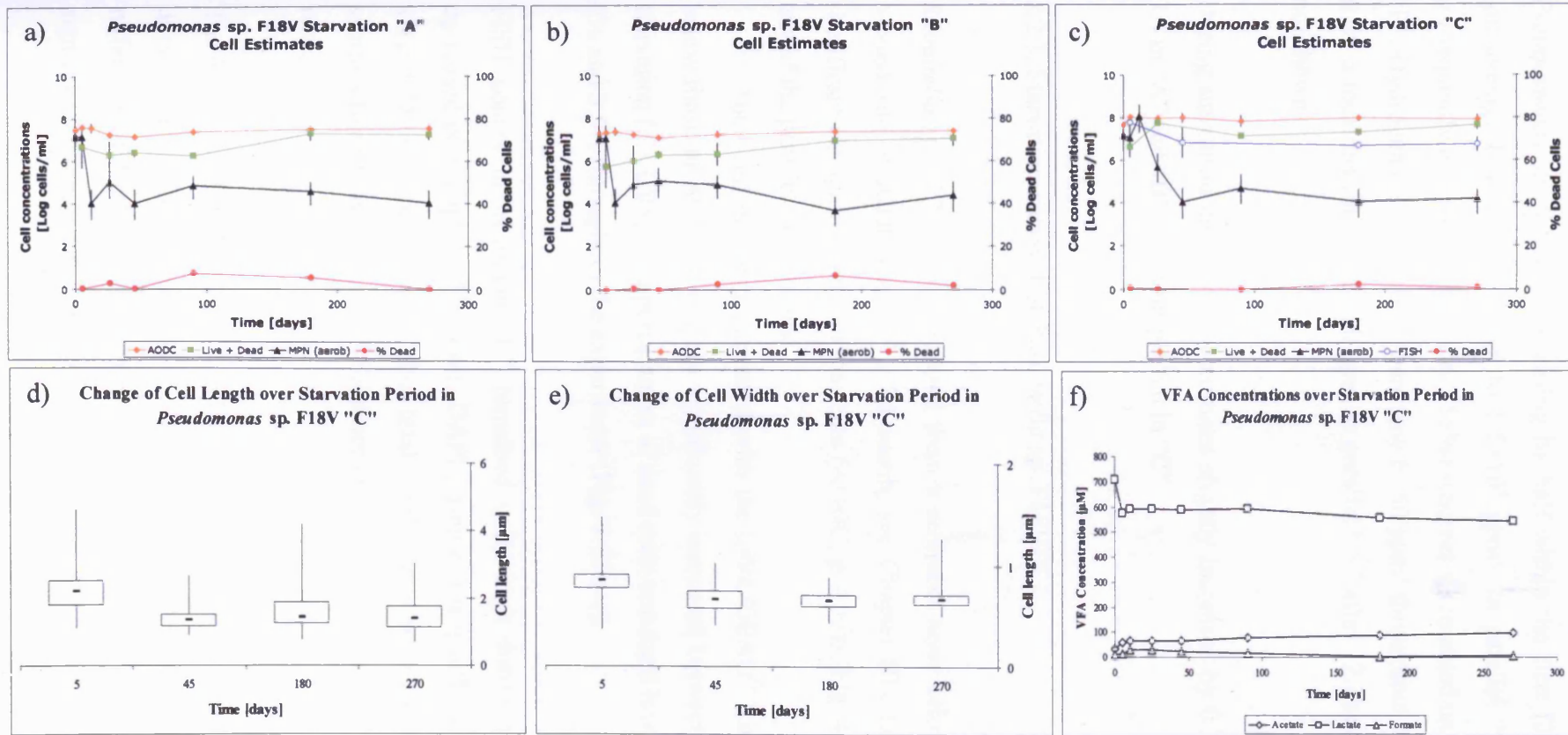
showed a local maximum at 180 days of starvation but only 2.3% dead cells were present.

FISH detectability in treatment “C” decreased within the first 45 days of starvation (from  $6.0 \times 10^7$  cells  $\text{ml}^{-1}$  to  $7.4 \times 10^6$  cells  $\text{ml}^{-1}$ ) and subsequently remained stable over time. Once more DAPI failed to stain cells at 180 and 270 days of starvation, hence the total count of hybridised cells is given (Fig. 4.14c). For the two samplings (5 and 45 days) where DAPI did stain cells, the FISH detectability was near 100%.

Cells of strain F18V were significantly longer and wider (2.2  $\mu\text{m}$  and 0.8  $\mu\text{m}$ , respectively) at the beginning of starvation than at any other stages of the experiment (Fig. 4.14 d-e). However, cells at 45 days of starvation were significantly shorter (1.4  $\mu\text{m}$ ) than at later time points (average 1.6  $\mu\text{m}$ ).

Culturability in aerobic MPNs decreased rapidly during the first 12 days of starvation from  $\sim 10^7$  cells  $\text{ml}^{-1}$  to around  $\sim 10^4$  cells  $\text{ml}^{-1}$  in parallels “A” and “B” and subsequently remained at this level. The loss of culturability in “C” was slower (45 days) but also resulted in  $\sim 10^4$  culturable cells  $\text{ml}^{-1}$  and subsequently remained at this level. The necessary incubation time of MPNs was six days on average and did not increase with prolonged starvation. No growth was detected in anaerobic MPNs. In parallel “C”, however, some activity was seen in the  $10^{-1}$  dilution (colour change of the redox indicator) for the first five days of starvation but not in later samples.

VFA concentration changes in the three parallels developed relatively similarly during starvation. Concentrations for lactate, however, were much lower in treatments “A” and “B” ( $\sim 1\%$ ) than “C” in. In “C”, lactate decreased within the first five days of starvation (from 710  $\mu\text{M}$  to 576  $\mu\text{M}$ ) coinciding with the increase in total cell numbers over the same period (Fig. 4.14c). Subsequently the concentration did not change for 85 days before starting to decrease slowly. Throughout the experiment acetate concentrations increased slowly and continuously (from 34  $\mu\text{M}$  to 97  $\mu\text{M}$ ) whereas formate initially increased (from 8  $\mu\text{M}$  at the beginning to 28  $\mu\text{M}$  after 10 days), then remained stable until 25 days before decreasing until the end of starvation (to  $\sim 5$   $\mu\text{M}$ ; Fig. 4.14f).



**Fig. 4.14: Physiological responses of *Pseudomonas* sp. F18V to starvation.** a-c) Cell number estimates for treatments "A", "B", and "C". Confidence limits are given for AODC, Live + Dead, and MPNs, if not visible they fall within the size of the data points. d-e) Change of size during starvation in "C". Shown are the 25 and 75 percentile (box), the median (horizontal dash) and the data range (vertical dashes). f) Changes in VFA concentrations during starvation in "C".

Changes in headspace carbon dioxide concentrations were similar to those in *Pseudomonas* sp. NA101, decreasing by half within the first 12 days of starvation (on average from  $1.1 \times 10^5$  ppm to  $6.5 \times 10^4$  ppm). In parallel “C”, carbon dioxide subsequently increased slowly (by 155%) whereas it remained unchanged in “A” and “B”. Hydrogen concentrations were low ( $< 50$  ppm) throughout the experiment apart from a local maximum of 290 ppm in parallel “A” after 12 days of starvation (data not shown).

During starvation the pH in the cultures slightly increased by 0.3 pH units from pH 7.4 in “A” and “B” and from pH 6.6 in “C”.

### 2.2.3. Starvation-survival of *Raoultella* sp. F17VIII

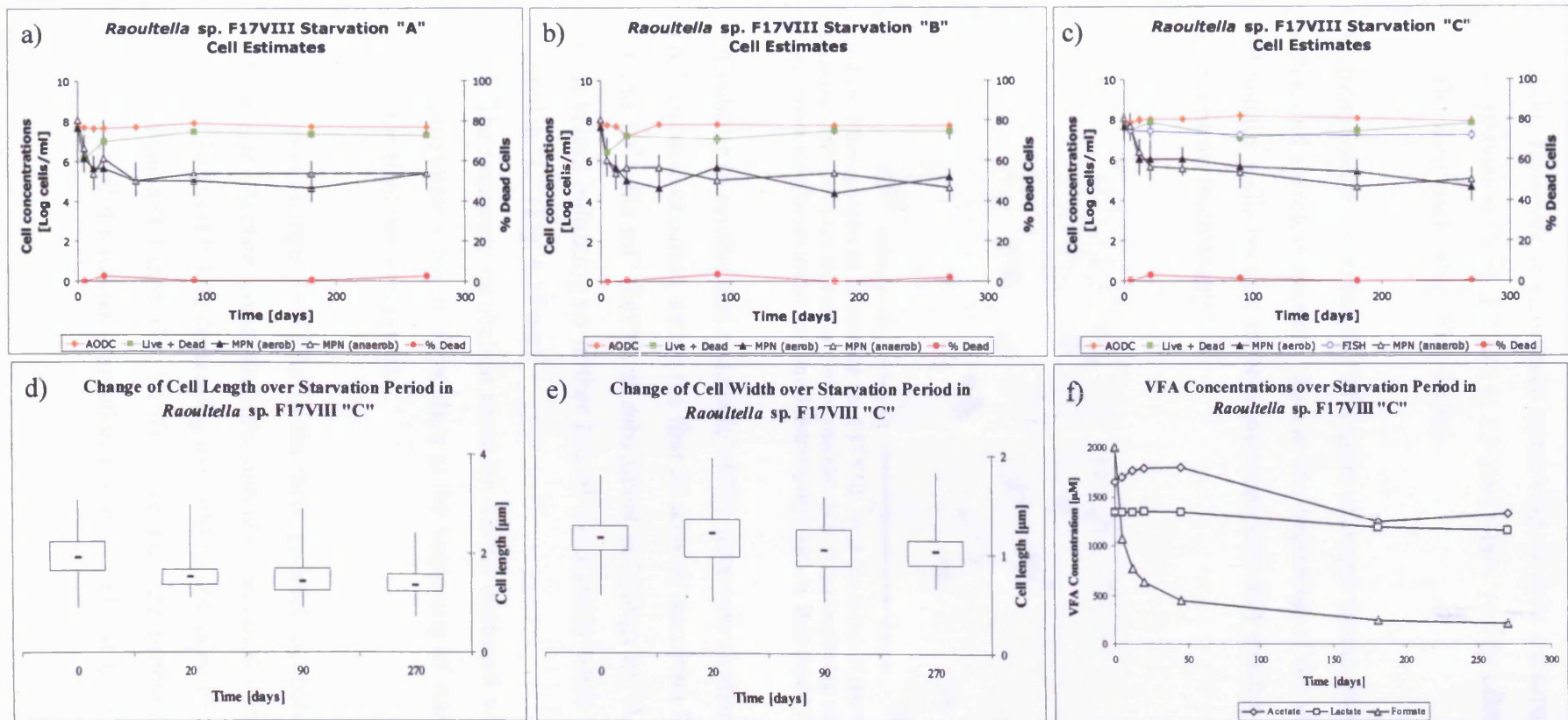
*Raoultella* sp. F17VIII was isolated from a sediment layer below the mound at the Mound site of IODP Leg 307 (-74 mamb, see Chapter III). During starvation no significant change in total cell numbers (AODC,  $p < 0.05$ ; Fig. 4.15 a-c) occurred in any of the parallel incubations.

Total cell numbers estimated with the LIVE/DEAD<sup>®</sup> Kit were continuously below those of AODC but again significantly increased between 5 and 270 days of starvation ( $p < 0.05$ ). The percentages of dead cells remained low and varied between 0% and 3.5% throughout the experiment (Fig. 4.15 a-c).

FISH counts in treatment “C” remained quite high during the experiment and decreased only slightly. However, DAPI staining was insufficient for samples from 90 and 270 days of starvation, thus total FISH counts are presented (Fig. 4.15c). For samples from 5 and 20 days, FISH detectability was at 88% and 53% of DAPI count, respectively.

The lengths of *Raoultella* sp. F17VIII cells decreased significantly between  $t_0$  and 20 days of starvation (from 1.9  $\mu\text{m}$  to 1.6  $\mu\text{m}$ ). Cells at 20 and 90 days (1.5  $\mu\text{m}$ ) did not differ significantly from each other but cells at both these samplings were significantly longer compared to cell lengths after 270 days (1.4  $\mu\text{m}$ ; Fig. 4.15d).

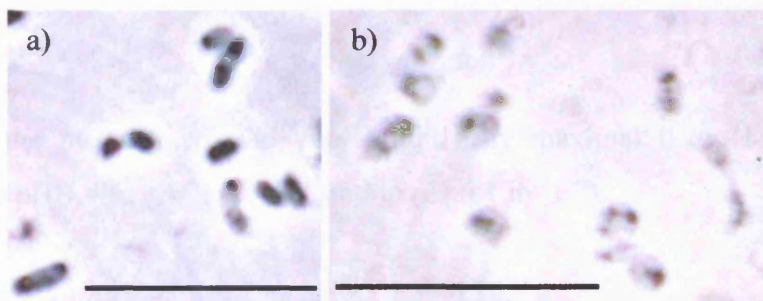




**Fig. 4.15: Physiological responses of *Raoutella* sp. F17VIII to starvation.** a-c) Cell number estimates for treatments "A", "B", and "C". Confidence limits are given for AODC, Live + Dead, FISH and MPNs, if not visible they fall within the size of the data points. d-e) Change of size during starvation in "C". Shown are the 25 and 75 percentile (box), the median (horizontal dash) and the data range (vertical dashes). f) Changes in VFA concentrations during starvation in "C".

Cell widths at the beginning of starvation did significantly change by 20 days of starvation. However, a significant narrowing of cells occurred between 20 and 90 days of starvation (from 1.2  $\mu\text{m}$  to 1.1  $\mu\text{m}$ ) while, widths after 90 and 270 days did not differ from each other (Fig. 4.15e).

The morphology of strain F17VIII cells changed during starvation. Cells became shorter, and therefore rounder, than at the beginning of the experiment (Fig. 4.16). Additionally, cells became more translucent with dark patches when observed under phase contrast microscopy.



**Fig. 4.16: Micrographs of *Raoultella* sp. F17VIII.** a) at the onset of starvation; b) after 270 days of starvation. Cells in b) are shorter and often rounder than at the beginning of the experiment. Cells also become more translucent under continued nutrient deprivation. Bars equal 10  $\mu\text{m}$ .

Culturability in aerobic and anaerobic MPNs were very similar in strain F17VIII. An initial decrease occurred within the first 20 days of starvation from  $10^7$ - $10^8$  cells  $\text{ml}^{-1}$  down to  $10^5$  cells  $\text{ml}^{-1}$ . MPNs at subsequent samplings in “A” and “B” resulted in similar values indicating no further loss of culturability while a continuing decrease occurred in “C” (Fig. 4.15 a-c).

The necessary incubation times for MPNs increased with ongoing starvation from approximately two to three days at the beginning of starvation to five and six days for the final two timepoints.

Concentration changes in VFAs in the three parallel incubations of strain F17VIII were similar. Acetate concentrations initially increased (from 1.75 mM at  $t_0$  to 1.9 mM at 20 days) before decreasing to 1 mM at 181 days. In the final sample it was slightly elevated (1.1 mM; Fig. 5.15f). A constant and almost linear slow decrease in lactate occurred throughout starvation (from 1.4 mM at  $t_0$  to 0.9 mM at 270 days)

whereas formate followed a logarithmic decrease from 2.1 mM at  $t_0$  to 0.2 mM after 270 days suggesting bacterial consumption.

Headspace gas analysis revealed a ten-fold increase in hydrogen concentrations (to  $\sim 10^2$  ppm in “A” and “B”, and to  $5 \times 10^4$  ppm in “C”) within the first five days of the experiment. In “B”, this was accompanied by an increase of carbon dioxide of 500% (to  $9 \times 10^4$  ppm) during the first five days while a 25% decrease in “A” and “C” occurred (to  $8 \times 10^4$  ppm, respectively). This can most likely be explained by the dissolution of the gas in the medium as part of buffer system. Hydrogen production during growth occurred for this strain during its phenotypic characterisation (Chapter III).

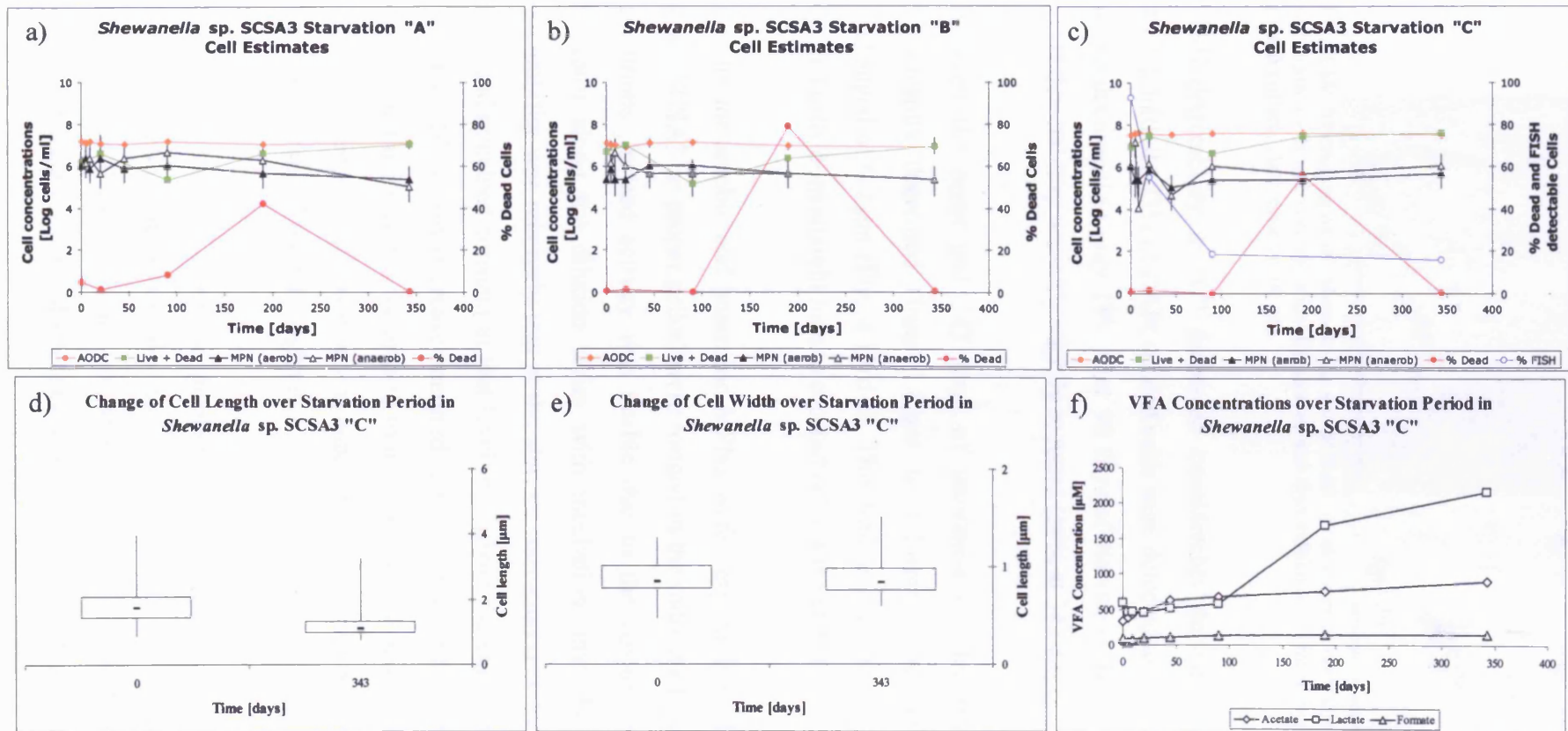
The pH of the media increased very slightly by maximal 0.2 pH units during starvation (to pH 7.4 in “A” and “B”, and to pH 6.1 in “C”).

#### 2.2.4 Starvation-survival of *Shewanella*

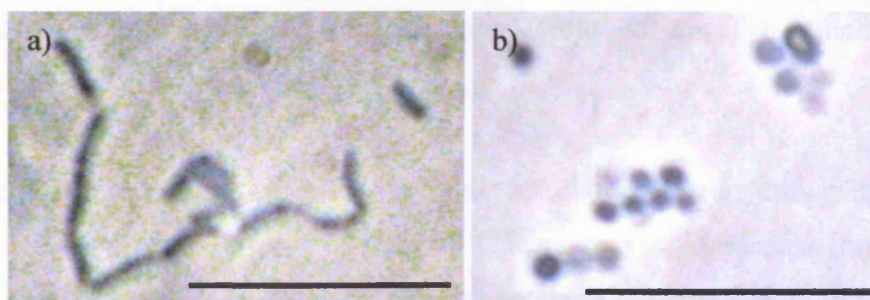
##### a) *Shewanella* sp. SCSA3

Strain SCSA3 was isolated from an intertidal surface sediment of the German Wadden Sea (Droege and Sass, unpublished). During starvation no significant loss in AODC total counts occurred ( $p < 0.05$ ; Fig. 4.17 a-c). Total cell numbers estimated with the LIVE/DEAD<sup>®</sup> Kit were variable and generally below the total cell counts obtained with AODC especially at the start of starvation (up to two orders of magnitude difference). However, after 90 days numbers slowly increased with time and were very close to the AODC counts in the final samples (between 80% and 130%; 343 days). For the proportion of dead cells a local maximum of up to 79% (in “B”) occurred in all three parallels in samples from 190 days of starvation. This result however seems unreliable as it does not coincide with a decrease in total cell numbers nor was an elevated number of dead cells present in previous or subsequent samples. This rather suggests an experimental error than an actual sudden change in the proportions of dead cells.





**Fig. 4.17: Physiological responses of *Shewanella* sp. SCSA3 to starvation.** a-c) Cell number estimates for treatments "A", "B", and "C". Confidence limits are given for AODC, Live + Dead, and MPNs, if not visible they fall within the size of the data points. d-e) Change of size during starvation in "C". Shown are the 25 and 75 percentile (box), the median (horizontal dash) and the data range (vertical dashes). f) Changes in VFA concentrations during starvation in "C".



**Fig. 4.18: Micrographs of *Shewanella* sp. SCSA3.** a) at onset of starvation; b) after 343 days of starvation. Cells in b) are significantly shorter and thus exhibit a different morphology compared with a fresh culture. Bars indicate 10  $\mu\text{m}$ .

FISH detectability in “C” decreased considerably during initial starvation (Fig. 4.17c). Initially 93% of DAPI stained cells were detected using the eubacterial probe, which decreased to only 19% after 90 days of starvation. In the subsequent sample FISH detectability was only slightly reduced (16% at 343 days).

Between the onset and 343 days of starvation cell lengths of strain SCSA3 significantly decreased (from 1.8  $\mu\text{m}$  to 1.2  $\mu\text{m}$ ) while cell widths remained unchanged at 0.9  $\mu\text{m}$  (Fig. 4.17 d-e). This led to a change in morphology of the starved cells from straight rods to coccoid cells at the end of starvation (Fig. 4.18).

Results for aerobic and anaerobic MPNs were very similar. However, in case of strain SCSA3 no proper pellets were formed in the individual wells under anaerobic conditions. Instead activity was visible due to the colour change of the redox indicator along the dilution series with incubation time. In the three parallels culturability was relatively low at the start of starvation (between  $10^5$  and  $10^6$  cells  $\text{ml}^{-1}$ ) and remained constant at that level throughout the experiment in “C” while in “A” and “B” a slow decrease occurred (still  $10^5$  cells  $\text{ml}^{-1}$  in final sample of 343 days). The time required for growth to be completed in aerobic MPNs and bacterial activity to reduce the conditions in anaerobic MPNs did not change with ongoing starvation being  $\sim 5$  days throughout.

The fate of VFAs in the three parallels was similar. Acetate, lactate, formate all increased during incubation. For acetate and formate the increase was slow (from 330  $\mu\text{M}$  to 890  $\mu\text{M}$  and from 90  $\mu\text{M}$  to 155  $\mu\text{M}$ , respectively between  $t_0$  and 343 days). For lactate an initial decrease occurred (from 600  $\mu\text{M}$  at  $t_0$  to 470  $\mu\text{M}$  at 5

days), followed by a slow increase (to 580  $\mu\text{M}$  after 90 days) and then a much steeper increase (to 2.2 mM after 343 days).

Hydrogen concentrations in parallels "A" and "B" did not change during incubation and remained at around  $10^2$  ppm whereas in "C" an initial rapid increase (from  $3 \times 10^2$  ppm to  $4.5 \times 10^3$  ppm after 45 days) was followed by a constant but slower increase (to  $5.7 \times 10^3$  ppm after 434 days). Headspace carbon dioxide concentrations in cultures "A" and "B" also did not change during starvation (on average  $7 \times 10^4$  ppm) but slightly increased in "C" (from  $8.5 \times 10^3$  ppm at  $t_0$  to  $1.6 \times 10^5$  ppm in the final sample).

The pH of the cultures varied between pH 7.0 and pH 7.5 throughout starvation with an average pH of 7.2 in "A" and "B" and 7.3 in "C".

b) *Shewanella* sp. F18III

*Shewanella* sp. F18III was isolated from 4.25 mbsf at the Reference site of IODP Leg 307 (see Chapter III). During starvation no significant decrease in AODC total counts occurred ( $p < 0.05$ ; Fig. 4.19 a-c). In parallel "C", however, an initial increase from  $1.8 \times 10^7$  cells  $\text{ml}^{-1}$  to  $5.7 \times 10^7$  cells  $\text{ml}^{-1}$  within the first 20 days of incubation occurred (Fig. 4.19c) indicating that the culture had not quite reached stationary phase. Subsequently, cell numbers remained stable until the end of the experiment. Total cell numbers using the LIVE/DEAD<sup>®</sup> Kit were comparable to AODC except at  $t_0$  in "A" and at 90 and 190 days in "B" and "C". The percentages of dead cells were highest at the onset of starvation for all parallels and around 10%. Subsequently, the proportion of dead cells decreased to below 1% after 20 days and subsequently remained low in "C" whereas a slight upwards trend occurred in "A" (3%) and "B" (2%) towards the end of starvation (Fig. 4.19 a-c).

FISH detectability in "C" was high at the beginning of starvation ( $> 100\%$ ) but decreased to  $\sim 70\%$  by 90 days (Fig. 4.19c). This is a much higher FISH detectability than occurred in *Shewanella* strain SCSA3 (Fig. 4.17c).

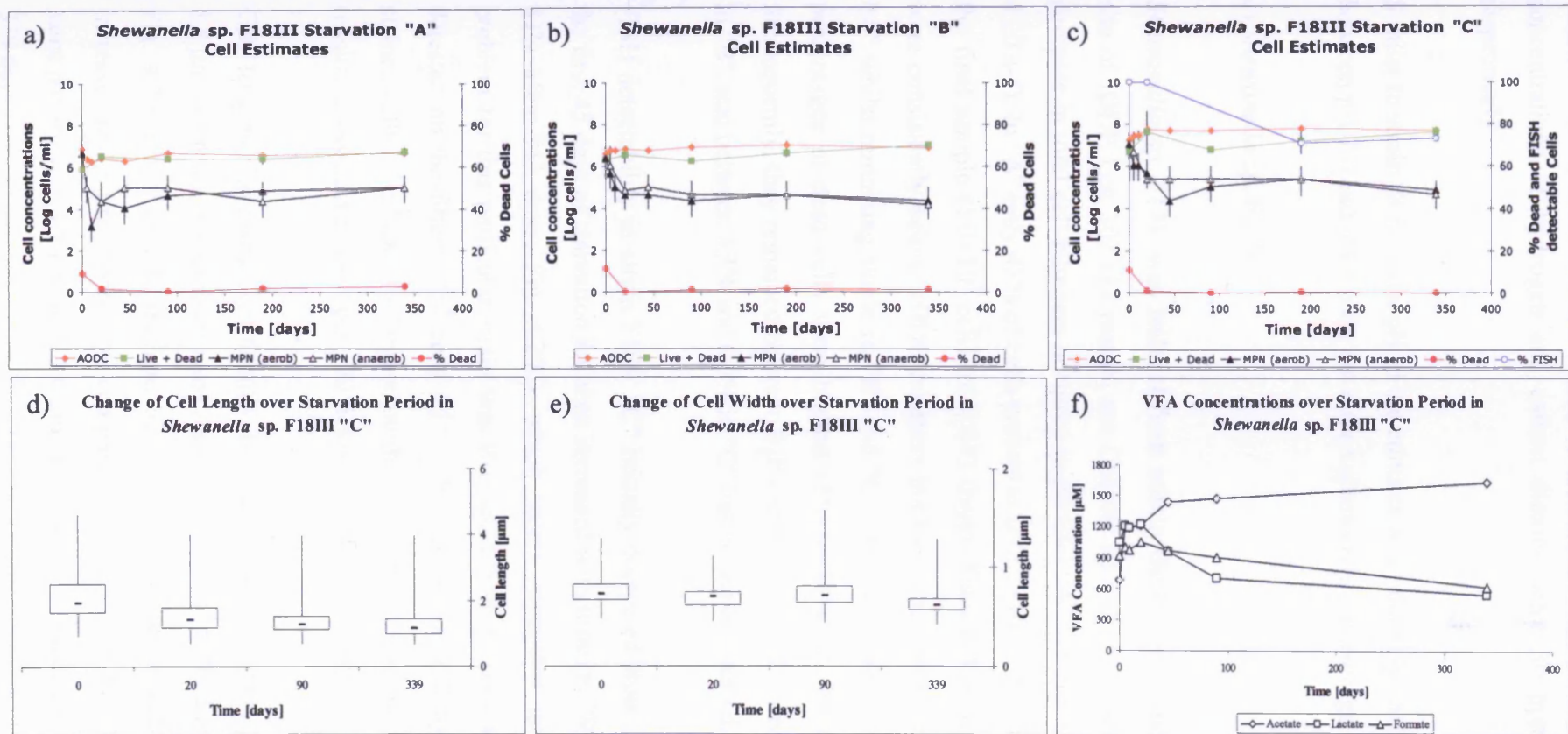
Cell lengths continuously decreased during starvation (from 2.1  $\mu\text{m}$  at  $t_0$  to 1.5  $\mu\text{m}$  after 20 days and to 1.4  $\mu\text{m}$  after 90 days). Cells were significantly longer at the onset of starvation than at any later samplings. Also, cells were significantly longer after 20 days of starvation than after 90 days. Although the mean of cell lengths decreased further between the final two samplings (1.3  $\mu\text{m}$  after 339 days), this change was not significant (Fig 4.19d). Results of cell widths measurements were more variable and, although some statistically significant changes occurred, no definitive trend occurred with mean cell widths throughout the experiment being 0.7  $\mu\text{m}$ . Similar to strain SCSA3 the cell morphology of strain F18III was more coccoid in later stages of starvation than at the beginning.

Culturability in aerobic MPNs decreased between the onset of starvation, in "A" from  $4.6 \times 10^6$  to  $1.5 \times 10^3$  cells  $\text{ml}^{-1}$  after 10 days, in "B" from  $2.4 \times 10^6$  to  $4.6 \times 10^4$  cells  $\text{ml}^{-1}$  after 20 days, and in "C" from  $1.1 \times 10^7$  to  $2.4 \times 10^4$  cells  $\text{ml}^{-1}$  after 45 days (Fig. 4.19 a-c). Afterwards, culturable cell numbers in "A" increased and in all cultures remained in the range of  $10^4$  to  $10^5$  cells  $\text{ml}^{-1}$  until the end of the experiment. As for *Shewanella* sp. SCSA3 anaerobic MPNs did not result in proper growth but activity was detected as indicated by a change of colour in the redox indicator. This was used as the basis for MPN calculations and gave almost identical results in "B" and "C" compared with the respective aerobic MPNs and was also similar in "A".

Incubation times for growth or activity to occur in the lowest dilutions of the MPN plates did not increase during starvation taking consistently three to five days.

The changes of VFA concentrations were similar in the three treatments of strain F18III. In parallel "C", acetate generally increased with time (from 0.7 mM at  $t_0$  to 1.6 mM after 339 days) while lactate and formate initially increased (from 1.0 mM to 1.2 mM after 5 days and from 0.9 mM to 1.0 mM after 20 days, respectively) before decreasing to 0.5 mM and 0.6 mM, respectively towards the end of starvation (Fig. 4.19f).

For treatment "C", headspace gas concentrations of hydrogen and carbon dioxide increased steeply within the first 5 days of the experiment (from  $1 \times 10^3$  to  $1 \times 10^4$  ppm and from  $1.3 \times 10^5$  to  $1.7 \times 10^5$ , respectively) and subsequently remained stable at this



**Fig. 4.19: Physiological responses of *Shewanella* sp. F18III to starvation.** a-c) Cell number estimates for treatments "A", "B", and "C". Confidence limits are given for AODC, Live + Dead, and MPNs, if not visible they fall within the size of the data points. d-e) Change of size during starvation in "C". Shown are the 25 and 75 percentile (box), the median (horizontal dash) and the data range (vertical dashes). f) Changes in VFA concentrations during starvation in "C".



level. In “A” and “B” no changes occurred for these two gases and average concentrations of hydrogen and carbon dioxide were 30 ppm and  $7 \times 10^4$  ppm, respectively.

Similar to strain SCSA3 the pH of the cultures was relatively variable. Values ranged between pH 7.0 and pH 7.6 for the three cultures with an average of pH 7.25 in each.

c) *Shewanella* sp. F17V

*Shewanella* sp. F17V was isolated from within the buried carbonate at the Mound site of IODP Leg 307 (17 mamb, see Chapter III). During starvation a significant decrease in total cell numbers occurred in parallel “A” but not in “B” and “C” (Fig. 4.20 a-c). In “A” only 45% of cells present at  $t_0$  ( $2.3 \times 10^7$  cells ml<sup>-1</sup>) were detected in the final sample ( $1.0 \times 10^7$  cells ml<sup>-1</sup>, 343 days). Total LIVE/DEAD<sup>®</sup> cell numbers were consistently below AODC numbers but the trends were similar, decreasing in “A” whilst remaining stable in “B” and “C”. As previously seen for strain F18III, percentages of dead cells were highest (5% to 19%) at the onset of starvation. Subsequently, they remained between 0.3% and 3% in “A”, between 0.8% and 1.7% in “B”, and between 0.3% and 0.9% in “C” but no trend occurred.

FISH detectability in strain F17V “C” initially decreased from 76% to 62% within the first 45 days of starvation but then increased with time (to 79% after 90 days and 83% after 343 days; Fig. 4.20c), which seems somewhat unlikely. A technical problem for this particular strain was that apart from  $t_0$ , only very few cells were detected on the filter ( $\sim 10^4$  cells ml<sup>-1</sup>). This applies to both hybridised and DAPI stained cells. The lack of a large enough sample population might have affected the results as less than 20 cells per 100 fields of view were counted.

Cell lengths decreased significantly between  $t_0$  and 20 days of starvation (from 1.8  $\mu\text{m}$  to 1.6  $\mu\text{m}$ , respectively) and also between 20 and 195 days (1.3  $\mu\text{m}$ ;  $p < 0.05$ , Fig. 4.20 d-e). Towards the end of starvation, however, cell sizes significantly increased to 1.5  $\mu\text{m}$  at 339 days compared with 195 days. Cell widths followed the same pattern with 1.1  $\mu\text{m}$  at  $t_0$ , 1.0  $\mu\text{m}$  at 20 days, 0.9  $\mu\text{m}$  at 195 days, and 1.0  $\mu\text{m}$  at 339 days.

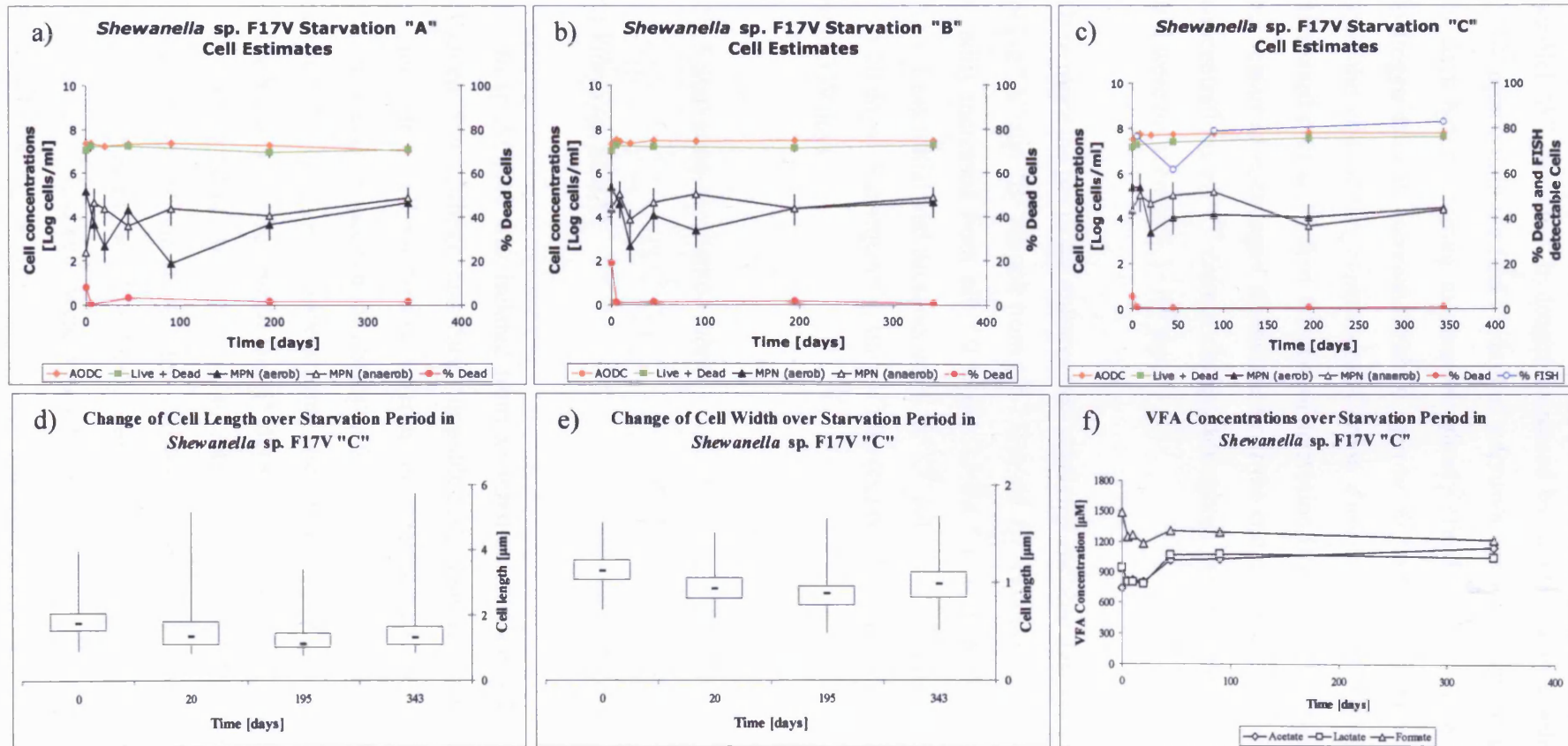
Culturability of strain F17V was relatively low at the start of the experiment with only  $\sim 10^5$  culturable cells  $\text{ml}^{-1}$  in aerobic incubations. Subsequently, the number of culturable cells decreased to between  $10^2$  and  $10^3$  cells  $\text{ml}^{-1}$  after 20 days before slowly increasing to approximately  $10^4$  cells  $\text{ml}^{-1}$  towards the end of the experiment (Fig. 4.20 a-c).

Unlike the other two *Shewanella* strains, strain F17V showed real growth under anaerobic conditions forming cell pellets. Culturable anaerobic cell numbers initially increased from  $10^2$  in “A” and  $10^3$  in “B” and “C” to  $\sim 10^5$  cells  $\text{ml}^{-1}$  after 10 days. Subsequently, they remained relatively stable in “C” until 90 days before decreasing to  $4.6 \times 10^3$  cells  $\text{ml}^{-1}$  followed by another increase to  $2.4 \times 10^4$  cells  $\text{ml}^{-1}$ . In “A”, culturable cell numbers decreased to  $3.8 \times 10^3$  cells  $\text{ml}^{-1}$  between 10 and 45 days followed by a slow increase towards the end of the experiment ( $7.5 \times 10^4$  cells  $\text{ml}^{-1}$  at 339 days). Culturability in “B” also decreased after 10 days to  $7.5 \times 10^3$  cells  $\text{ml}^{-1}$  after 20 days. Subsequently, it increased relatively continuously to  $7.5 \times 10^4$  cells  $\text{ml}^{-1}$  after 339 days. Incubation times did not increase during starvation but varied between 2 and 6 days.

VFA concentrations in treatments “A” and “B” were below the detection limit for the first 20 days of starvation. Subsequently, acetate increased from  $43 \mu\text{M}$  at 45 days to  $128 \mu\text{M}$  after 339 days in “A” but remained around  $25 \mu\text{M}$  in “B”. Lactate marginally increased from  $2 \mu\text{M}$  in “A” and  $5 \mu\text{M}$  in “B” after 45 days to  $8 \mu\text{M}$  in both treatments after 339 days. In contrast, formate concentrations decreased in both “A” and “B” from  $25 \mu\text{M}$  after 45 days to  $5$  after 339 days.

In treatment “C” acetate was relatively stable for the first 20 days at around  $0.8 \text{ mM}$ . Then it increased steeply to  $1.0 \text{ mM}$  after 45 days followed by a slow steady increase to  $1.1 \text{ mM}$  after 339 days (Fig. 4.20c). Lactate initially decreased from  $0.9 \text{ mM}$  to  $0.8 \text{ mM}$  after 5 days, remained at this level until 20 days before also steeply increasing until 45 days ( $1.0 \text{ mM}$ ). Subsequently it remained at this concentration until the end of the experiment. Formate concentrations initially decreased from  $1.5 \text{ mM}$  to  $1.1 \text{ mM}$  after 20 days. This was followed by an increase to  $1.3 \text{ mM}$  after 45 days and a subsequent slow decrease to  $1.2 \text{ mM}$  after 339 days.

Headspace hydrogen and carbon dioxide concentrations remained unchanged in



**Fig. 4.20: Physiological responses of *Shewanella* sp. F17V to starvation.** a-c) Cell number estimates for treatments "A", "B", and "C". Confidence limits are given for AODC, Live + Dead, and MPNs, if not visible they fall within the size of the data points. d-e) Change of size during starvation in "C". Shown are the 25 and 75 percentile (box), the median (horizontal dash) and the data range (vertical dashes). f) Changes in VFA concentrations during starvation in "C".



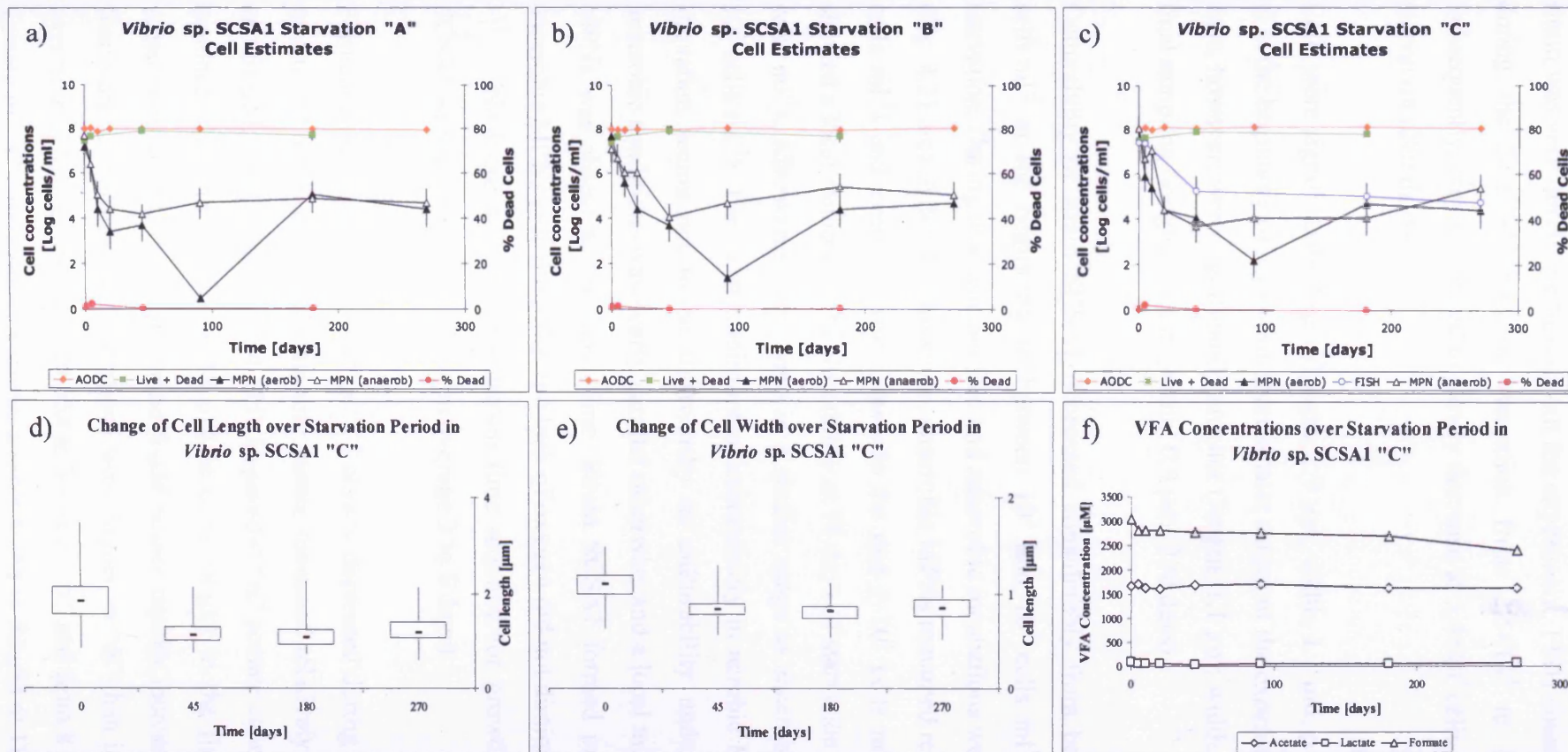
parallel “C” (average 30 ppm and  $1.5 \times 10^5$  ppm, respectively) during starvation. In parallel “A”, however, hydrogen increased by more than one order of magnitude to  $2 \times 10^3$  ppm within the first 10 days of starvation and continued to almost double until 90 days before starting to decrease slowly (final concentration 80 ppm). In “B”, hydrogen initially increased from 6 ppm to 50 ppm after 5 days where it remained until the end of the experiment. Carbon dioxide concentrations in “A” and “B” decreased during the first 10 days of starvation (from  $1.2 \times 10^5$  ppm to  $6.7 \times 10^4$  ppm) but remained unchanged afterwards until the end of the experiment. Carbon dioxide concentrations in “C” did not change throughout the entire lengths of the experiment and were on average  $1.5 \times 10^5$  ppm.

Once more the pH in the cultures was relatively variable. During the first 20 days the pH in “A” and “B” ranged from pH 7.0 to pH 7.7. However, from 45 days onwards it steadily increased from pH 7.0 to pH 7.2 for “A” and to pH 7.4 for “B” after 339 days. Less initial variation occurred in “C” (pH between pH 7.0 and pH 7.2 for the first 20 days). Subsequently, the pH also increased from pH 7.0 at 45 days to pH 7.4 after 339 days.

## 2.2.5 Starvation-survival of *Vibrio*

### a) *Vibrio* sp. SCSA1

*Vibrio* sp. SCSA1 was isolated from an intertidal surface sediment of the German Wadden Sea (Droege and Sass, unpublished). During starvation, no significant change in total cell numbers occurred in any treatment (AODC;  $p < 0.05$ ; Fig. 4.21 a-c) with average concentrations being in  $9 \times 10^7$  cells  $\text{ml}^{-1}$  “A” and “B”  $1 \times 10^8$  cells  $\text{ml}^{-1}$  and in “C”. Total cell numbers using the LIVE/DEAD<sup>®</sup> Kit increased by 250% to 374% between the beginning and 45 days of starvation (to  $7.8 \times 10^7$  cells  $\text{ml}^{-1}$  in “A”, to  $8.5 \times 10^7$  cells  $\text{ml}^{-1}$  in “B”, and to  $7.4 \times 10^7$  cells  $\text{ml}^{-1}$  in “C”). Subsequently, they decreased by 16% to 46% after 180 days. After five days of starvation a local maximum of up to 3% of dead cells were present in the cultures. In other samples the percentages of dead cells were below 1%, (for “B” and “C” on average 0.7%, “A” on average 0.9%; Fig. 4.21c).



**Fig. 4.21: Physiological responses of *Vibrio sp. SCSA1* to starvation.** a-c) Cell number estimates for parallel "A", "B", and "C". Confidence limits are given for AODC, Live + Dead, FISH and MPNs, if not visible they fall within the size of the data points. d-e) Change of size during starvation in "C". Shown are the 25 and 75 percentile (box), the median (horizontal dash) and the data range (vertical dashes). f) Changes in VFA concentrations during starvation in "C".

The FISH total counts for “C” are given (Fig. 4.21c) because DAPI staining for this strain was very unreliable throughout the experiment. FISH counts decreased rapidly during the first 45 days of starvation from  $2.5 \times 10^7$  to  $1.9 \times 10^5$  cells ml<sup>-1</sup>. Subsequently, they continued to slowly decrease to  $5.4 \times 10^4$  cells ml<sup>-1</sup> until the end of starvation (270 days).

Cells were significantly larger (length, 1.9  $\mu$ m; width, 1.1  $\mu$ m;  $p < 0.05$ ; Fig. 4.21 d-e) at the beginning of starvation than at later stages of the experiment. Cells after 180 days, however, were significantly smaller (length, 1.1  $\mu$ m; width, 0.8  $\mu$ m) than at the final sampling (length, 1.2  $\mu$ m; width, 0.9  $\mu$ m; 270 days).

Culturability of strain SCSA1 decreased considerably from between  $10^7$  and  $10^8$  cells ml<sup>-1</sup> at the beginning to between  $10^3$  and  $10^4$  cells ml<sup>-1</sup> after 45 days of starvation. During this time aerobic and anaerobic incubations were relatively similar (Fig. 4.21 a-c). After this, however, anaerobic MPNs remained relatively stable ( $\sim 10^4$  cells ml<sup>-1</sup>) and even increased towards the end ( $\sim 10^5$  cells ml<sup>-1</sup>). Aerobic MPNs showed a local minimum of culturability at 91 days of starvation (between 3 and 150 cells ml<sup>-1</sup>). Afterwards, they were in a similar range as anaerobic incubations ( $10^4$ - $10^5$  cells ml<sup>-1</sup>). The local minimum of culturability in aerobic MPNs at 91 days of starvation seems not to be trustworthy as culturability under both aerobic and anaerobic conditions was nearly parallel otherwise and a local minimum in anaerobic MPNs was absent at the same time. Strain SCSA1 formed proper cell pellets in anaerobic MPNs indicating that the lack of oxygen did not diminish its culturability.

No increase in the incubation time required for growth occurred in strain SCSA1 with ongoing starvation (on average 3 to 5 days).

Formate concentrations in parallel “C” slowly decreased during starvation (from 3.0 mM to 2.4 mM) whereas acetate and lactate remained relatively constant at 1.7 mM and 61  $\mu$ M, respectively (Fig. 4.21f). In parallel “A” acetate concentrations increased continuously with time from 11  $\mu$ M at  $t_0$  to 48  $\mu$ M in the final sample. In “B”, acetate remained constant at around 5  $\mu$ M before rapidly increasing to 57  $\mu$ M in the final sample. Formate concentrations were higher in “A” than in “B” but decreased over time in both cases (from 24  $\mu$ M to 3  $\mu$ M in “A” and from 8  $\mu$ M to 2  $\mu$ M in “B”). Lactate in “B” continuously increased from 5  $\mu$ M to 52  $\mu$ M at 180 days of starvation

before rapidly dropping to 7  $\mu\text{M}$  in the final sample. Concentrations of lactate in “A” remained at low levels throughout (4  $\mu\text{M}$ ).

Headspace carbon dioxide initially increased by approximately one order of magnitude from  $10^4$  to  $10^5$  ppm in all parallels and remained within this range until the end of the experiment. Hydrogen continuously increased from below the detection limit of 2 ppm at the beginning to 65 - 82 ppm at the end of starvation (270 days) in all parallels.

The pH of the cultures decreased by approximately 0.6 pH units during the first 20 days to pH 7.3 in “A” and “B”, and to pH 7.1 in “C”. Subsequently the pH of the media remained constant until the end of starvation.

b) *Vibrio* sp. F17IV

*Vibrio* sp. F17IV was isolated from within the buried carbonate mound at the Mound site of IODP Leg 307 (17 mamb, see Chapter III). No significant change in total cell counts occurred during starvation under any treatment (on average  $7.8 \times 10^7$  cells  $\text{ml}^{-1}$  in “A”,  $7.3 \times 10^7$  cells  $\text{ml}^{-1}$  in “B”, and in  $1.0 \times 10^8$  cells  $\text{ml}^{-1}$  “C”; AODC,  $p < 0.05$ ; Fig. 4.22 a-c). Total cell numbers estimated with the LIVE/DEAD<sup>®</sup> Kit were generally below those using AODC and more variable. Interestingly, the percentages of dead cells were slightly elevated in all samples from 5 days of starvation with up to 17%. A similar observation was made for strain SCSA1 albeit the proportions of dead cells did not exceed 3% (Fig. 4.21). The average of dead cells at other timepoints was below 1% in “B” and “C” and at 2% in “A” where additionally a slight increase was observed towards the end of the experiment (4% in final sample; Fig. 4.22).

In “C”, total cell counts for FISH decreased with time from  $1.9 \times 10^7$  cells  $\text{ml}^{-1}$  at  $t_0$  to  $3.0 \times 10^5$  cells  $\text{ml}^{-1}$  after 45 days and subsequently varied between  $1.5 \times 10^5$  and  $2.2 \times 10^5$  cells  $\text{ml}^{-1}$  until the end of the experiment (Fig. 4.22c). These results are very similar to strain SCSA1.

Cell sizes significantly decreased between  $t_0$  (length, 2.4  $\mu\text{m}$ ; width, 1.2  $\mu\text{m}$ ) and 45 days of starvation (length, 1.5  $\mu\text{m}$ ; width, 0.9  $\mu\text{m}$ ; ; $p < 0.05$ ). Subsequently, no further reduction in cell lengths occurred whereas there was some variation for widths (between 0.9 and 1.0  $\mu\text{m}$ ; Fig. 4.22 d-e).

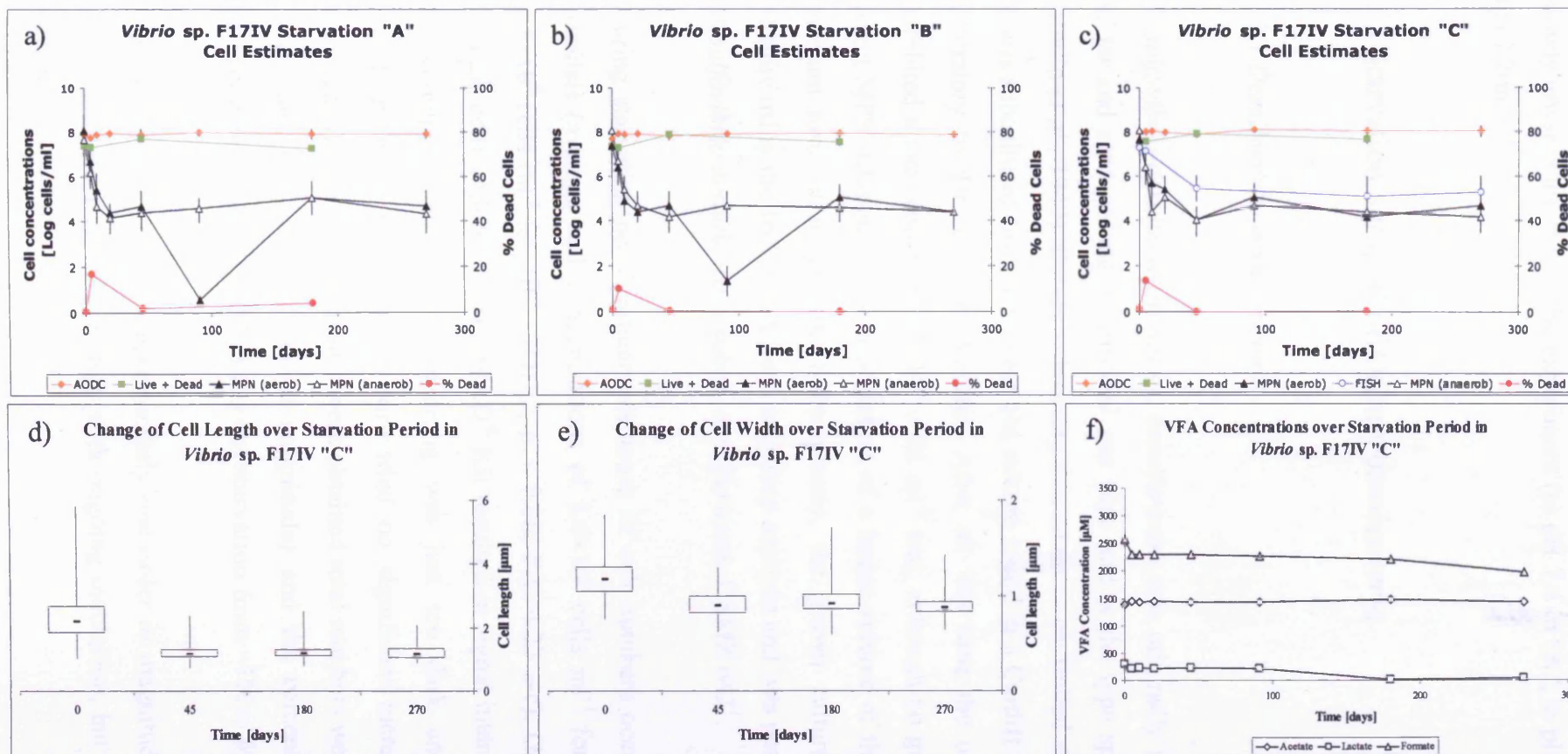
Culturability was very similar in aerobic and anaerobic incubations (Fig. 4.22 a-c) and also compared to strain SCSA1. An initial decrease of up to four orders of magnitude occurred within the first 20 days of starvation (from between  $10^7$  and  $10^8$  to  $10^4$  and  $10^5$  cells  $\text{ml}^{-1}$ ). Subsequently, MPNs remained stable at around  $10^4$  cells  $\text{ml}^{-1}$  except for aerobic culturability at 91 days in parallels “A” and “B” (local minimum of 4 to 23 cells  $\text{ml}^{-1}$ ). This also occurred in strain SCSA1 (Fig. 4.21).

With continued starvation a slight increase of incubation time required for growth occurred in the MPNs. While growth was completed after 2 days for the first two timepoints up to seven days were needed for the final sample for both aerobic and anaerobic incubations.

Changes in VFA concentrations were very similar in the three parallel incubations. In “C”, acetate concentrations initially increased slightly (from 1.38 mM at the beginning to 1.43 mM after 91 days) but then remained relatively stable throughout the experiment (Fig. 4.22f). Lactate and formate generally decreased with time although three stages occurred. An initial decrease until 5 days of starvation (from 310  $\mu\text{M}$  to 230  $\mu\text{M}$  for lactate, and from 2.58 mM to 2.3 mM for formate) was followed by a stable period until 91 days. Subsequently, a further decrease in concentrations occurred (to  $\sim 30$   $\mu\text{M}$  for lactate, and to 2.0 mM for formate).

Hydrogen headspace concentrations increased from 13 ppm to 82 ppm over the starvation period in parallel “C” while no changes occurred in the other two parallels apart from a local peak in “A” of 235 ppm after 20 days of starvation (mean concentrations of  $\sim 35$  ppm in “A” and “B”). Carbon dioxide concentrations remained close to  $1 \times 10^5$  ppm in “A” and “B” during starvation while concentrations were twice as high for “C” but also stable.

The pH of the cultures decreased sharply by 0.7 pH units during the first 20 days of



**Fig. 4.22: Physiological responses of *Vibrio* sp. F17IV to starvation.** a-c) Cell number estimates for treatments "A", "B", and "C". Confidence limits are given for AODC, Live + Dead, FISH, and MPNs, if not visible they fall within the size of the data points. d-e) Change of size during starvation in "C". Shown are the 25 and 75 percentile (box), the median (horizontal dash) and the data range (vertical dashes). f) Changes in VFA concentrations during starvation in "C".

incubation (to pH 7.2 in “A” and “B” and to pH 7.0 in “C”) and then increased slowly towards the end of the experiment (to pH 7.4 in “A”, to pH 7.3 in “B”, and to pH 7.2 in “C”).

## 2.3 Starvation-survival of *Deltaproteobacteria*

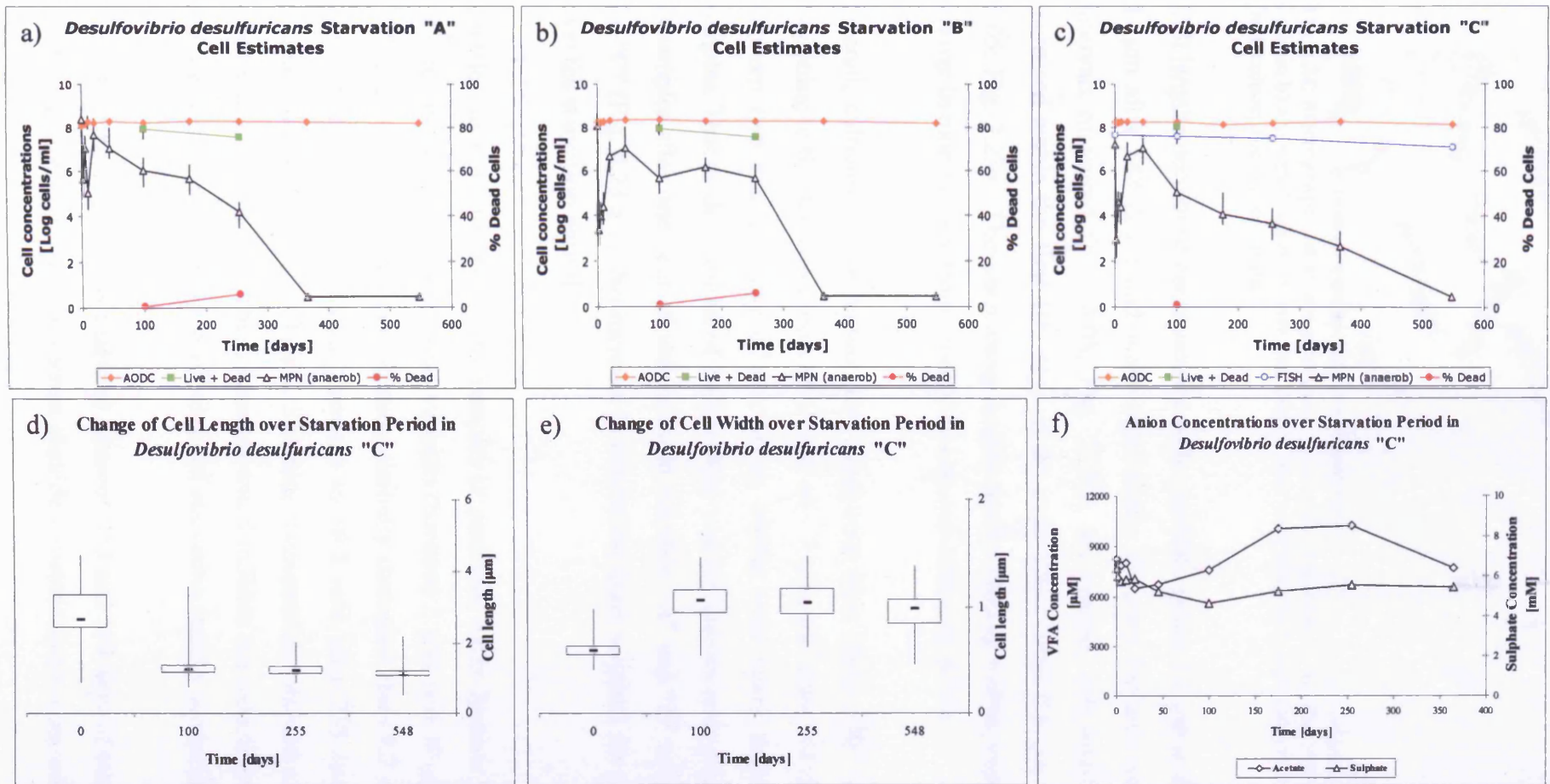
### 2.3.1 *Desulfovibrio desulfuricans*

*Desulfovibrio desulfuricans* subsp. *desulfuricans* was originally isolated from a mix of tar and sand around a corroded gas pipe and is the type species of the genus (Butlin *et al.*, 1949). Before subjecting this strain to controlled starvation conditions it was subcultured from a 12 year old culture found in a Cardiff University teaching laboratory by Dr. Gordon Webster. After all this time the original culture still exhibited a total count of  $3.7 \times 10^6$  cells ml<sup>-1</sup> and, although no growth occurred in a direct MPN dilution series, inoculation of a larger volume of the culture into fresh medium resulted in growth. Subsequently, the grown culture was checked for identity using the 16S rDNA gene sequence analysis and was positively identified as *Desulfovibrio desulfuricans* subsp. *desulfuricans*. DSMZ 642<sup>T</sup>.

During starvation no significant decrease of cell numbers occurred in any of the parallels (average cell concentrations of  $1.6 \times 10^8$  cells ml<sup>-1</sup> for “A” and “C” and  $1.8 \times 10^8$  cells ml<sup>-1</sup> for “B”; AODC;  $p < 0.05$ ; Fig. 4.23 a-c). Only few attempts of staining cells with the LIVE/DEAD<sup>®</sup> Kit resulted in signal intensities bright enough for counting. Generally the staining was just too dark and although several modifications to the protocol were tried no significant increase in quality was achieved. Where positive results were obtained total numbers were below the AODC total count (up to one order of magnitude) and the percentages of dead cells increased between 100 and 255 days of starvation from ~1% to 6% in “A” and “B”.

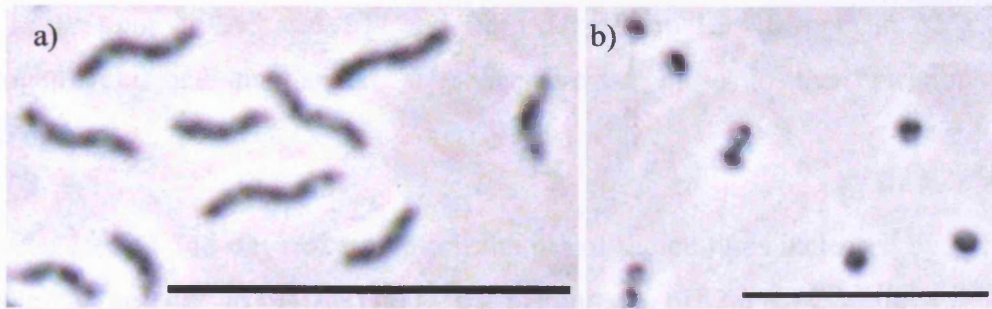
FISH counts in “C” were approximately one order of magnitude below the AODC count and decreased only slightly with ongoing incubation, but remained above  $10^7$  cells ml<sup>-1</sup>.





**Fig. 4.23: Physiological responses of *D. desulfuricans* to starvation.** a-c) Cell number estimates for treatments "A", "B", and "C". Confidence limits are given for AODC, Live + Dead, FISH and MPNs, if not visible they fall within the size of the data points. d-e) Change of size during starvation in "C". Shown are the 25 and 75 percentile (box), the median (horizontal dash) and the data range (vertical dashes). f) Changes in acetate and sulphate concentrations during starvation in "C".





**Fig. 4.24: Micrographs of *D. desulfuricans*.** a) at onset of starvation; b) after 548 days of starvation. Cells in b) are significantly shorter and thicker thus exhibiting a coccoid morphology compared to a fresh culture. Bars equal 10 µm.

Cell lengths decreased continuously with starvation (from 2.8 µm at the beginning to 1.1 µm after 548 days) and were significantly different between each of the time intervals measured ( $p < 0.05$ ; Fig. 2.23d). In contrast, cell widths significantly increased within the first 100 days of the experiment (from 0.6 µm to 1.0 µm;  $p < 0.05$ ; Fig. 2.23e). The decreasing lengths and increasing widths were reflected in a change in morphology from vibrioid to coccoid cells (Fig. 4.24).

Overall, culturability decreased with incubation time (from  $\sim 10^8$  cells ml<sup>-1</sup> at the beginning to the detection limit of growth of  $< 3$  cells ml<sup>-1</sup> after 548 days). Bizarrely, between two and 10 days of starvation, MPNs were lower than in subsequent samples. That aside, the loss of culturability was continuous and no growth occurred in samples after one year of starvation in parallels “A” and “B” and after 1.5 years for “C” (Fig. 4.23 a-c). No increase in incubation times required for growth occurred over the starvation period.

Lactate was not detected in any samples or parallels, while formate was sometimes detected but concentrations were variable (between 5 µM and 40 µM). In parallel “C” (Fig. 4.23f), acetate concentrations initially decreased from 8.2 mM to 6.5 mM after 20 days, followed by an increase to 10.3 mM after 255 days and another decrease to 7.6 mM after 548 days. Sulphate concentrations showed a similar trend if slightly less pronounced. These changes could indicate the reduction of endogenous substrates using sulphate up to 100 days of starvation thereby producing acetate.

The culture headspace was analysed between 255 and 548 days of starvation. During this time a slight decrease in carbon dioxide concentrations occurred (from  $1.6 \times 10^5$

to  $1.1 \times 10^5$  ppm in “A” and “B” and from  $2.0 \times 10^5$  to  $1.3 \times 10^7$  ppm in “C”) while hydrogen remained unchanged at concentrations close to the detection limit ( $< 35$  ppm).

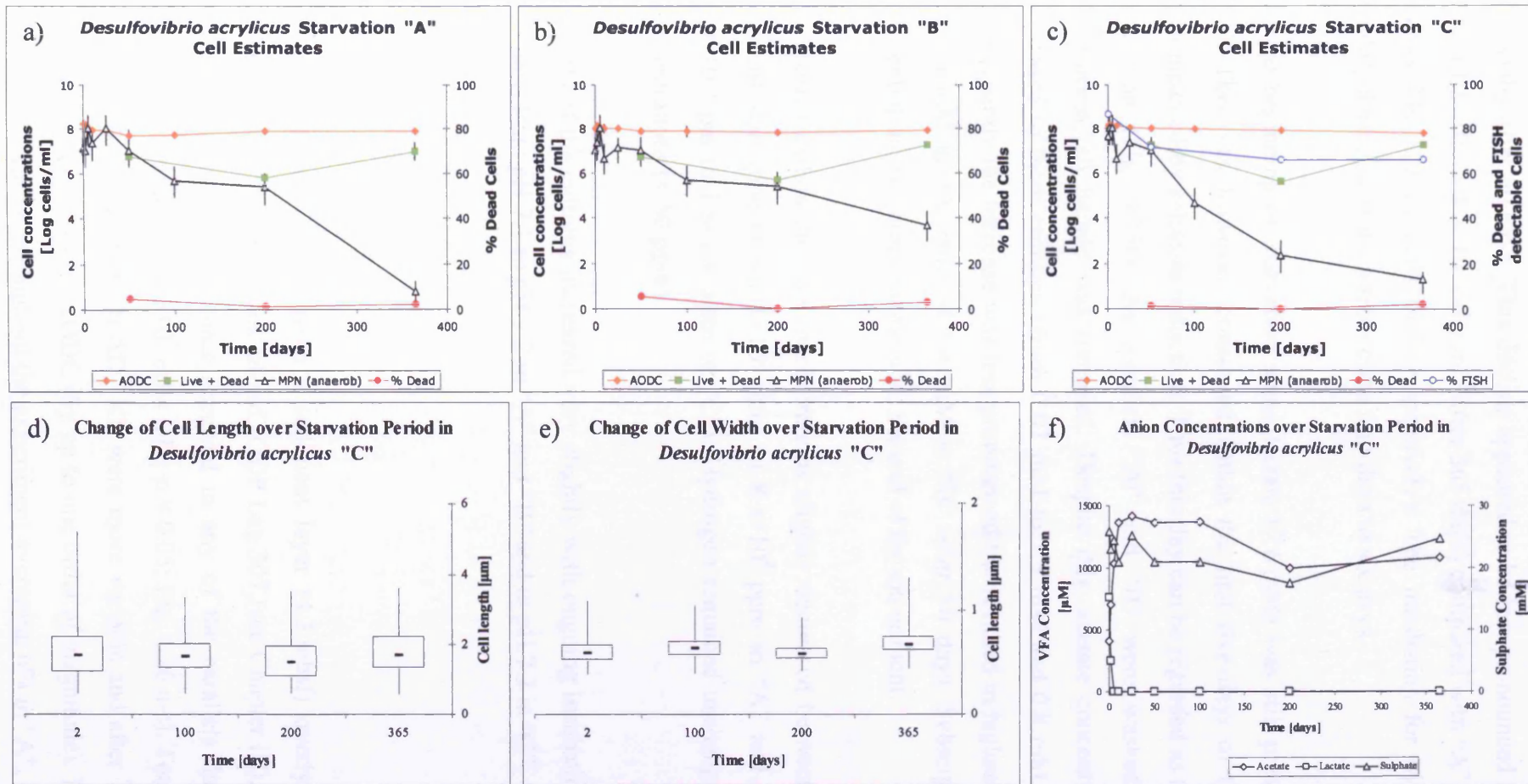
Between 10 and 548 days of starvation the pH of the cultures increased by 0.25 pH units in “A” and “B” to pH 7.5 and by 0.3 pH units to pH 7.6 in “C”.

### 2.3.2 *Desulfovibrio acrylicus*

*D. acrylicus* was isolated from an intertidal surface sediment of the Dutch Wadden Sea (van der Maarel *et al.*, 1996). During starvation total cell counts did not significantly decrease in parallels “A” and “B” (on average  $9.9 \times 10^7$  and  $9.6 \times 10^7$  cells  $\text{ml}^{-1}$ ) but in “C” (from  $1.4 \times 10^8$  cells  $\text{ml}^{-1}$  to  $6.3 \times 10^7$  cells  $\text{ml}^{-1}$  after 365 days). The final sample of “C” only contained 42% of the original cell concentration (AODC,  $p < 0.05$ ; Fig. 4.25 a-c). The LIVE/DEAD<sup>®</sup> Kit worked better on this strain than on *D. desulfuricans*, but total cell numbers remained below AODC total counts (between  $10^5$  and  $10^7$  cells  $\text{ml}^{-1}$ ). The proportions of dead cells varied but were generally below 5% and generally lower in “C” than in “A” and “B”. The lack of dead cells in “C” contradicts the above mentioned significant decrease of total cell numbers in this culture.

Percentages of FISH detectable cells in “C” decreased rapidly within the first 200 days (from 87% to 66%) of the experiment and remained at this value until the end of starvation (365 days).

Cell sizes in *D. acrylicus* did not change as dramatically as observed in other strains (e.g. *D. desulfuricans*; Fig. 4.25 d-e). Some changes, however, were calculated to be statistically significant ( $p < 0.05$ ), for example cell lengths between two and 200 days decreased from  $1.9 \mu\text{m}$  to  $1.6 \mu\text{m}$ . The microscopic morphology of starving cells also did not change as strikingly as observed for *D. desulfuricans* (from rod-shaped to coccoid).



**Fig. 4.25: Physiological responses of *Desulfovibrio acrylicus* to starvation.** a-c) Cell number estimates for treatments "A", "B", and "C". Confidence limits are given for AODC, Live + Dead, and MPNs, if not visible they fall within the size of the data points. d-e) Change of size during starvation in "C". Shown are the 25 and 75 percentile (box), the median (horizontal dash) and the data range (vertical dashes). f) Changes in VFA and sulphate concentrations during starvation in "C".

Anaerobic culturability of *D. acrylicus* decreased with continued incubation, especially after 50 days. This decline appeared to be less pronounced in parallel “B” (from  $1.1 \times 10^7$  to  $4.3 \times 10^3$  cells ml<sup>-1</sup> after 365 days) compared with “A” and “C” (final MPNs 240 and 21 cells ml<sup>-1</sup>, respectively). The incubation for MPNs increased slightly during starvation from circa four days to six days.

At the beginning of starvation some lactate (7.6 mM) was still present in parallel “C”. This was, however, consumed within the first five days of the experiment accompanied by sulphate reduction. Thus this day can be regarded as the real start of starvation (Fig. 4.25f). As parallels “A” and “B” were washed prior to the experiment, all lactate was removed. Despite this, acetate concentrations steeply increased in these cultures (from 0.07 mM to 1.3 mM and 0.8 mM, respectively). Subsequently the increase was less pronounced but resulted in highest concentration of 2.0 mM in “A” and of 1.4 mM in “B” after 50 days. Subsequently, acetate concentrations remained stable until the end of the experiment.

Headspace carbon dioxide concentrations slightly decreased between 100 and 365 days of starvation (from  $1 \times 10^5$  ppm to  $8.3 \times 10^4$  ppm in “A” and “B” and from  $1.9 \times 10^5$  ppm to  $1.6 \times 10^5$  ppm in “C”). Hydrogen remained unchanged at very low concentrations (< 50 ppm).

The pH of the cultures increased very slightly with ongoing incubation by up to 0.2 pH units from pH 7.1 to pH 7.2 in “A” and “B” and to pH 7.3 in “C”.

### 2.3.3 *Desulfovibrio* sp. F16I

Strain F16I was isolated from a sediment layer (4.3 mbsf) overlying the buried carbonate mound at the Flank site of IODP Leg 307 (see Chapter III). No significant decline in AODC total counts occurred in any of the parallels during starvation (average cell numbers  $\sim 2 \times 10^7$  cells ml<sup>-1</sup>;  $p < 0.05$ ; Fig. 4.26 a-c). Total cell numbers estimated with the LIVE/DEAD<sup>®</sup> Kit were more variable and after 100 days lower than those obtained using AODC (by up to one order of magnitude). The proportions of dead cells varied throughout the experiment averaging 6% in “A”, 4% in “B”, and

11% in “C”. The elevated average of dead cells in “C” is caused by a local maximum of 41% dead cells at 175 days. The proportion of dead cells in “A” and “B” was also elevated for this timepoint albeit lower (at 11%). Local maxima of dead cells contradict the stable total counts.

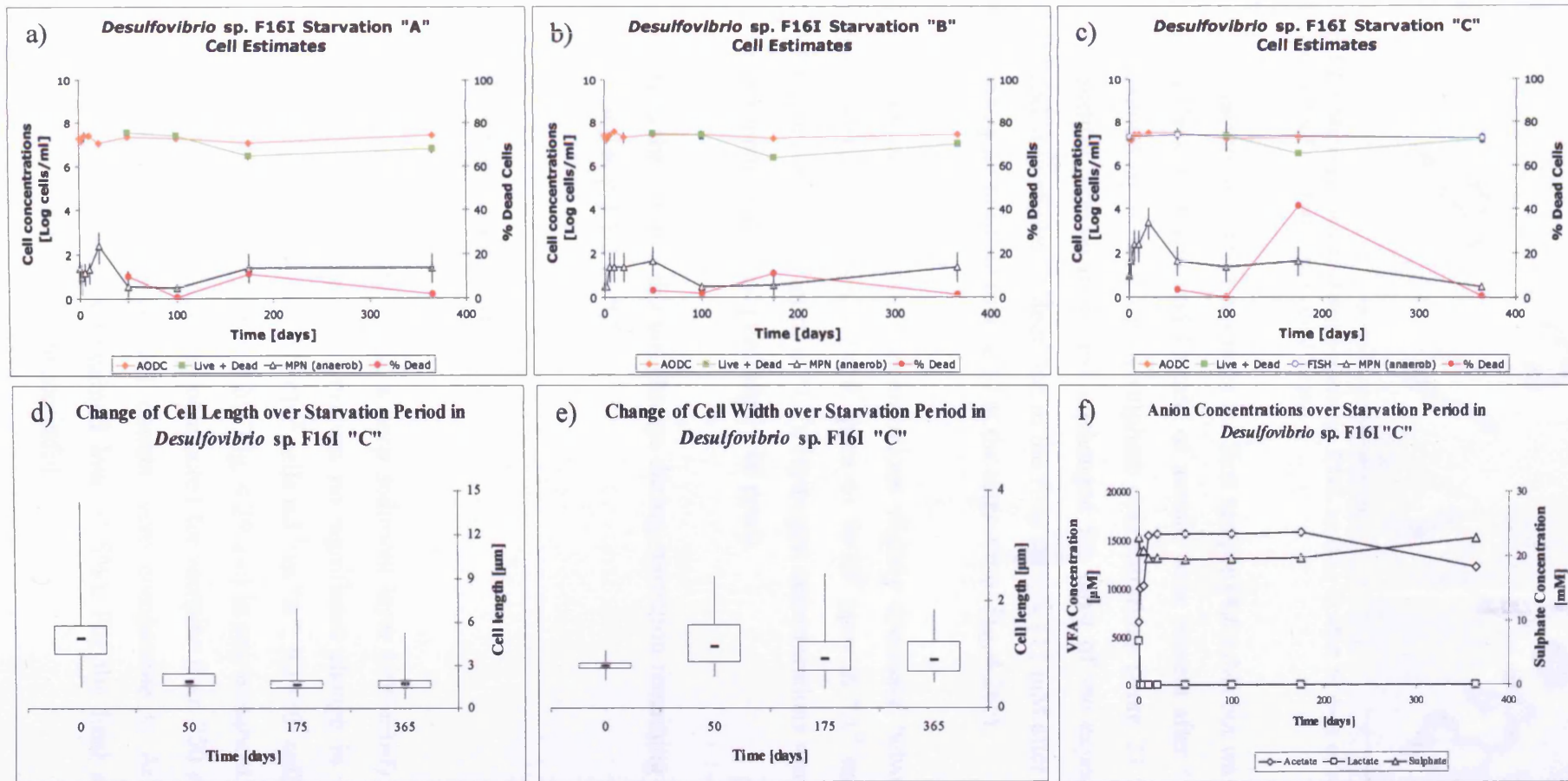
Total FISH counts in parallel “C” did not decrease with starvation and were similar to the AODC total counts. However, DAPI staining was very unreliable resulting in cell numbers of one order of magnitude below the FISH counts for samples from  $t_0$  and 50 days of starvation (FISH detectability 333% and 421%, respectively). In the final sample DAPI did not stain any cells. Thus, total numbers of FISH detected cells are given rather than percentages of DAPI count (Fig. 4.26c).

Cell lengths of strain F16I continuously decreased during starvation. There was a significant decline between  $t_0$  (5.0  $\mu\text{m}$ ) and subsequent samples but also between 50 days (2.3  $\mu\text{m}$ ) of starvation and later stages (1.8  $\mu\text{m}$ ; Fig. 4.26d). Cell widths initially increased significantly (from 0.8  $\mu\text{m}$  at  $t_0$  to 1.3  $\mu\text{m}$  after 50 days;  $p < 0.05$ ; Fig. 4.26e) before decreasing again (to 1.0  $\mu\text{m}$  after 175 days). This can be explained by the formation of spheroblasts in the cultures (Fig. 4.27b), which so far had not occurred in any other culture. Previously, dwarfing occurred resulting in round cells (e.g. *D. desulfuricans* and *Shewanella* sp. SCSA3) but spheroblasts were not observed.

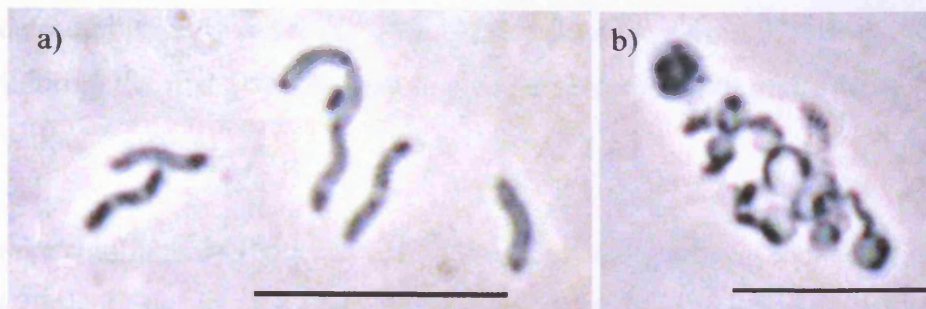
Culturability in strain F16I was very low and variable throughout starvation (Fig. 4.26 a-c) never exceeding  $10^3$  culturable cells  $\text{ml}^{-1}$ . It is unclear why this was the case. FeS precipitation occurred in the medium and growth was confirmed by SGI analysis of the MPN plates. The low culturability contradicts the stable AODC total counts, the relatively low percentages of dead cells, and the high detection of viable cells using FISH.

During starvation acetate concentrations continuously increased in parallels “A” and “B” but were about three times higher in “A” than in “B” with 1.6 mM compared to 0.57 mM after 365 days, respectively. Acetate production occurred despite the apparent absence of lactate in these washed cultures. However, sulphate concentrations remained stable in these cultures.





**Fig. 4.26: Physiological responses of *Desulfovibrio* sp. F161 to starvation.** a-c) Cell number estimates for treatments "A", "B", and "C". Confidence limits are given for AODC, Live + Dead, and MPNs, if not visible they fall within the size of the data points. d-e) Change of size during starvation in "C". Shown are the 25 and 75 percentile (box), the median (horizontal dash) and the data range (vertical dashes). f) Changes in VFA and sulphate concentrations during starvation in parallel "C".



**Fig. 4.27: Micrographs of *Desulfovibrio* sp. F16I.** a) at  $t_0$ ; b) after 50 days of starvation. Cells in b) had formed spheroblasts. Bars equal 10  $\mu\text{m}$ .

In “C”, lactate was still present in the first sample (4.6 mM) but was depleted by two days of the experiment and 15 mM of acetate were present after 10 days. This was accompanied by a decrease in sulphate concentrations (from 23 mM to 20 mM). Subsequently, acetate remained unchanged for most of the experiment (until 175 days) and only showed a decrease in the final sample (12 mM after 365 days), which is surprising as sulphate increased at the same time (Fig. 4.26c).

Headspace carbon dioxide concentrations slightly decreased between 100 and 365 days of starvation (from  $1.8 \times 10^5$  ppm to  $9 \times 10^5$  ppm in “A” and “B” and from  $3.4 \times 10^5$  ppm to  $1.6 \times 10^5$  ppm in “C”). Hydrogen concentrations were low throughout the experiment in all three parallels ( $< 40$  ppm).

The pH in the cultures did not change during starvation remaining at pH 7.1 in “A” and “B” and at pH 7.2 in “C”.

#### 2.3.4 *Desulfovibrio profundus*

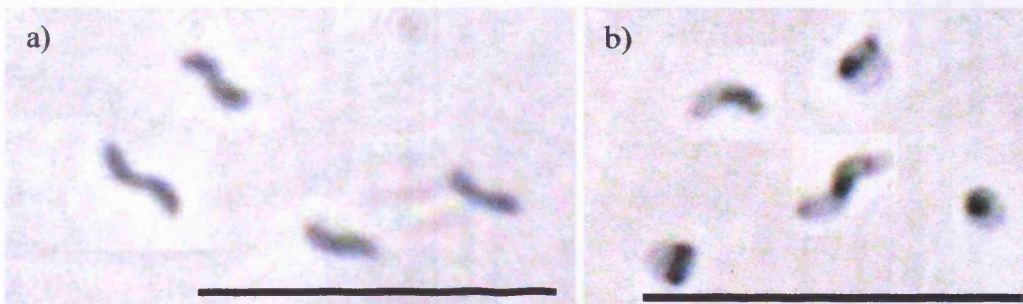
*D. profundus* was isolated from a deep sediment layer (500 mbsf) of the Japan Sea (Bale *et al.*, 1997). During starvation no significant change in AODC total cell counts occurred (on average  $1.0 \times 10^8$  cells  $\text{ml}^{-1}$  in “A”,  $9.1 \times 10^7$  cells  $\text{ml}^{-1}$  in “B” and  $1.7 \times 10^8$  cells  $\text{ml}^{-1}$  in “C”;  $p < 0.05$ ; Fig. 4.29 a-c) in any treatment. Staining of cells with the LIVE/DEAD<sup>®</sup> Kit was conducted for samples from 250 days of starvation onwards. Results for total cell counts were comparable to AODC counts and percentages of dead cells remained low ( $< 5\%$ ). For the final sample, however, LIVE/DEAD<sup>®</sup> staining was unsuccessful.



FISH detectability in parallel “C” (Fig. 4.29c) decreased from 97% at the beginning to 25% during the first 100 days and then varied between 10% and 20% until the end of starvation.

Cells were significantly longer at  $t_0$  (2.3  $\mu\text{m}$ ) than at subsequent samplings ( $p < 0.05$ ; Fig. 4.29d). Cells in samples from 100 days of starvation, however, were significantly shorter (1.2  $\mu\text{m}$ ) than at later stages (1.4  $\mu\text{m}$ ). Cell widths of starving *D. profundus* increased slightly with time, but the only significant increase was between  $t_0$  and 100 days of starvation (from 0.7  $\mu\text{m}$  to 0.8  $\mu\text{m}$ ; Fig. 4.29e).

A change in morphology and the formation of spheroblasts occurred in the cultures after 250 days of starvation (Fig. 4.28 a-b). Cells also became more translucent.

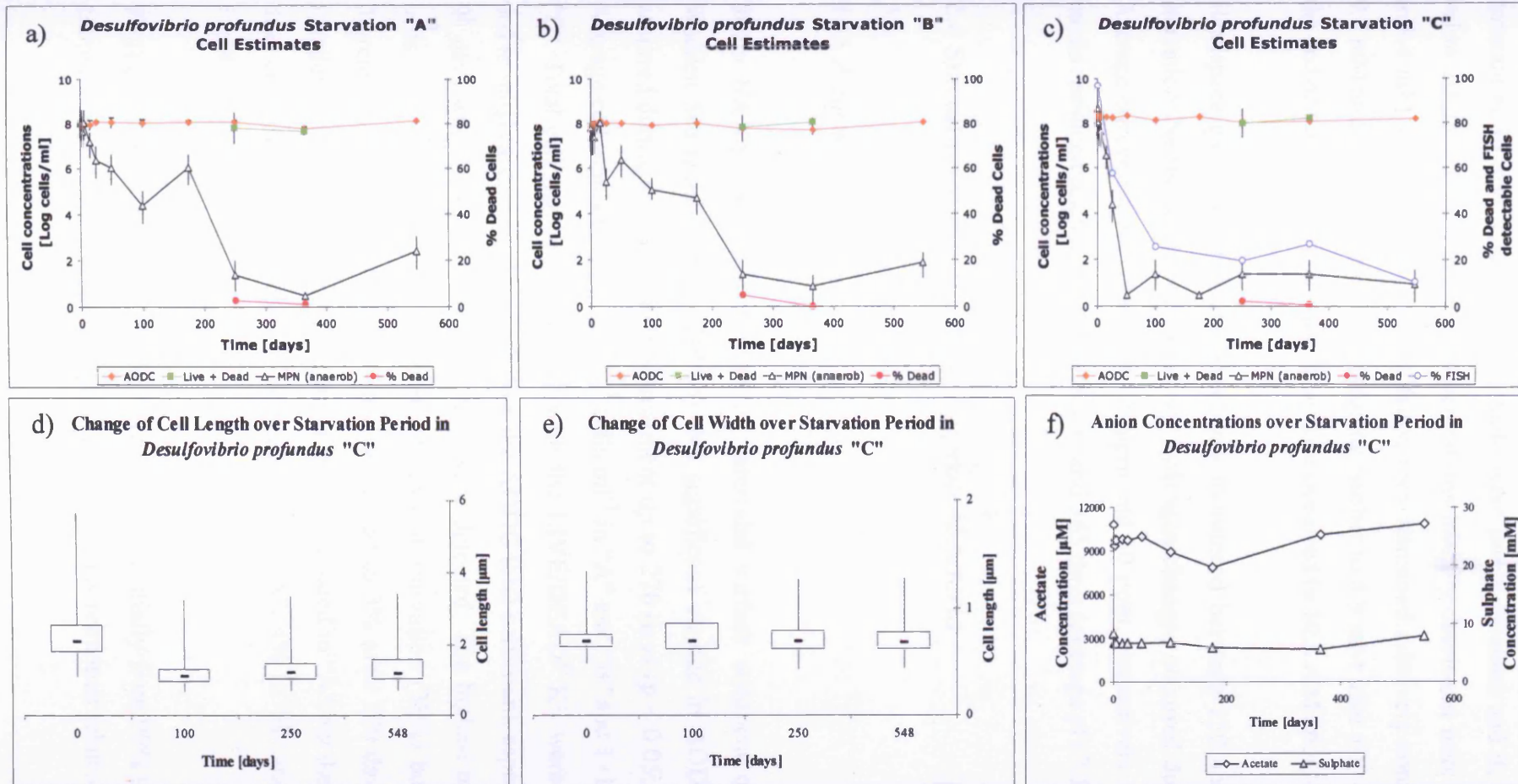


**Fig. 4.28: Micrographs of *D. profundus*.** a) at  $t_0$ ; b) after 250 days of starvation. Cells in b) are more translucent and exhibit formation of spheroblasts. Bars equal 10  $\mu\text{m}$ .

Of all strains investigated, culturability decreased most rapidly during starvation in *D. profundus*. In “A” and “B” a slow decrease occurred from  $\sim 10^8$  cells  $\text{ml}^{-1}$  at  $t_0$  to  $10^6$  cells  $\text{ml}^{-1}$  and  $10^4$  cells  $\text{ml}^{-1}$ , respectively, after 175 days. This was followed by a rapid loss of culturability until 250 days (down to 23 cells  $\text{ml}^{-1}$ ). Subsequently, cell concentrations ranged between the detection limit ( $< 3$  cells  $\text{ml}^{-1}$ ) and 240 cells  $\text{ml}^{-1}$ . In “C”, the decrease of culturable cells occurred more rapidly. After 50 days of starvation less than 3 cells  $\text{ml}^{-1}$  were detected ( $10^8$  cells  $\text{ml}^{-1}$  at  $t_0$ ). Subsequently culturable cell numbers ranged between the detection limit and 23 cells  $\text{ml}^{-1}$  until the end of starvation. The loss of culturability seems to have preceded the loss of FISH detectability (Fig. 4.29c).

Acetate concentrations in “A” and “B” increased by a factor of three during





**Fig. 4.29: Physiological responses of *D. profundus* to starvation.** a-c) Cell number estimates for treatments "A", "B", and "C". Confidence limits are given for AODC, Live + Dead, and MPNs, if not visible they fall within the size of the data points. d-e) Change of size during starvation in "C". Shown are the 25 and 75 percentile (box), the median (horizontal dash) and the data range (vertical dashes). f) Changes in acetate and sulphate concentrations during starvation in parallel "C".

starvation to  $\sim 90 \mu\text{M}$  in both cultures, while sulphate concentrations remained constant at 30 mM. Lactate and formate were rarely detected and if, they remained below  $10 \mu\text{M}$ . In “C”, acetate concentrations initially decreased from 10.8 mM at  $t_0$  to 9.4 mM after one day. Subsequently, they remained relatively constant (between 9.7 mM and 10.0 mM) before decreasing further to 8.9 mM after 100 days. Towards the end of starvation an increase in acetate occurred to 10.8 mM after 548 days.

Headspace gas concentrations were only monitored between 250 and 548 days of starvation. Neither carbon dioxide nor hydrogen changes occurred during that time. Average concentrations were  $1.4 \times 10^5$  ppm and 10 ppm, respectively. The pH of the media remained unchanged between 100 and 365 days (average pH 7.1).

## 2.4 Starvation-survival of *Epsilonproteobacteria*

### 2.4.1 *Arcobacter* sp. NA105

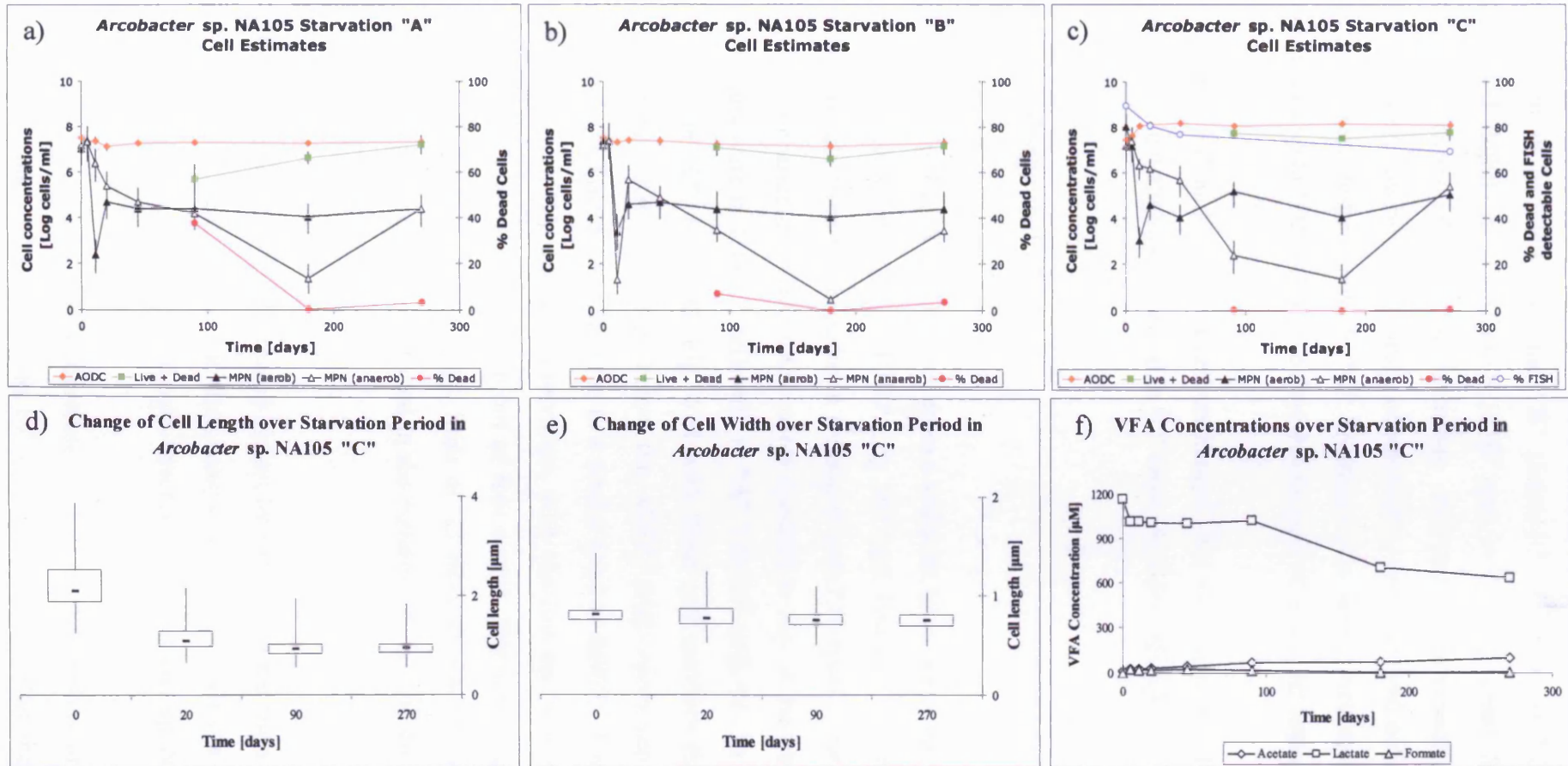
Strain NA105 was isolated from an intertidal surface sediment of the German Wadden Sea (Freese *et al.*, 2008b). No significant change in AODC total counts occurred during starvation in any treatment up to 270 days ( $p < 0.05$ ; Fig. 4.30 a-c). Average cell numbers were  $2.2 \times 10^7$  cells  $\text{ml}^{-1}$  in “A” and “B” and  $1 \times 10^8$  cells  $\text{ml}^{-1}$  in “C”. Total cell numbers estimated with the LIVE/DEAD<sup>®</sup> Kit were more variable and in most cases considerably below the AODC total cell count, especially in “A” at 90 days where only  $5.2 \times 10^5$  cells  $\text{ml}^{-1}$  were detected. The highest number of dead cells occurred in parallel “A” after 90 days of starvation (38%) but subsequently decreased to 0.3% after 180 days and increased to 3% after 270 days (Fig. 4.30a). Parallels “B” and “C” showed a similar trend compared to “A” but the proportions of dead cells were much lower (on average 14% in “A”; 4% in “B” and 0.3% in “C”; Fig. 4.30 b-c).

FISH detectability in “C” decreased inverse exponentially from 90% to 69% over the entire duration of the experiment (270 days) and thus remained relatively high.

Cell lengths of strain NA105 significantly decreased between  $t_0$  and subsequent samplings (from 2.2  $\mu\text{m}$  to 1.0  $\mu\text{m}$ ;  $p < 0.05$ ; Fig. 4.30d). Additionally, cells were also significantly longer after 20 days (1.2  $\mu\text{m}$ ) than at later stages of starvation (1.0  $\mu\text{m}$ ). Cell widths also continuously decreased with incubation and cells were significantly wider at  $t_0$  (0.8  $\mu\text{m}$ ) than at any other time (0.7  $\mu\text{m}$ ;  $p < 0.05$ ; Fig. 4.30e). The decrease in cell size resulted in a change from the initial curved rod-like to a coccoid morphology.

Culturability in aerobic MPNs decreased rapidly during the first 11 days of starvation (from between  $10^7$  and  $10^8$  cells  $\text{ml}^{-1}$  to  $10^2$  and  $10^3$  cells  $\text{ml}^{-1}$ ; Fig. 4.30 a-c). Subsequently, MPNs recovered slightly and remained between  $10^1$  and  $10^5$  culturable cells  $\text{ml}^{-1}$  until the end of the experiment. Anaerobically incubated MPNs did not always show pellet formation. This was the case especially at later stages of starvation. However, metabolic activity was still present (as indicated by a change in the redox indicator) and MPNs were calculated on the basis of this activity. The trend of culturability in anaerobic MPNs was similar to aerobic MPNs with an exception at 180 days where in all treatments significantly lower culturable cell numbers were present (between  $< 3$  and 23 cells  $\text{ml}^{-1}$ ) compared to aerobic MPNs here ( $1.1 \times 10^4$  cells  $\text{ml}^{-1}$ ). An increase in incubation time for growth from less than four to more than nine days occurred during starvation.

Concentrations for acetate and lactate in parallels "A" and "B" were variable and often below the detection limit. Average concentrations for acetate were 5.4  $\mu\text{M}$  and 2.5  $\mu\text{M}$  and for lactate 7.4  $\mu\text{M}$  and 4.2  $\mu\text{M}$ , respectively. Formate concentrations, however, were higher in "A" than in "B" but continuously decreased in both cultures (from 13  $\mu\text{M}$  to 2  $\mu\text{M}$  in "A" and from 7  $\mu\text{M}$  to 2  $\mu\text{M}$  in "B"). Parallel "C" also showed decreasing formate concentrations (from 23  $\mu\text{M}$  to 4  $\mu\text{M}$ ; Fig 4.30f) as well as a general decline in lactate. This decrease can be divided into four stages, an initial decrease from 1.1 mM at  $t_0$  to 1.0 mM after five days, where concentrations remained until 90 days. This was followed by a steep decrease to 0.7 mM after 180 days and another slow decrease to 0.6 mM until the end of starvation (270 days). Acetate concentrations on the other hand slowly increased from 14  $\mu\text{M}$  to 105  $\mu\text{M}$  during starvation.



**Fig. 4.30: Physiological responses of *Arcobacter* sp. NA105 to starvation.** a-c) Cell number estimates for treatments "A", "B", and "C". Confidence limits are given for AODC, Live + Dead, and MPNs, if not visible they fall within the size of the data points. d-e) Change of size during starvation in "C". Shown are the 25 and 75 percentile (box), the median (horizontal dash) and the data range (vertical dashes). f) Changes in VFA concentrations over starvation period in parallel "C".

Headspace carbon dioxide concentrations halved within the first 20 days of incubation in parallels "A" and "B" (from  $1.5 \times 10^5$  ppm to  $7.2 \times 10^4$  ppm) and decreased slightly in "C" (from  $8.3 \times 10^4$  ppm to  $6.6 \times 10^4$  ppm). Subsequently, no changes occurred in any of the treatments. Hydrogen concentrations in parallels "A" and "B" were low at the beginning of starvation (~60 ppm) and decreased to 30 ppm and < 10 ppm, respectively. In "C", hydrogen detection varied more showing local maxima of up to 150 ppm at five days but no general trend was apparent.

The pH in "A" and "B" remained unchanged over the duration of the experiment at pH 7.3 while it slightly increased in "C" from pH 6.2 to pH 6.4.

#### 2.4.2 *Arcobacter* sp. F17IX

*Arcobacter* sp. F17IX was isolated from a sediment layer below the buried carbonate mound at the Mound site of IODP Leg 307 (see Chapter III) and represents the deepest isolate from this expedition (obtained from 250 mbsf). During starvation no significant change in AODC total counts occurred in any of the treatments (average cell counts were in  $1.4 \times 10^8$  cells ml<sup>-1</sup> in "A",  $1.8 \times 10^8$  cells ml<sup>-1</sup> in "B" and  $2.4 \times 10^8$  cells ml<sup>-1</sup> in "C";  $p < 0.05$ ; Fig. 4.31 a-c). Total cell numbers estimated with the LIVE/DEAD<sup>®</sup> Kit were often below the AODC total counts (up to one order of magnitude) but increased with time to a similar level as AODC. Parallel "C" differed somewhat from this general observation, with the two methods matching for the majority of the samples (in three out of four cases). The percentages of dead cells were generally low (< 2%) apart from at 45 days of starvation where elevated numbers of dead cells were detected in the cultures (5% in "A" and 4% in "B"), the highest being 20% in "C".

FISH detectability in "C" decreased almost linearly with time from 77% at  $t_0$  to only 6% of DAPI stained cells in the final sample (Fig. 4.31c). This is much lower than the final proportion of hybridised cells observed in *Arcobacter* sp. NA105 (69%).

Culturability in aerobic MPNs decreased rapidly by two orders of magnitude from between  $10^7$  and  $10^8$  cells ml<sup>-1</sup> to  $10^4$  and  $10^5$  cells ml<sup>-1</sup> after the first 11 days of

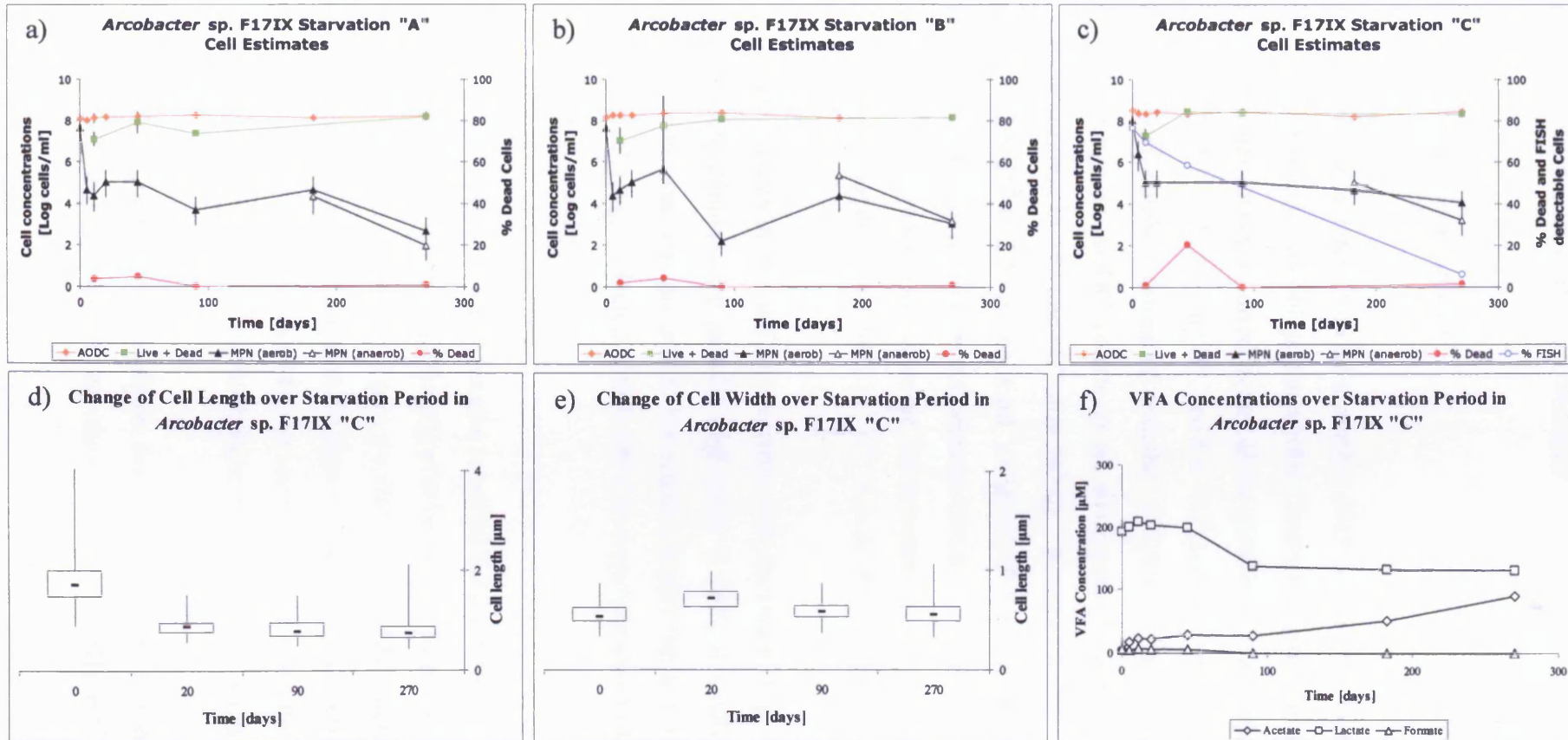
starvation in all treatments (Fig. 4.31 a-c). Subsequently, parallel "C" had a relatively stable number of culturable cells ( $10^5$  cells  $\text{ml}^{-1}$ ) present followed by a slight decrease in the final two samples ( $10^4$  cells  $\text{ml}^{-1}$ ). After the initial decrease culturable cell numbers in "A" and "B" recovered slightly (from  $10^4$  to  $10^5$  cells  $\text{ml}^{-1}$ ) but showed a local minimum after 90 days of starvation ( $4.3 \times 10^3$  cells  $\text{ml}^{-1}$  and  $1.5 \times 10^2$  cells  $\text{ml}^{-1}$ , respectively) before increasing again to  $10^4$  cells  $\text{ml}^{-1}$ , followed by another decrease to  $4.6 \times 10^2$  and  $1.1 \times 10^3$  cells  $\text{ml}^{-1}$ , respectively in the final sample (270 days). Anaerobic MPNs were only conducted for the final two samplings and resulted in comparable numbers to aerobic MPNs. Like strain NA105, cell pellets were not formed, but a colour change of the redox indicator suggested metabolic activity. No increase in incubation time for growth occurred with starvation.

In treatment "C", lactate concentrations remained stable at 200  $\mu\text{M}$  until 45 days of starvation. Subsequently, a steep decrease occurred to 137  $\mu\text{M}$  after 90 days followed by a slower decrease to 130  $\mu\text{M}$  after 270 days (Fig. 4.31f). Formate concentrations were on average 6  $\mu\text{M}$  but below the detection limit (0.2  $\mu\text{M}$ ) at 90 days and beyond. Acetate almost linearly increased during starvation from 5  $\mu\text{M}$  at  $t_0$  to 88  $\mu\text{M}$  after 207 days. In "A" and "B", VFA concentrations generally remained below 10  $\mu\text{M}$  but no trend was apparent.

Headspace carbon dioxide in all parallels decreased within the first 11 days (from  $1.2 \times 10^5$  ppm to  $9.4 \times 10^4$  ppm in "A" and "B" and from  $8.2 \times 10^4$  ppm to  $4.7 \times 10^4$  ppm in "C") but remained stable afterwards until the end of starvation. Hydrogen in "A" decreased by 90% from 134 ppm within five days of starvation. Subsequently, a local maximum of 74 ppm occurred at 20 days followed by another decrease to below the detection limit (2 ppm). Hydrogen in "B" decreased from 50 to 10 ppm in the first 11 days and remained at this low level throughout the remaining starvation period. Treatment "C" had low levels of hydrogen at all times (< 25 ppm).

The pH of the cultures decreased by  $\sim 0.3$  units within the first 5 days of starvation from pH 7.7 to pH 7.4 in "A" and "B" and from pH 7.0 to 6.8 in "C". Subsequently it remained relatively unchanged.





**Fig. 4.31: Physiological responses of *Arcobacter* sp. F171X to starvation.** a-c) Cell number estimates for treatments "A", "B", and "C". Confidence limits are given for AODC, Live + Dead, and MPNs, if not visible they fall within the size of the data points. d-e) Change of size during starvation in "C". Shown are the 25 and 75 percentile (box), the median (horizontal dash) and the data range (vertical dashes). f) Changes in VFA concentrations over starvation period in parallel "C".

## 2.5 Starvation-survival of *Firmicutes*

### 2.5.1 *Acetobacterium malicum*

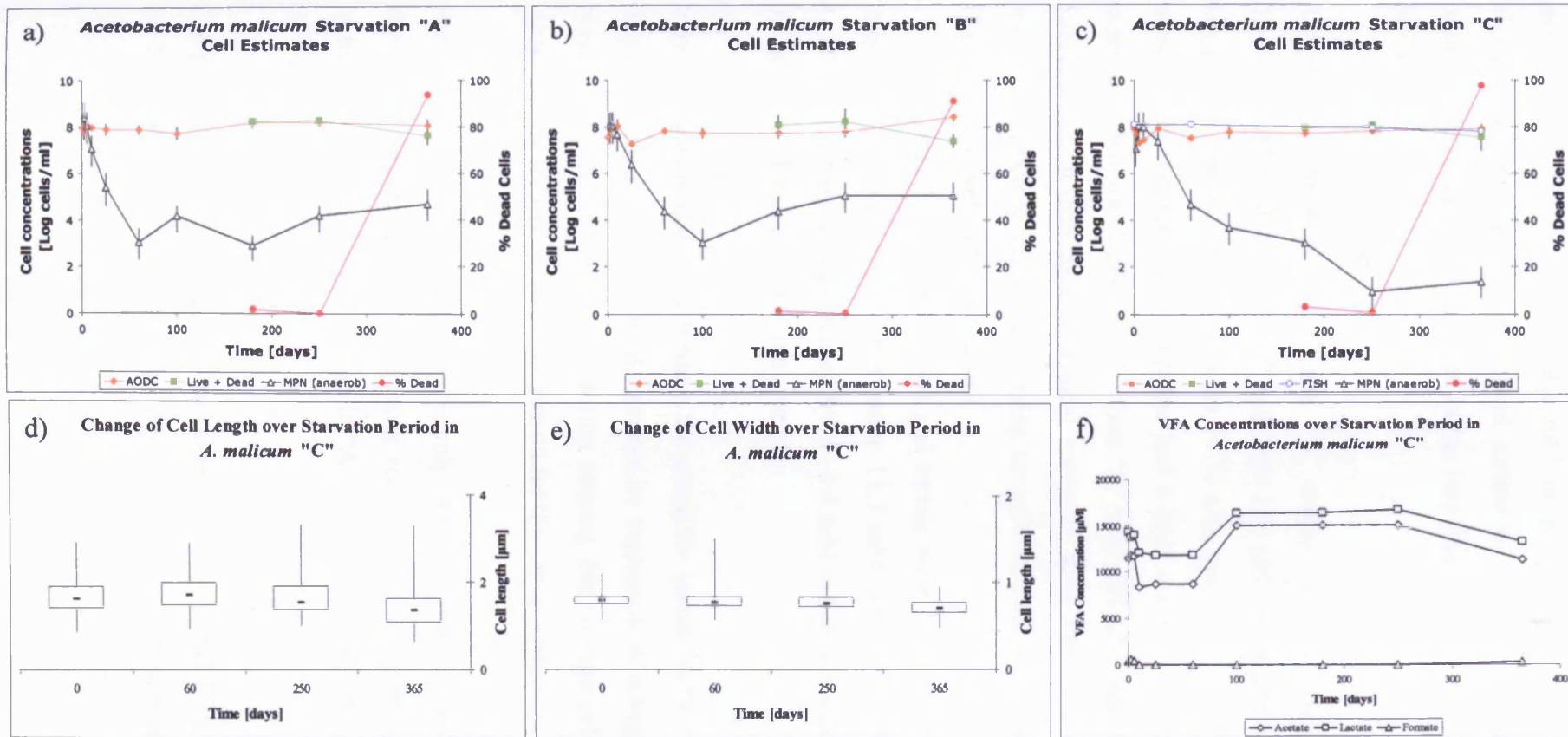
*A. malicum* was isolated from an anaerobic ditch sediment in Germany (Tanaka and Pfennig, 1988) and is an obligate anaerobe. During starvation no significant decrease in AODC total counts occurred in any of the parallels (on average  $9.5 \times 10^7$  cells ml<sup>-1</sup> in “A”,  $8.2 \times 10^7$  cells ml<sup>-1</sup> in “B”, and  $5.6 \times 10^7$  cells ml<sup>-1</sup> in “C”;  $p < 0.05$ ; Fig. 4.32 a-c). Total cell numbers estimated with the LIVE/DEAD<sup>®</sup> Kit varied but were within a similar range as AODC counts in all treatments. However, a decrease in cell numbers occurred in “A” and “B” after 365 days (from  $\sim 1.8 \times 10^8$  to  $4.3 \times 10^7$  cells ml<sup>-1</sup> and from  $1.6 \times 10^8$  to  $2.5 \times 10^7$  cells ml<sup>-1</sup>, respectively). The percentages of dead cells were low ( $< 4\%$ ) in all treatments between 180 and 250 days of starvation. For the final timepoint (365 days), however, an increase in the proportion of dead cells occurred in all parallels with 94% in “A”, 91% in “B”, and 98% in “C”.

Total FISH counts in “C” did not decrease with starvation (Fig. 4.32c). Percentages of FISH detectability were initially high (96% to 98% of DAPI) but unfortunately DAPI staining was only successful for earlier samples (up to 60 days of starvation) before less cells were detected with DAPI than were hybridised ( $4.1 \times 10^7$  and  $6.6 \times 10^7$  cells ml<sup>-1</sup>, respectively).

No significant decrease in cell lengths occurred for the first 250 days of starvation, but cells at 365 days were significantly shorter than at previous samplings (decrease from an average of 1.7  $\mu\text{m}$  to 1.4  $\mu\text{m}$ ;  $p < 0.05$ ; Fig. 4.32d). There was no significant change in cell widths for the first 50 days of starvation but between cells at  $t_0$  (0.8  $\mu\text{m}$ ) compared with 365 days of starvation (0.7  $\mu\text{m}$ ). Despite these changes cell morphology remained relatively unchanged throughout starvation being rod-shaped.

Anaerobic culturability in *A. malicum* decreased by up to five orders of magnitude within the first 100 days of starvation (from  $10^8$  cells ml<sup>-1</sup> to  $10^4$  cells ml<sup>-1</sup>





**Fig. 4.32: Physiological responses of *Acetobacterium malicum* to starvation.** a-c) Cell number estimates for treatments "A", "B", and "C". Confidence limits are given for AODC, Live + Dead, FISH, and MPNs, if not visible they fall within the size of the data points. d-e) Change of size during starvation in "C". Shown are the 25 and 75 percentile (box), the median (horizontal dash) and the data range (vertical dashes). f) Changes in VFA concentrations over starvation period in parallel "C".

in "A" and to  $10^3$  cells  $\text{ml}^{-1}$  in "B" and "C"). Culturability subsequently slowly increased to  $\sim 10^5$  culturable cells  $\text{ml}^{-1}$  in parallels "A" and "B" despite large increases in dead cells, it decreased further in "C" until only approximately 10 culturable cells  $\text{ml}^{-1}$  were detected in the two final samples of this parallel (250 and 365 days).

Acetate concentrations in "A" and "B" continuously increased during starvation (from 168  $\mu\text{M}$  to 494  $\mu\text{M}$  in "A" and from 185  $\mu\text{M}$  to 538  $\mu\text{M}$  in "B") while lactate was present only in minute amounts ( $< 30 \mu\text{M}$ ) until 10 days of starvation and was subsequently not detectable. Formate had a local maximum after two days into the experiment but was higher in "A" than "B" with 101  $\mu\text{M}$  and 18  $\mu\text{M}$ , respectively. Subsequently, it decreased to a local minimum after 180 days (11  $\mu\text{M}$  and 2  $\mu\text{M}$ , respectively) before slightly increasing towards the end of starvation (to 37  $\mu\text{M}$  and 18  $\mu\text{M}$ , respectively after 365 days).

In parallel "C" both acetate and lactate were present in high concentrations throughout the experiment (on average 11.3 mM and 14.0 mM, respectively; Fig. 4.32f). Formate initially decreased from  $\sim 0.4$  mM to below the detection limit before 0.3 mM were detected in the final sample.

Why *A. malicum* did not consume the available lactate in "C" is unclear. Lactate concentrations as high as 16 mM cannot be explained by a high substrate affinity. Maybe end product inhibition, quorum sensing due to high cell concentrations, or other limiting factors could be the reason for this occurrence.

Headspace gas concentrations were only determined between 180 and 365 days. During this time neither carbon dioxide (on average  $\sim 10^5$  ppm in all treatments) nor hydrogen (on average  $1.5 \cdot 10^3$  ppm in "A",  $6.2 \cdot 10^2$  ppm in "B", and  $2.3 \cdot 10^3$  ppm in "C") concentrations changed.

The pH of the cultures varied around average values of pH 7.36 in "A" and "B" and pH 7.15 in "C" between 60 and 365 days of starvation but no trend occurred.

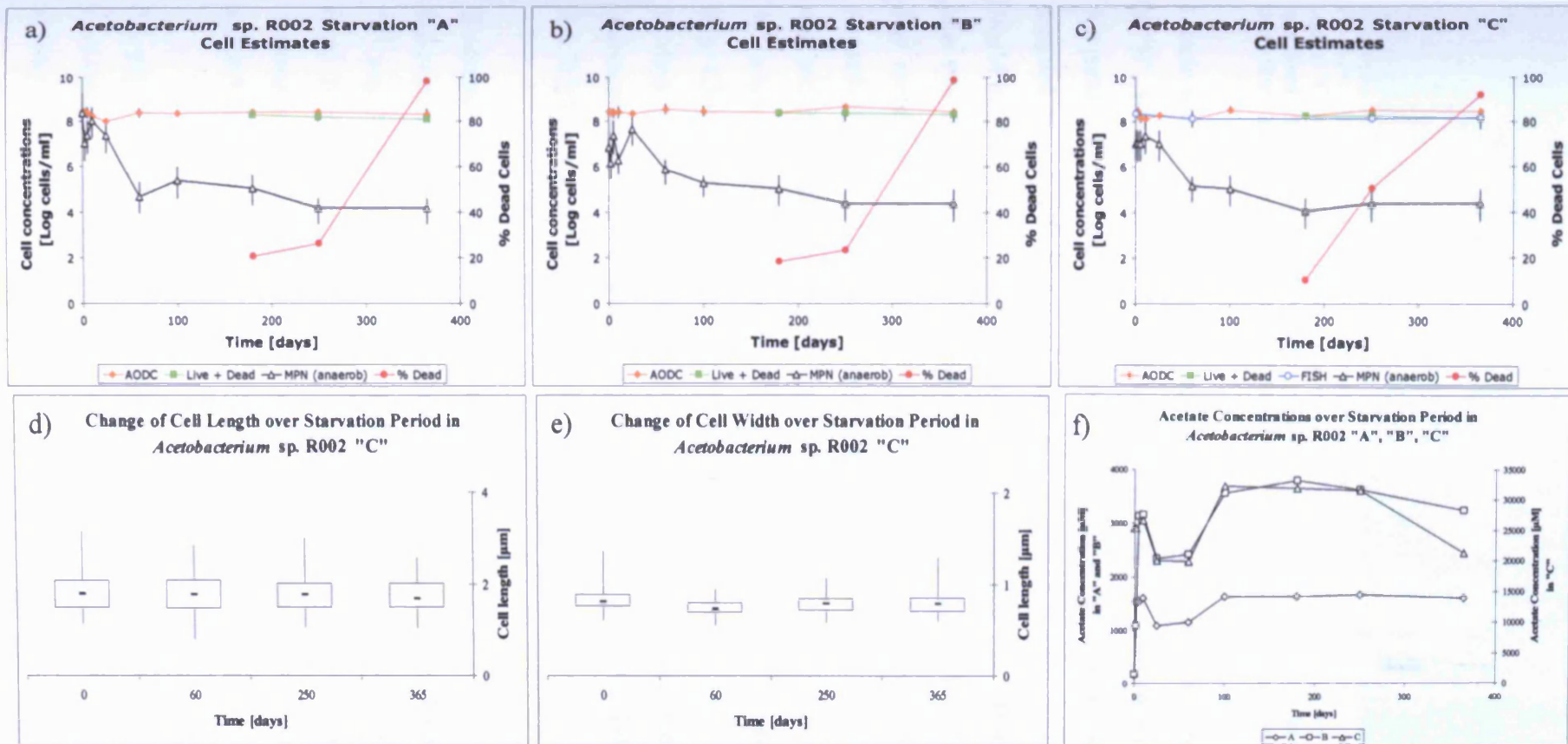
### 2.5.2. *Acetobacterium* sp. R002

*Acetobacterium* sp. R002 was isolated by Dr. Chloe Heywood during her PhD project from samples of the New Zealand Debits lignite drilling programme (Fry *et al.*, 2009). This strain has a 98% rDNA sequence similarity to *A. malicum*, is of terrestrial origin, and was isolated from 144.3 m depth. During starvation no significant decrease in AODC total counts occurred in any treatment (on average  $2.3 \cdot 10^8$  cells ml<sup>-1</sup> in "A",  $2.9 \cdot 10^8$  cells ml<sup>-1</sup> in "B", and  $2.2 \cdot 10^8$  cells ml<sup>-1</sup> in "C";  $p < 0.05$ ; Fig. 4.33 a-e). Total cell numbers estimated with the LIVE/DEAD<sup>®</sup> Kit from 170 days onwards were in the same order of magnitude as AODC counts (between 53% and 103%). The proportions of dead cells in all cultures were relatively high after half a year of starvation (20% in "A", 18% in "B", and 10% in "C"). After 250 days they were slightly increased in "A" and "B" (26% and 23% respectively) and significantly higher in "C" (50%). In the final samples at 365 days more than 90% of dead cells were present in the three parallels (Fig. 4.33 a-e and Fig. 4.34).

DAPI staining worked well for samples of the first 60 days of starvation and FISH detectability in "C" was high (~97%). At later stages DAPI failed to stain cells effectively (Fig. 4.34c). Thus the total FISH count is presented (Fig. 4.33c), which is not significantly different from the AODC total counts until 60 days of starvation but subsequently decreased significantly (to 40% and 55% of AODC after 250 and 365 days, respectively;  $p < 0.05$ ).

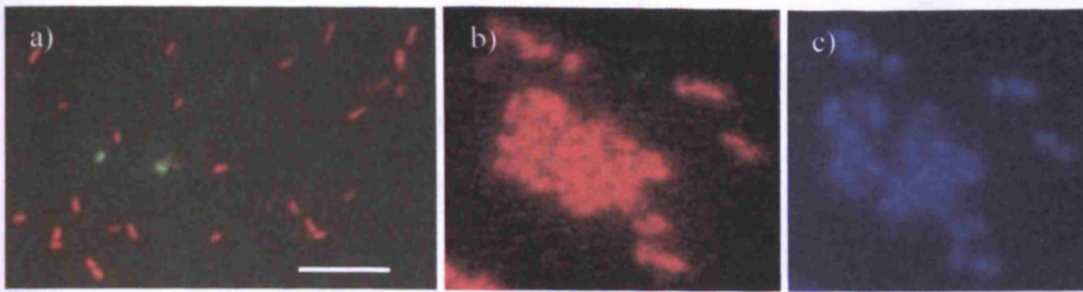
No significant change in cell lengths occurred during starvation for strain R002 (on average 1.8  $\mu$ m). Cell widths, however, significantly decreased between  $t_0$  (0.82  $\mu$ m) and subsequent timepoints (on average 0.77  $\mu$ m).

Anaerobic culturability decreased slowly in all three incubations. In "A", it decreased from  $10^8$  to  $10^4$  cells ml<sup>-1</sup>, in "B" from  $10^7$  to  $10^6$  cells ml<sup>-1</sup> and in "C" from  $10^7$  to  $10^5$  cells ml<sup>-1</sup> after 60 days. Subsequently, culturable cell counts stabilised and ranged between  $10^4$  and  $10^5$  cells ml<sup>-1</sup>.



**Fig. 4.33: Physiological responses of *Acetobacterium* sp. R002 to starvation.** a-c) Cell number estimates for treatments "A", "B", and "C". Confidence limits are given for AODC, Live + Dead, FISH, and MPNs, if not visible they fall within the size of the data points. d-e) Change of size during starvation in "C". Shown are the 25 and 75 percentile (box), the median (horizontal dash) and the data range (vertical dashes). f) Changes in acetate concentrations during starvation in "A", "B", and "C".





**Fig. 4.34: Micrographs of starving *Acetobacterium* sp. R002.** All pictures taken from subsamples of parallel “C” after 1 year of starvation. a) stained with the *BacLight*® Kit; Bar indicates 10  $\mu\text{m}$ ; b) cells hybridised with Cy3-labeled eubacterial probe (EUB338I); c) the same field of view as in b) but with DAPI filter. It is apparent that not all of the hybridised cells give a signal with DAPI.

Lactate was not detected in any the samples of any parallels. The only exception is  $t_0$  in “B”, which contained 19  $\mu\text{M}$  Lactate, roughly the added amount of substrate to this parallel at  $t_0$ . Formate was also not detected in “C”. In “A” and “B” formate only occurred sporadically and concentrations remained below 12  $\mu\text{M}$  in “A” and below 66  $\mu\text{M}$  in “B”. Acetate initially increased in treatment “A” (from 0.1 mM to 1.5 mM after two days) and in “B” (from 0.1 mM to 3.1 mM after 5 days). Subsequently, they remained stable until 10 days before decreasing to 1.0 mM and 2.3 mM, respectively after 25 days. This was followed by another increase at 100 days (1.6 mM and 3.5 mM, respectively) another stable period until the end of starvation for “A” whilst another decrease to 3.2 mM occurred in “B” between 270 and 365 days (Fig. 4.33f). Acetate concentrations in “C” were on average 25 mM during the first 10 days of starvation. Then the pattern was almost identical as in “B” albeit concentrations were generally eight times higher and the final decrease between 270 and 365 days was more pronounced.

Headspace gas analysis was conducted between 180 and 365 days. Carbon dioxide concentration did not change during this time (on average  $1.0 \times 10^5$  ppm in “A” and “B” and  $3.0 \times 10^5$  ppm in “C”). Hydrogen concentrations slowly decreased in “A” (from 20 to 11 ppm) and in “C” (from 49 to 14 ppm). In “B” no hydrogen was detected at 180 days but a slight decrease was observed between 270 days (19 ppm) and 365 days (13 ppm).

The pH of the cultures was measured between 60 and 365 days of starvation and did not change during this time. In “A” and “B” the average was pH 7.3 while it was slightly lower in “C”, pH 7.0.

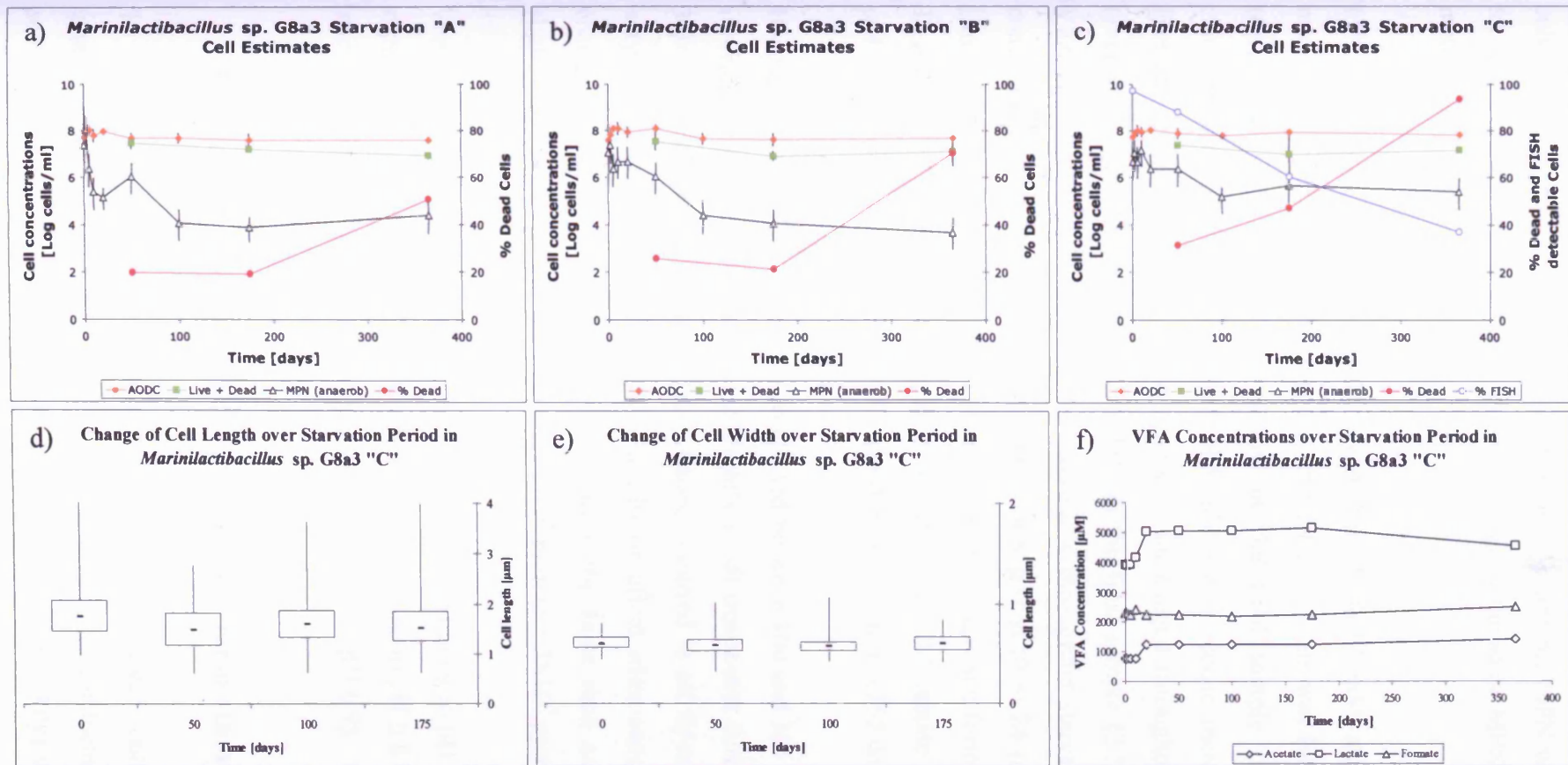
### 2.5.3 *Marinilactibacillus* sp. G8a3

*Marinilactibacillus* sp. G8a3 was isolated by Dr. Gerard Sellek from a gas hydrate containing sediment layer in the Gulf of Mexico (227 mbsf; Parkes *et al.*, 2009). During starvation no significant change in AODC total counts occurred (on average  $6.5 \times 10^7$  cells ml<sup>-1</sup> in “A”,  $7.3 \times 10^7$  cells ml<sup>-1</sup> in “B”, and  $8.2 \times 10^7$  cells ml<sup>-1</sup> in “C”;  $p < 0.05$ ; Fig. 4.35). Total cell numbers using the LIVE/DEAD<sup>®</sup> Kit were up to one order of magnitude below AODC total counts for individual timepoints but on average comparable ( $1.8 \times 10^7$  cells ml<sup>-1</sup> in “A”,  $1.9 \times 10^7$  cells ml<sup>-1</sup> in “B”, and  $1.6 \times 10^7$  cells ml<sup>-1</sup> in “C”). The trends were also very similar in all treatments. The proportions of dead cells in this strain during initial starvation (up to 175 days) were higher (19% to 47%) than in any other strains investigated and increased even further with time to 51% in “A”, 70% in “B”, and 94% in “C” in the final sample after 365 days (Fig. 4.35c). These high numbers of dead cells contradict unchanging total cell counts determined with AODC and the LIVE/DEAD<sup>®</sup> Kit.

FISH detectability in “C” decreased almost linearly with time from 97% of the DAPI count at  $t_0$  to 37% in the final sample for parallel “C” (Fig. 4.35c), mirroring the increase in proportions of dead cells.

A significant decrease in cell lengths occurred between  $t_0$  and subsequent timepoints of starvation (from 1.8  $\mu\text{m}$  to an average of 1.6  $\mu\text{m}$ ;  $p < 0.05$ ; Fig. 4.35d). Cell widths remained relatively stable at around 0.6  $\mu\text{m}$  throughout the experiment.

Culturability for *Marinilactibacillus* sp. G8a3 was mainly tested for anaerobic growth. An initial increase of up to one order of magnitude occurred in each treatment within the first two days of starvation (from  $2.4 \times 10^7$  to  $1.1 \times 10^8$  cells ml<sup>-1</sup> in “A”, from  $1.1 \times 10^7$  to  $2.4 \times 10^7$  cells ml<sup>-1</sup> in “B”, and from  $4.6 \times 10^6$  to  $1.1 \times 10^7$  cells ml<sup>-1</sup> in “C”). Subsequently, in parallel “A”, a steep exponential decrease occurred with the loss of three orders of magnitude within the first 20 days of the experiment (to  $1.4 \times 10^5$  cells ml<sup>-1</sup>). The loss of culturability in “B” and “C” were less rapid (to  $1.1 \times 10^6$  cells ml<sup>-1</sup> after 50 days in “B” and to  $2.4 \times 10^6$  cells ml<sup>-1</sup> after 20 days in “C”).



**Fig. 4.35: Physiological responses of *Marinilactibacillus* sp. G8a3 to starvation.** a-c) Cell number estimates for treatments "A", "B", and "C". Confidence limits are given for AODC, Live + Dead, FISH, and MPNs, if not visible they fall within the size of the data points. d-e) Change of size during starvation in "C". Shown are the 25 and 75 percentile (box), the median (horizontal dash) and the data range (vertical dashes). f) Changes in VFA concentrations during starvation in "C".

MPNs stabilised after 100 days of starvation and varied between  $10^3$  and  $10^5$  culturable cells  $\text{ml}^{-1}$  depending on the parallel. An aerobic MPN was only conducted for the final sample and resulted in almost exactly the same MPNs as the anaerobic incubation.

In "C", an initial increase in acetate (from 0.8 mM to 1.2 mM) and lactate (from 3.9 mM to 5.0 mM) occurred during the first 20 days. This was followed by a stable period until 175 days of starvation. In the final sample (365 days) lactate concentrations slightly decreased to 4.5 mM while acetate increased to 2.5 mM. Formate concentrations remained relatively unchanged throughout the experiment (average 2.3 mM) but increased slightly in the final sample (2.5 mM; Fig. 4.35f). VFA concentrations in "A" and "B" were low throughout starvation but increased continuously. In "A", acetate increased from 9  $\mu\text{M}$  at  $t_0$  to 24  $\mu\text{M}$  after 365 days. During this time lactate increased from 23  $\mu\text{M}$  to 61  $\mu\text{M}$  and formate from 13  $\mu\text{M}$  to 32  $\mu\text{M}$ . In "B", acetate increased from 11  $\mu\text{M}$  to 41  $\mu\text{M}$ , lactate from 35  $\mu\text{M}$  to 72  $\mu\text{M}$ , and formate from 15  $\mu\text{M}$  to 182  $\mu\text{M}$  during starvation (365 days).

Headspace gas analysis was only conducted between 100 and 365 days of starvation. Hydrogen concentrations decreased slightly in all treatments during this time (from  $3.0 \times 10^3$  ppm to  $6.6 \times 10^2$  ppm). This change occurred in all three parallels and was very synchronous, and thus might be a dilution effect when sampling rather than a change caused by microbial activity. During the same time carbon dioxide also slightly decreased from an average of  $2.7 \times 10^5$  ppm to  $1.2 \times 10^5$  ppm in all treatments.

The pH in "A" and "B" slightly decreased by 0.1 pH units to pH 7.1 throughout the experiment while the pH in "C" decreased by 0.5 units to pH 6.8 within 100 days of starvation before slightly increasing towards the end (to pH 6.9).

### **3. Potential metabolic activities of long-term starved cultures**

To assess the potential metabolic activities of starved cultures, time series experiments using radioactively labelled substrates were conducted. For this, several subsamples (5 ml each) of selected strains (treatment "C" only) were amended with



substrate and anaerobically incubated in parallel at the respective starvation temperature. The final concentrations of  $^{14}\text{C}$ -glucose and  $^{14}\text{C}$ -lactate were 0.4 nM and 3.8 nM, respectively. After certain timepoints individual parallels were analysed while the remaining parallels were incubated for later processing (“destructive sampling”).

In this section percentages of substrate removal and product formation are given. These percentages, which were detected in the different fractions collected (e.g. substrate, VFAs, carbon dioxide, and cells), relate to the total radioactivity (in DPM) present in the respective sample.

### 3.1 Metabolic activity of *Actinobacteria* after prolonged starvation

The two representatives of the phylum *Actinobacteria* were assessed in regard to their response to the addition of minute substrate concentrations under anaerobic conditions. These experiments were conducted after 256 days of starvation in *S. marinus* (Fig. 4.36a) and after 315 days in *Ornithinimicrobium* sp. F18IV (Fig. 4.36b).

In both strains glucose was not metabolised within the first 240 hours of incubation. In the final samples (after 2788 hours, 116 days) glucose was at least partly consumed, 53% of the radioactivity (DPM) were removed in *S. marinus* (at a rate of  $0.002 \text{ pmol ml}^{-1} \text{ d}^{-1}$ ) and 20% in *Ornithinimicrobium* sp. F18IV (at a rate of  $0.001 \text{ pmol ml}^{-1} \text{ d}^{-1}$ ). *S. marinus* produced mainly  $\text{CO}_2$  (38%, 9% bicarbonate/succinate [B/S], and 5% in ALF), while in *Ornithinimicrobium* sp. F18IV more radioactive signal was detected in acetate, lactate, and formate (ALF Fraction, 11%) than in any other fraction (4% in  $\text{CO}_2$ , 3% in B/S, 2% in cells).

The long lag phase before metabolism is consistent with previous experiments. Both these strains grew best (pellet formation) and quickest in aerobic MPNs, however strain F18IV showed activity in anaerobic MPNs throughout starvation although decreasing with time (Fig. 4.3). *S. marinus* was described to be strictly aerobic (Yie *et al.*, 2004) thus the consumption of glucose in the final vials could be linked to oxygen penetration. This is supported by the fact that in *S. marinus* 40% of the radioactivity was detected in the  $\text{CO}_2$  fraction and only very little in the ALF fraction (5%; Fig. 4.36a), which suggests aerobic respiration.

However, if this was the case surely all of the glucose would have been converted to CO<sub>2</sub> after 116 days of incubation. Although obligate aerobic, at the beginning of starvation *S. marinus* formed cell pellets in anaerobically incubated MPNs (up to a dilution of 10<sup>-3</sup>) and at later stages was still able to reduce conditions in the 10<sup>-1</sup> dilution as indicated by a change in the redox indicator in the medium. Although this “anaerobic” activity decreased with continued starvation it was still present in the final samples of “A” and “B” but not in “C” (270 days). Hence, it cannot be ruled out that *S. marinus* “C” eventually used the available glucose under anaerobic conditions (after 116 days of incubation).

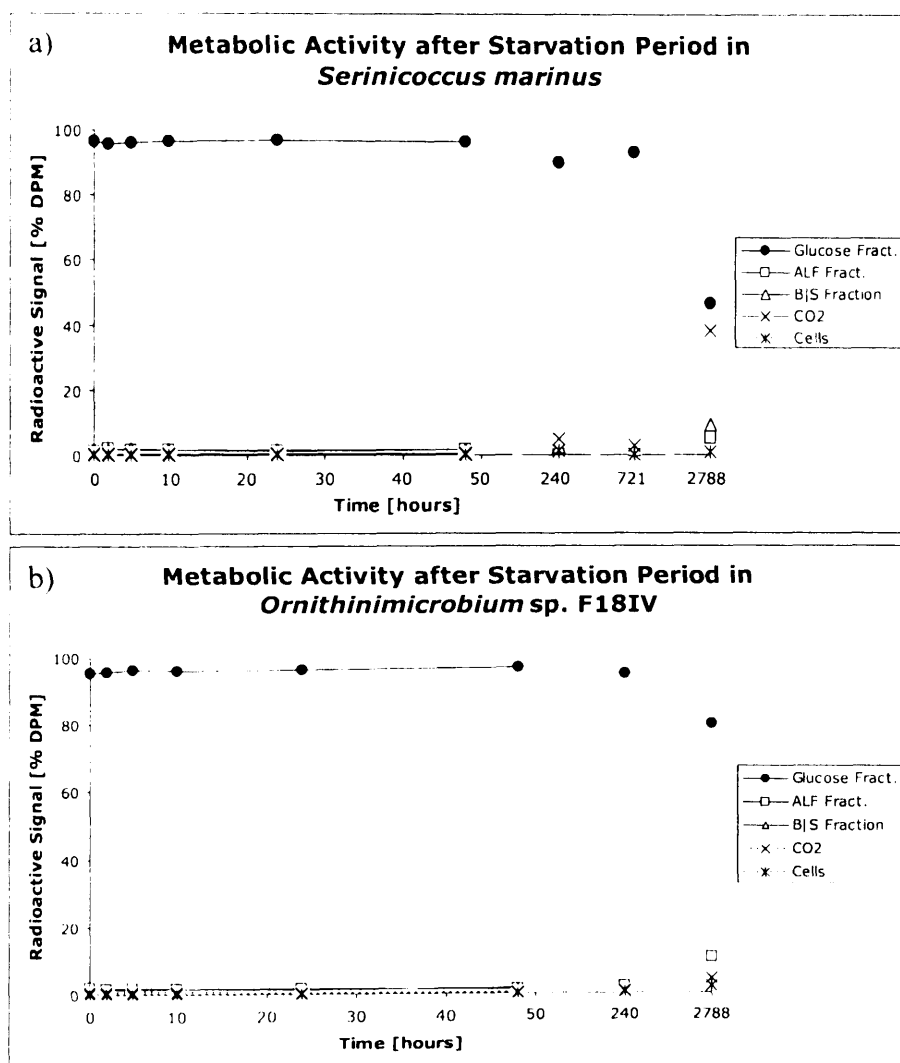


Fig. 4.36: Metabolic activity of *Actinobacteria* after starvation. a) *S. marinus*; b) *Ornithinimicrobium* sp. F18IV Legend: ALF, acetate, lactate, formate; B|S, bicarbonate, succinate. These compounds were not recovered in separate fractions due to their close retention times.

## 3.2 Metabolic activity of $\gamma$ -*Proteobacteria* after prolonged starvation

### 3.2.1 *Photobacterium* strains

Strains of *Photobacterium* from different origins and starvation lengths were subjected to glucose radiotracer experiments. At the time these tests were conducted strain SAMA2 had been starving for 970 days, strain S10 for 754 days, and strain F18I for 469 days.

The rate at which glucose was consumed varied markedly in the three strains. It was removed quickest in strain F18I (by 48 hours; 94% glucose removed after 10 days; Fig. 4.37c) with no apparent lag phase. This strain always grew well and quickly in anaerobic MPNs during starvation (see 2.2.1c). Strain S10 showed a lag phase of 10 hours before it started to consume glucose, which was nearly depleted between 10 and 30 days of incubation (96% of glucose consumed after 80 days; Fig. 4.37b). Strain SAMA2 never grew in anaerobic MPNs, despite having been isolated under anaerobic conditions. In the radiotracer experiments, however, a decline in glucose concentrations occurred after 240 hours of incubation (46% of glucose removed in after 110 days).

The highest rate of substrate utilisation ( $0.7 \text{ pmol ml}^{-1} \text{ d}^{-1}$ ) occurred in strain F18I, followed by strain S10 ( $0.06 \text{ pmol ml}^{-1} \text{ d}^{-1}$ ), and strain SAMA2 ( $0.003 \text{ pmol ml}^{-1} \text{ d}^{-1}$ ). All *Photobacterium* strains metabolised the glucose to  $\text{CO}_2$  and VFAs (Fig. 4.37 a-c). However, different ratios of products were present in the final samples for the respective strains. In strain SAMA2 mainly ALF was produced (15%, followed by 12% in  $\text{CO}_2$ , 5% in B/S and in cells), in strain S10 the major product was  $\text{CO}_2$  (48%, followed by 20% in cells, 16% in ALF, and 12% in B/S), and in strain F18I highest radioactivity was found within the cells (34%, followed by 22% in ALF, 20% in B/S, and 18% in  $\text{CO}_2$ ).

The data suggest that the initial lag phase could be linked to the length of the preceding starvation period. However, as no increase in incubation time for growth with ongoing starvation occurred (MPNs), it is also possible that the strains have different physiological responses to metabolism after prolonged starvation.

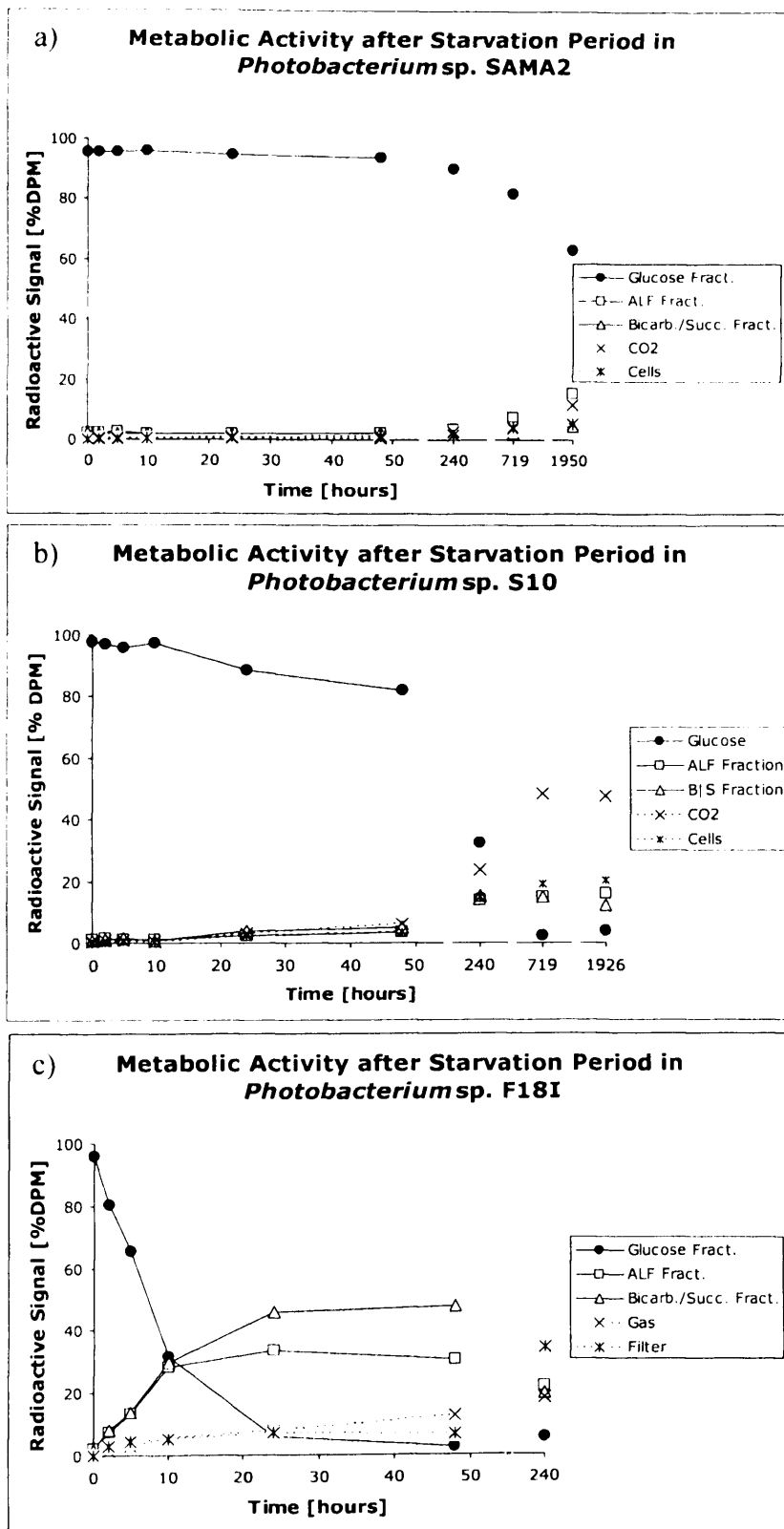
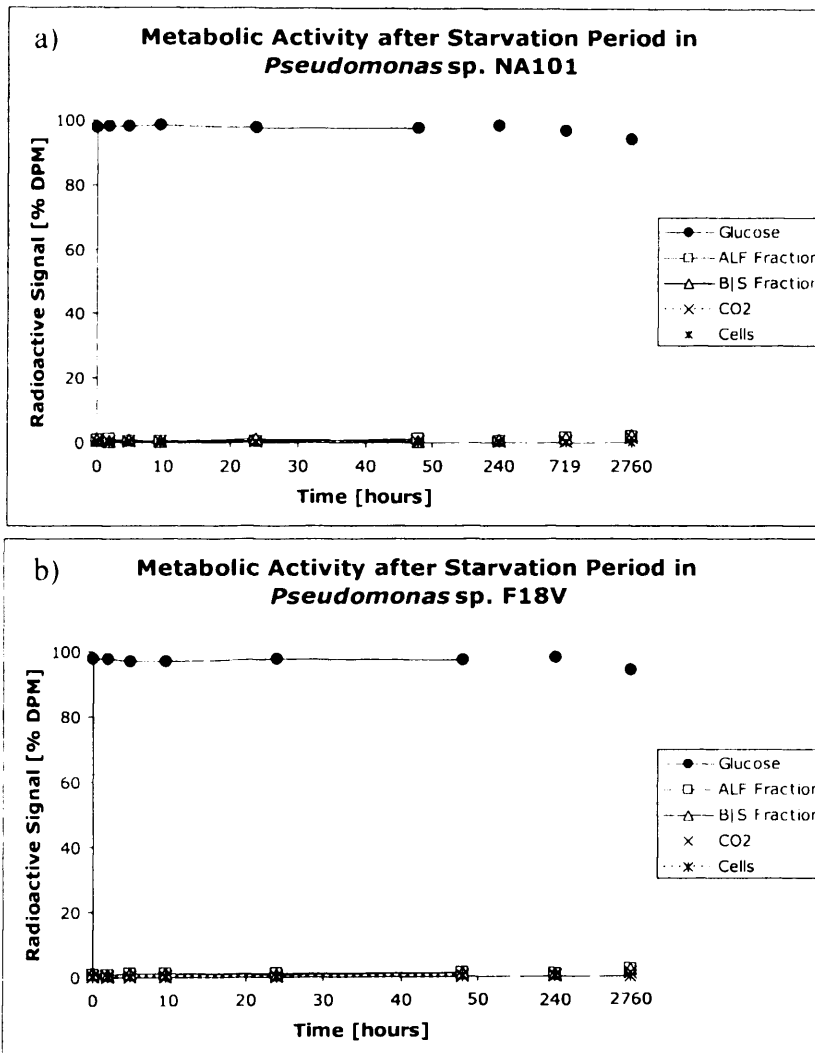


Fig. 4.37: Metabolic activity of *Photobacterium* strains after starvation. a) SAMA2; b) S10; c) F18I. Legend: ALF, acetate, lactate, formate; B|S, bicarbonate, succinate. These compounds were not recovered in separate fractions due to their close retention times

### 3.2.2 *Pseudomonas* strains

The two *Pseudomonas* strains were subjected to radiotracer experiments after 313 days of starvation. Neither of them showed growth in MPNs under anaerobic conditions, although the metabolic activity of both strains sometimes caused a colour change of the redox indicator in anaerobic medium.



**Fig. 4.38: Metabolic activity of *Pseudomonas* strains after starvation.** a) NA101; b) F18V. Legend: ALF, acetate, lactate, formate; B|S, bicarbonate, succinate. These compounds were not recovered in separate fractions due to their close retention times

In the radiotracer experiments it became apparent that neither of the strains was able to either take up or metabolise the available glucose even after 115 days of incubation (Fig. 4.38). This is consistent with the lack of growth in anaerobic MPNs but does not mean that these strains were not viable as they still grew in aerobic MPNs at the final sampling of the starvation-survival experiments (see 2.2.2). This

suggests that these *Pseudomonas* strains require oxygen for glucose metabolism and, more importantly, that oxygen penetration into the vials was absent.

### 3.2.3. *Raoultella* sp. F17VIII

Strain F17VIII was subjected to glucose radiotracer experiments after 330 days of starvation. This strain rapidly utilised the available glucose and did so within only five hours of incubation without a measurable lag phase (96%; Fig. 4.39). The rate of glucose consumption between  $t_0$  and two hours of incubation was  $3.6 \text{ pmol ml}^{-1} \text{ d}^{-1}$ . Mainly VFAs and B/S were produced (37% and 48%, respectively) while 10% of the glucose were metabolised to  $\text{CO}_2$  and only little signal was retained in the cells throughout (maximum 10% after 2 hours after incubation, average 6%). These results are reproducible and thus robust as indicated by constant contributions of products to the total radioactivity in samples between 10 and 719 hours. This clearly indicates that the same metabolic processes must have happened in all parallel incubations.

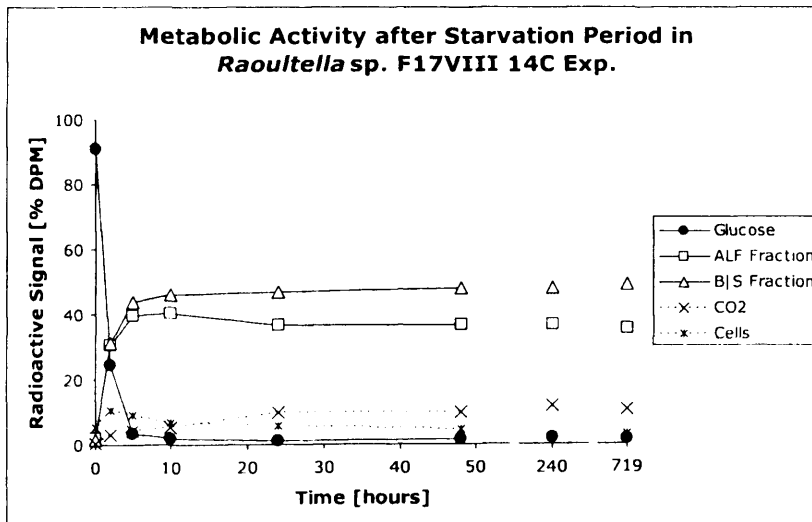


Fig. 4.39: Metabolic activity of *Raoultella* sp. F17VIII after starvation. Legend: ALF, acetate, lactate, formate; B|S, bicarbonate, succinate. These compounds were not recovered in separate fractions due to their close retention times

### 3.2.4 *Shewanella* strains

Two of the *Shewanella* strains were subjected to radiotracer experiments after ~340 days of starvation. Strain SCSA3 never formed cell pellets in anaerobic MPNs but

began utilising the added glucose by 240 hours of incubation (Fig. 4.40a). After 30 days, 28% of the glucose were consumed at a rate of  $0.004 \text{ pmol ml}^{-1} \text{ d}^{-1}$ . Mainly VFAs were produced (25%) followed by B/S (2%), and  $\text{CO}_2$  (1%). Only very small amounts of the radioactivity were present in the cells after 30 days (0.2%).

Strain F17V on the other hand showed strong growth in anaerobic MPNs (always formed cell pellets) and consumed the majority of the radioactively labelled glucose within two hours, again without an apparent lag phase (90%; Fig. 4.40b). In the samples from 10 and 30 days an average of 98% of the glucose was consumed. F17V also produced mainly VFAs (48%) followed by B/S (26%) and  $\text{CO}_2$  (17%) similar to strain SCSA1. Also, only a minor proportion of the radioactive signal was incorporated into the cells (5% between 2 and 30 days).

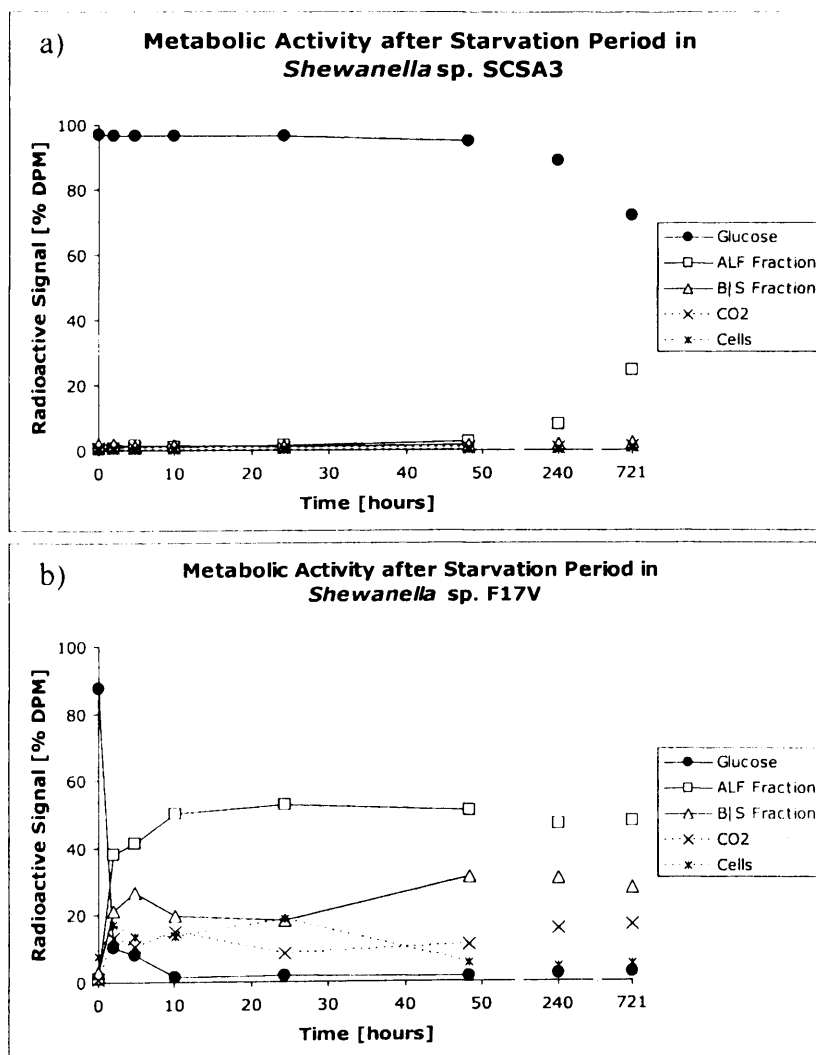
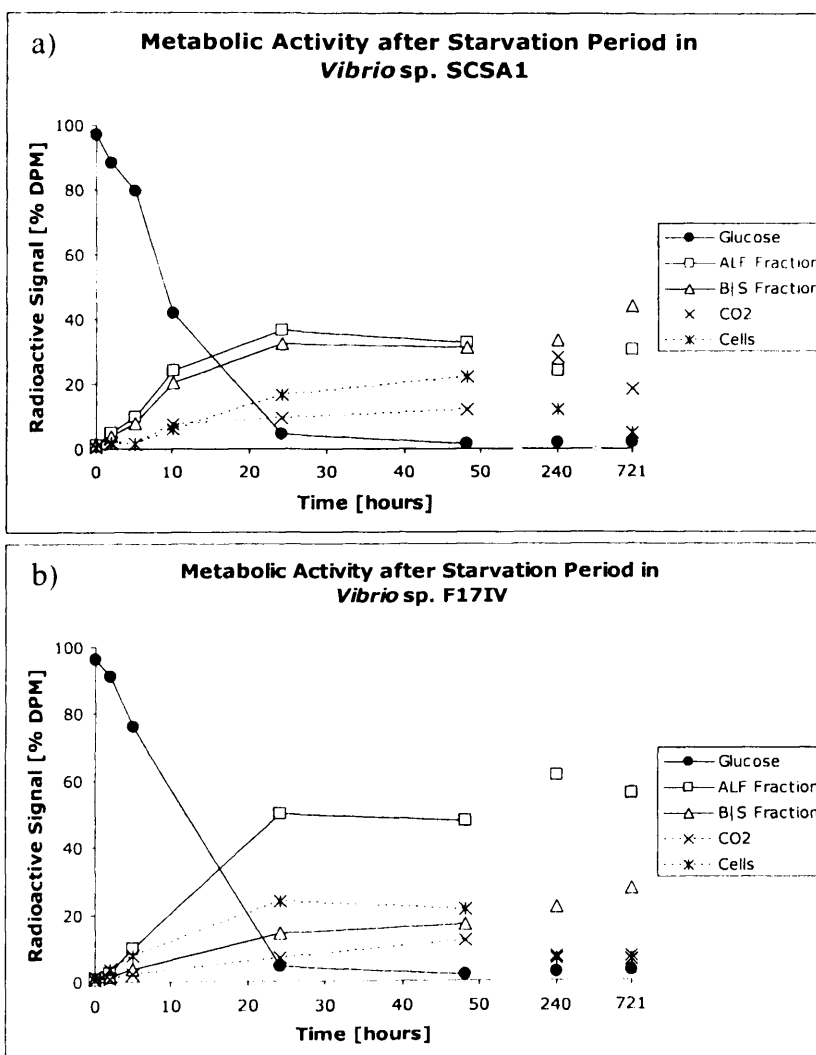


Fig. 4.40: Metabolic activity of *Shewanella* strains after starvation. a) SCSA3; b) F17V. Legend: ALF, acetate, lactate, formate; B|S, bicarbonate, succinate. These compounds were not recovered in separate fractions due to their close retention times

3.2.5. *Vibrio* strains

The two *Vibrio* strains were starved for 385 days prior to radiotracer experiments and culturability was high in the final anaerobic MPNs (Fig. 4.21c and 4.22c). It was thus not surprising that they consumed the available glucose quickly and without a lag phase (Fig. 4.41). In both strains almost all glucose was removed by 24 hours (95%) and at similar a rates ( $0.7 \text{ pmol h}^{-1} \text{ d}^{-1}$  for strain SCSA1 and  $0.4 \text{ pmol h}^{-1} \text{ d}^{-1}$  for strain F17IV).



**Fig. 4.41: Metabolic activity of *Vibrio* strains after starvation.** a) SCSA1; b) F17IV. Legend: ALF, acetate, lactate, formate; B|S, bicarbonate, succinate. These compounds were not recovered in separate fractions due to their close retention times

After 30 days, ~97% of the glucose had been consumed by both strains, however product formation differed. In strain SCSA1 most of the glucose was converted to B/S (44%), followed by ALF (31%), CO<sub>2</sub> (19%), and 5% were incorporated into



cells. In contrast, the majority of the signal was present in the ALF fraction (56%), followed by B/S (27%), CO<sub>2</sub> (7%), and a similar 6% was incorporated into cells of strain F17IV.

### 3.3 Metabolic activity of $\delta$ -*Proteobacteria* after prolonged starvation

Four representative strains of the genus *Desulfovibrio* (class: *Deltaproteobacteria*, obligate anaerobic) were subjected to radiotracer experiments using lactate after different starvation periods (*D. desulfuricans*, 506 days; *D. acrylicus*, 568 days; strain F16I, 595 days; *D. profundus*, 762 days).

As a reminder, anaerobic culturability in all four strains was very low at the end of starvation with only *D. acrylicus* having limited culturability (9 cells ml<sup>-1</sup> in “C”; Fig. 4.25c) for the final sampling. Therefore, it was not surprising that the quickest response to added lactate occurred in *D. acrylicus* and it was metabolised within 10 hours of incubation with a lag phase of two hours at a rate of 6 pmol ml<sup>-1</sup> d<sup>-1</sup> (Fig. 4.42a). The main products between 10 and 240 hours were B/S (46%) and CO<sub>2</sub> (54%). However, with continued incubation the B/S increased to 60% while CO<sub>2</sub> decreased to 40% after 88 days. No acetate was detected at any of the timepoints.

The response of *D. desulfuricans* was much slower, with degradation of the available substrate not starting until 48 hours (Fig. 4.42b). It is noteworthy that again the radioactivity did not increase in the acetate fraction but mainly in the B/S and CO<sub>2</sub> components with 20% and 14%, respectively after 88 days. The rate of lactate consumption was 0.01 pmol ml<sup>-1</sup> d<sup>-1</sup>.

*D. profundus* showed indications of activity at some of the timepoints (Fig. 4.42c) but no consistent trend occurred. This might indicate that a very small proportion of cells were still active, but that these cell numbers are so small that activity was only present in two or three of the experimental subsamples (vials). Interestingly, different metabolic products were formed. Unlike in the above strains, acetate was produced from lactate after five and 10 hours (13% and 10%, respectively) while after 48 hours CO<sub>2</sub> was slightly increased (4%), and after 88 days some signal was present in the B/S fraction (6%) and in the CO<sub>2</sub> (3%).

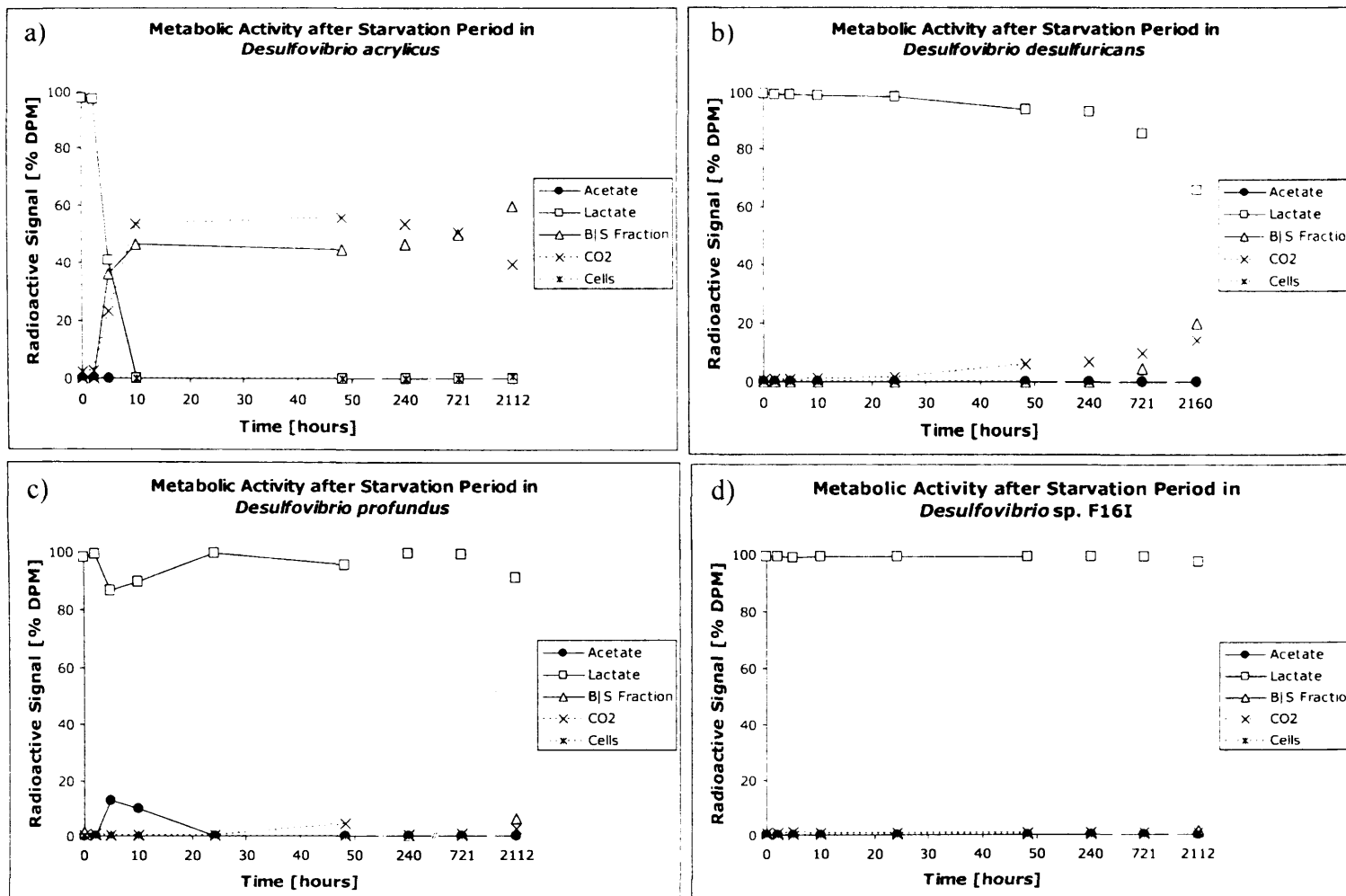


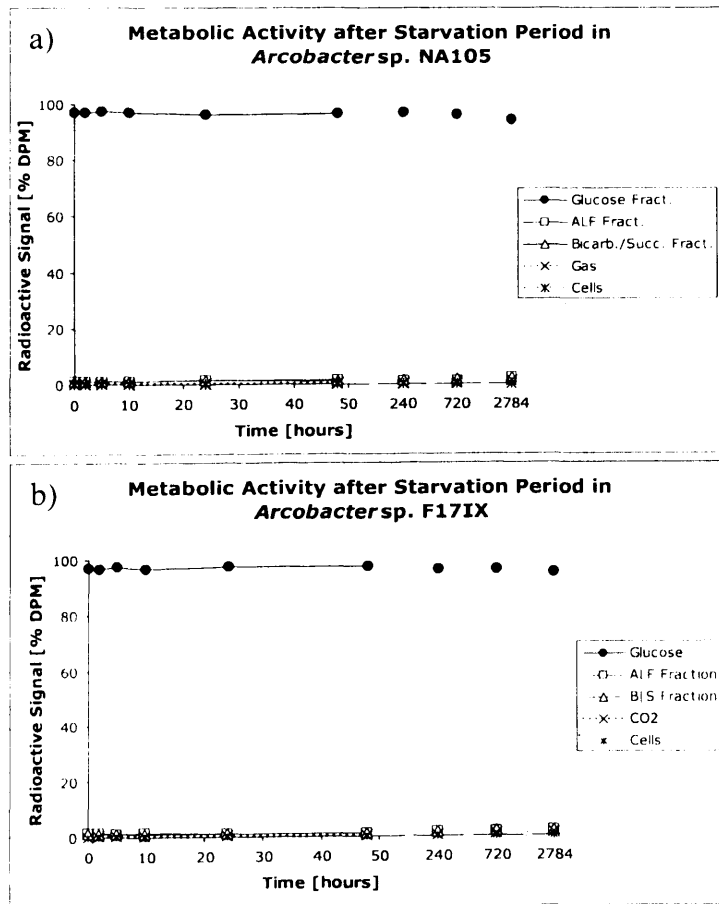
Fig. 4.42: Metabolic activity of *Desulfovibrio* strains after starvation. a) *D. acrylicus*; b) *D. desulfuricans*; c) *D. profundus* d) *Desulfovibrio* sp. F16I. Legend: B|S, bicarbonate, succinate. These compounds were not recovered in separate fractions due to their close retention times.

Strain F16I showed no indication of metabolic activity, which is not surprising considering the weak growth of this strain in MPNs throughout starvation (Fig. 4.42d) but both these observations are contradicted by high FISH counts (Fig. 4.26c).

These examples show that for closely related species the length of starvation time is not a key factor in the loss of activity or maybe culturability, as the duration of starvation for these strains were quite similar. It is also interesting to see that whilst *D. desulfuricans* did not grow anymore in the final MPN (Fig. 4.23c) it was able to utilise the added lactate with a limited lag phase (48 hours). Hence, the strain has characteristics of being viable but nonculturable, so it was quite lucky to have been able to revive it from a 12 year old culture.

### 3.4 Metabolic activity of $\epsilon$ -*Proteobacteria* after prolonged starvation

Two representatives of the genus *Arcobacter* (class: *Epsilonproteobacteria*) were



**Fig. 4.43: Metabolic activity of *Arcobacter* strains after starvation.** a) NA105; b) F17IX;. Legend: ALF, acetate, lactate, formate; B|S, bicarbonate, succinate. These compounds were not recovered in separate fractions due to their close retention times.

investigated for their starvation responses. After 315 days for strain NA105 and 237 days for strain F17IX they were subjected to glucose radiotracer experiments.

Both strains had reasonable anaerobic culturability after starvation (Fig. 4.30c and 4.31c). Although strain NA105 is generally able to use glucose under anaerobic conditions (Dr. Henrik Sass, personal communication) it did not utilise the trace amounts added in this experiment (Fig. 4.43a). Strain F17IX never metabolised glucose and even trace amounts were not utilised after starvation (Fig. 4.43b),

### 3.5 Metabolic activity of *Firmicutes* after prolonged starvation

#### 3.5.1 *Acetobacterium* strains

Lactate radiotracer experiments with *Acetobacterium* strains were conducted after 675 days of starvation. Culturability was present in *A. malicum* (22 cells ml<sup>-1</sup>; Fig. 4.32c) after 365 days of starvation but much higher for strain R002 (2.4×10<sup>4</sup> cells ml<sup>-1</sup>; Fig. 4.33c).

*A. malicum* showed limited signs of activity in two subsamples (48 and 2160 hours). However, the two vials in which lactate concentrations were lowered differed in the metabolic products (Fig. 4.44a). At 48 hours a clear increase in acetate occurred (9%) while in the 90 day sample the radioactivity was present in the B/S fraction (35%) and the headspace CO<sub>2</sub> (23%). As previously suggested for *D. profundus* with similar variability (Fig. 4.42c) this result might be due to the presence of very few viable cells being unequally distributed in the replicate vials. However, the variation in metabolites in both cases is both puzzling and interesting.

In comparison to *A. malicum*, strain R002 exhibited a very rapid lactate metabolism depleting the substrate within two hours of incubation and therefore the highest rate of substrate removal of all strains was calculated for this strain (33 pmol ml<sup>-1</sup> d<sup>-1</sup>; Fig. 4.44b). Main metabolic products were B/S and CO<sub>2</sub> and between two hours and 30 days contributed on average 32% and 67%, respectively to the total radioactivity. For the final sample (90 days), however, 53% were present in the B/S and 47% in CO<sub>2</sub>. Only a minor proportion of the radioactivity was present within the cells at any time (on average 0.5%)

These examples show once more that the length of starvation is probably not as important as the genetic, physiological characteristics of the individual species or strains themselves

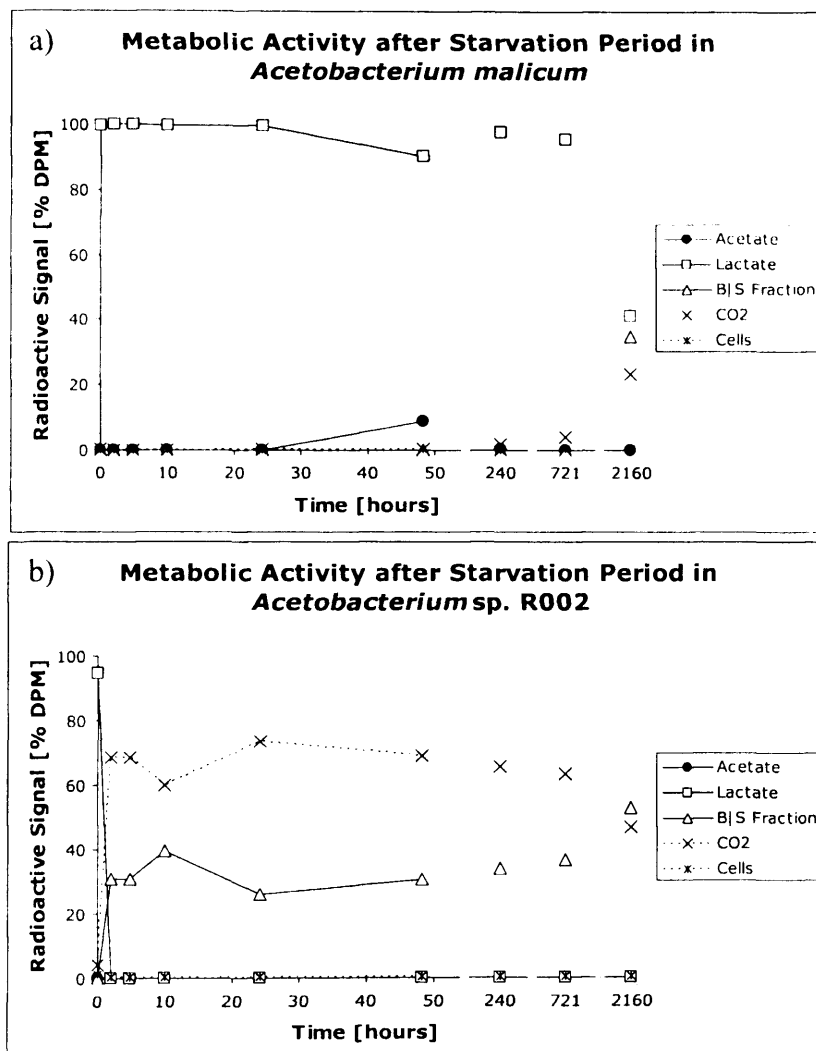


Fig. 4.44: Metabolic activity of *Acetobacterium* strains after starvation. a) *A. malicum*; b) R002. Legend: B|S, bicarbonate and succinate. These compounds were not recovered in separate fractions due to their close retention times

### 3.5.2 *Marinilactibacillus* sp. G8a3

*Marinilactibacillus* sp. G8a3 had starved for 519 days before glucose was added for radiotracer experiments. Culturability in this strain was high after 365 days of starvation with the estimates for culturable cell numbers in the aerobic and anaerobic MPN being identical ( $2.5 \times 10^5$  cells  $\text{ml}^{-1}$ ; Fig. 4.34c). Without an apparent lag phase glucose was consumed (by 98%; Fig. 4.45) within 48 hours of incubation at a rate of  $1.3 \text{ pmol ml}^{-1} \text{ d}^{-1}$ . As the name suggests most of the radioactivity was detected in the

ALF fraction (63%) and was probably predominantly present in the lactate. Other metabolites were CO<sub>2</sub> (21%) and B/S (12%). On average only 3% of the signal was detected within the cells.

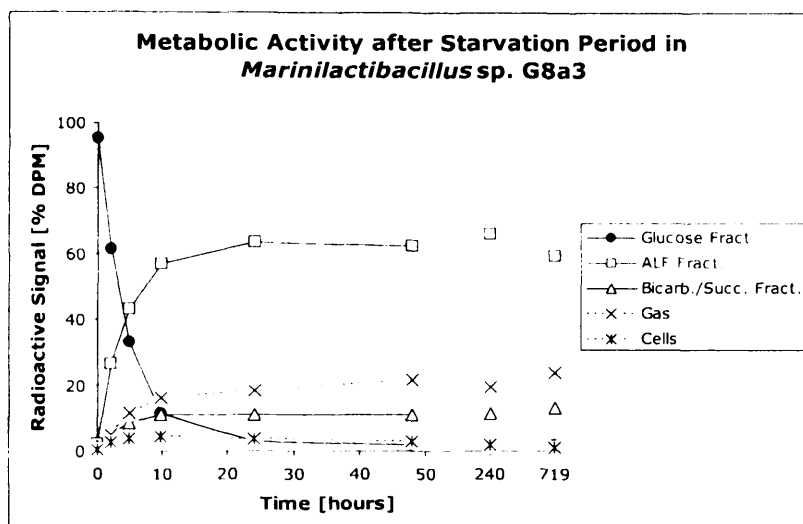


Fig. 4.45: Metabolic activity of *Marinilactibacillus* sp. G8a3 after starvation. Legend: ALF, acetate, lactate, formate; B|S, bicarbonate, succinate. These compounds were not recovered in separate fractions due to their close retention times

#### 4. Discussion

The survival of bacteria during long-term starvation was investigated and all strains tested showed their ability to survive extended periods of substrate limitation. During the experiments the total cell counts (AODC) generally remained unchanged, the number of dead cells increased slightly while the number of FISH detectable cells and culturability decreased with time. The potential metabolic activity of certain strains after long periods of starvation was tested using radiotracer experiments with minute substrate concentrations (0.4 nM glucose, 3.8 nM lactate) and showed a variety of responses, from rapid utilisation to no activity.

The methods used during this project (or a subset thereof) are routinely applied to sediment samples from the deep biosphere. Microorganisms inhabiting this environment constantly suffer low substrate availability and thus starvation. There is, however, a lack of knowledge regarding how starved deep biosphere prokaryotes are detected by these different measures of viability/activity. Without this information it is difficult to effectively interpret measurements of viable cells and biomass, culturability or to relate activity to viability.

## 4.1 Comparison of different measures of cell viability

During starvation-survival experiments different measures were applied to assess the viability of pure cultures including total cell counts (AODC), LIVE/DEAD<sup>®</sup> count, FISH, MPN, and heterotrophic activity. The experimental results of these techniques shed light on different aspects of survivability but were also puzzling in certain cases.

### 4.1.1 The reliability and efficiency of dyes and rRNA hybridisation during starvation

#### a) AODC total counts versus LIVE/DEAD<sup>®</sup> total counts

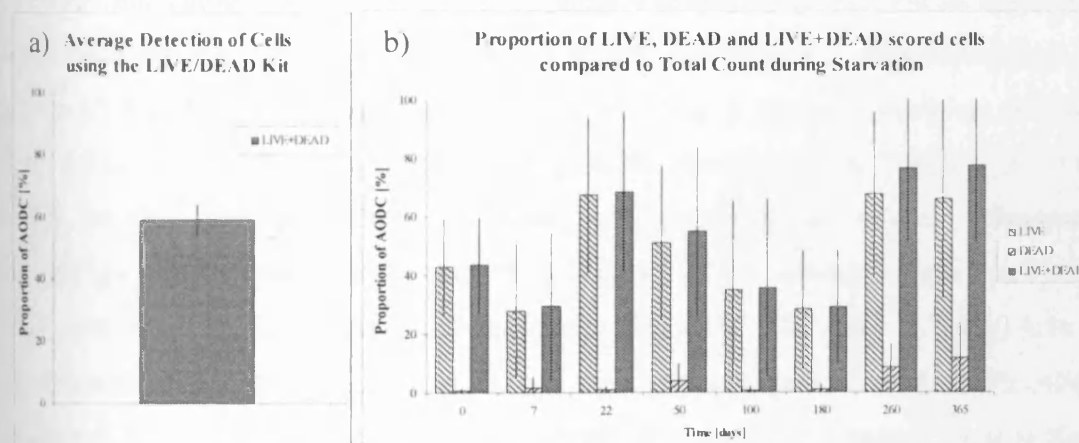
The question whether a microbial cell is alive or dead has been asked for a long time (Knaysi, 1935) and there does not seem to be an easy answer. The development from viability to death is gradual in microbes, probably involving a number of different phases differentially measured by various viability measures.

The LIVE/DEAD<sup>®</sup> Kit consists of two dyes. Syto<sup>®</sup>9 is a cell membrane permeant dye, which stains all cells green. Propidium iodide (PI) is a non-permeant dye, only staining cells that have a compromised cell membrane. These cells appear red under the microscope and are interpreted as dead cells as a permeable membrane would lead to the loss of the membrane potential and subsequently to the collapse of cell energetics and active transport systems (Gregori *et al.*, 2001), and furthermore to leakage of important cell constituents (Boulos *et al.*, 1999). Green stained cells are therefore interpreted as live cells.

During the starvation-survival experiments it was often noted that the total number of cells estimated with the LIVE/DEAD<sup>®</sup> Kit remained below the total AODC count. To investigate the efficiency of the LIVE/DEAD<sup>®</sup> Kit the data of starving pure cultures were compiled and averaged over the entire length of the starvation periods (Fig. 4.46a). It was found that the LIVE/DEAD<sup>®</sup> Kit on average only detects 59% of the cells counted with AODC (Fig. 4.46a; n = 291). During the experiments quite a range of detectability was observed, between 0.5% and 260% of the AODC total count. This leads to the conclusion that the LIVE/DEAD<sup>®</sup> Kit can give an idea of cell viability as the data also suggest that starvation does not affect

the sum of live and dead scored cells, although it has an effect on the proportion of dead cells (Fig. 4.46b and more detailed 4.47). However, it is concerning that only 59% of AODC cells are detected.

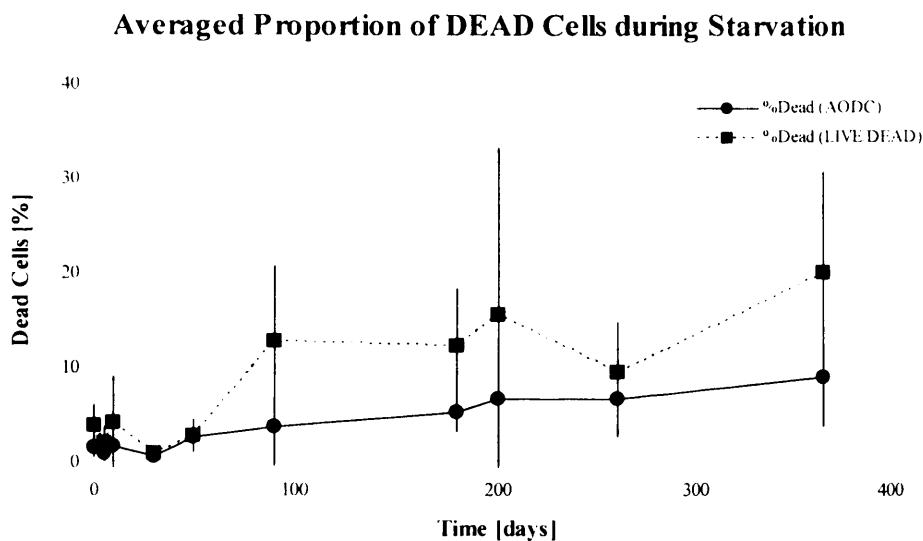
It is left to speculation why approximately 40% of AODC total counts are not detected with the LIVE/DEAD<sup>®</sup> Kit and what category these missing cells belong to: live, dead, or both. Boulos *et al.* (1999) observed no significant difference between total counts determined with acridine orange and the LIVE/DEAD<sup>®</sup> Kit. They argued that despite the reduction of cellular nucleic acid contents during starvation sufficient amounts of nucleic-acid-binding stains (AO, DAPI, Syto<sup>®</sup>9, or PI) can still be accumulated within cells to detect fluorescence. This is true unless Syto<sup>®</sup>9 and/or PI are more affected by nucleic acid reduction than AO but at least for Syto<sup>®</sup>9 the data presented here does not support this hypothesis (Fig. 4.46b) as a higher proportion of the AODC counts are also detected as “live” after starvation.



**Fig. 4.46: Staining efficiency of the LIVE/DEAD<sup>®</sup> Kit.** a) Averaged proportion of live + dead scored, starved cells compared to AODC count (all data, n = 292). b) Averaged proportion of live, dead and live + dead scored, starved cells compared to AODC count, broken down for respective starvation times. 95% Confidence limits are given.

The proportion of dead cells slowly increased with starvation (Fig. 4.47). Depending on whether this is calculated as a percentage of AODC total counts or of the sum of live and dead scored cells the R<sup>2</sup> values differ, 0.95 and 0.76 respectively. The same is true for the variation of the data. The confidence limits calculated for the percentage of dead cells based on AODC total counts are smaller than when based on the LIVE/DEAD<sup>®</sup> Kit. Nonetheless, there is a significant, positive correlation (p < 0.05) between time and the concentration of dead cells in the cultures.





**Fig. 4.47: Average proportion of dead cells during starvation.** The percentage of dead scored cells using the LIVE/DEAD<sup>®</sup> Kit is calculated based on AODC total counts and on the sum of live and dead scored cells. 95% confidence limits are given.

Another intriguing observation is that in some cultures local maxima of dead cells were detected (e.g. *Ornithinimicrobium* sp. F18IV "B", Fig. 4.3; *Photobacterium* sp. SAMA2, Fig. 4.4; *Pseudomonas* sp. NA101 "A", Fig. 4.12; *Shewanella* sp. SCSA3, Fig. 4.17; *Desulfovibrio* sp. F16I "C", Fig. 4.26; *Arcobacter* sp. NA105 "A", Fig. 4.30) or that high proportions of dead cells persisted for several subsequent samplings (e.g. *S. marinus* "A" and "C", Fig. 4.1; *Ornithinimicrobium* sp. F18IV "A", and "C", Fig. 4.3; *Marinilactibacillus* sp. G8a3 "A", "B", and "C", Fig. 4.34 a-c; *Acetobacterium* sp. R002, Fig. 4.33) yet no significant loss in total cell numbers occurred. It is noteworthy that the first group above contains mainly near-surface isolates whereas the second group contains mainly gram positive strains.

The reliability and pitfalls of the LIVE/DEAD<sup>®</sup> Kit have been discussed in the literature. Some studies have come to the conclusion that PI is not a reliable marker for dead cells during starvation (Lopez-Amoros *et al.*, 1995) or microbial cells at all (Kaneshiro *et al.*, 1993). Others argued that some species might be able to accumulate PI without having a compromised cell membrane (Gregori *et al.*, 2001).

Furthermore it was reported that cultivation conditions can affect the staining ability of PI. Boulos *et al.* (1999), for example, argued that growth rates and incubation temperature have an effect on cell membrane permeability. Low temperature causes the membrane to be less fluid (Russell, 1990) and potentially less permeable. This would suggest that there should be lower percentages of dead cells

in *Photobacterium* cultures, which were grown and starved at 10°C while all other strains were starved at 25°C. This was, however, not the case (see Appendix).

Additionally, Kjelleberg *et al.* (1987) suggested that starving cells change their membrane composition, which affects the permeability to facilitate nutrient uptake. A more permeable membrane could allow PI to enter cells that are not dead. This phenomenon could explain the observed cases of high percentages of dead cells despite stable AODC total counts. However, some studies have shown that starvation does not affect membrane permeability and therefore PI penetration (Lopez-Amoros *et al.*, 1995; Rigsbee *et al.*, 1997). In these publications, starvation-survival was only investigated for relative short periods of time (< 70 days). During this time starvation might not have had a sufficient effect on the cell membrane to cause permeability but the data presented here suggest that after extended periods of starvation (> 90 days; Fig. 4.47) the permeability of the membrane increased or that it was indeed compromised thus leading to a rise of dead scored cells in the cultures.

#### b) AODC total counts versus FISH and DAPI counts

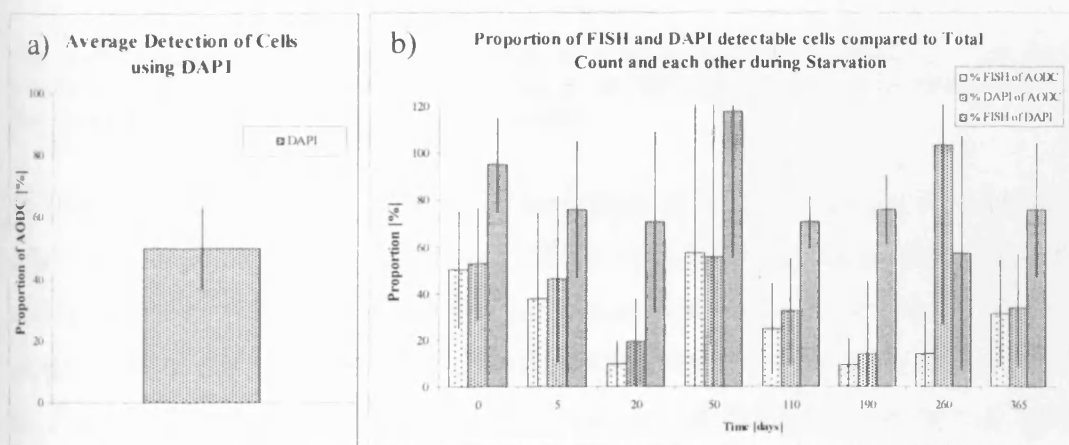
In their original method description of the FISH technique Glöckner *et al.* (1996) reported of a cell loss of 10% during the procedure. During the course of this project a difference between DAPI counter-stained cells and AODC total counts was observed. The loss of cells was approximately 47% (Fig. 4.48b). This estimation is based on all DAPI counts conducted at the onset of starvation when cultures were fully grown but not yet starving (Fig. 4.48b; n = 27). If starvation does not influence the staining ability of DAPI, the average loss of cells was approximately 50% (Fig. 4.48a; n = 106). This discrepancy of cell concentrations can be attributed to centrifugation steps after fixation prior to storage and/or to the loss of cells from filters during the actual hybridisation procedure.

The proportions of FISH and DAPI detectable cells based on AODC total counts decreased within the first 20 days of starvation from ~50% to 10% and 19%, respectively (Fig. 4.48b). After 50 days the respective proportions are higher than at the beginning of starvation but exhibit very high variability despite a reasonable sample size (n = 12). If one is to overlook this timepoint because of the high confidence limits, FISH and DAPI proportions during starvation were consistently

below the values determined for non-starving cells (0 days) on average 18% and 25% of the total AODC count, respectively. The only exception is DAPI at 260 days, which again shows very high confidence limits ( $n = 17$ ) and was therefore not included in this calculation.

FISH and DAPI proportions of AODC were quite similar in most cases. This could be an indication that starvation affects both FISH and DAPI detectability (see 4.1.1c) or that a greater proportion of starved cells were lost during hybridisation and centrifugation. The latter could be caused by a decrease in cell density during starvation (Zweifel and Hagstrom, 1995).

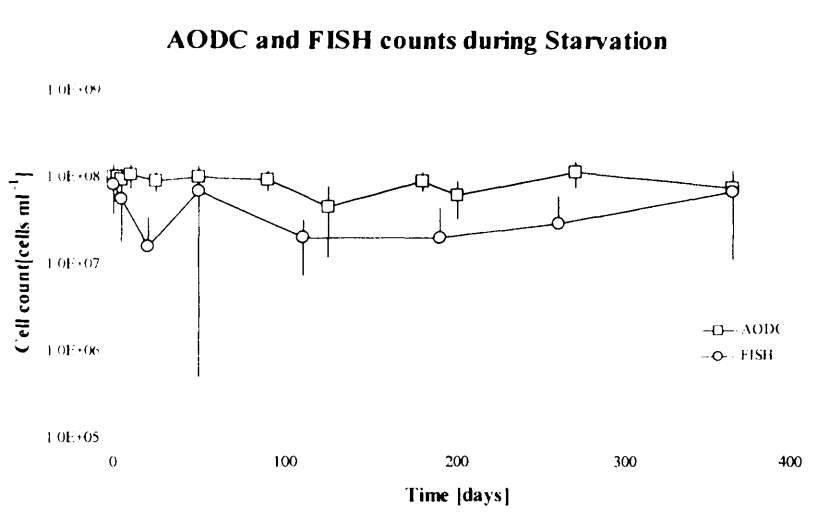
If we assume an equal loss of hybridisable and non-hybridisable cells during the FISH procedure, an average of 36% of total cells were detected with FISH during starvation (between 20 and 365 days.). If non-hybridisable cells exhibit lower cell densities and therefore more of these are lost during sample preparation than their hybridisable counterparts, FISH detectability of starving cells would hence be lower than the averaged 36%.



**Fig. 4.48: Detectability of starving cells using FISH and DAPI.** a) Proportion of DAPI total count compared to AODC total count averaged over starvation period. b) Proportions of FISH and DAPI counts compared to AODC total count broken down for respective starvation times. 95% Confidence limits are given.

Due to the above mentioned loss of cells (with potentially variable quantities) during the FISH procedure the counterstaining of cells after hybridisation is of great importance. The proportion of FISH detectable cells based on the DAPI total count decreased within the first 20 days of starvation from 95% to 71% (Fig. 4.48b). At 50 days of starvation the percentage of hybridised cells exceeds the DAPI count but again variability is very high. Subsequently, the average percentages of FISH

detectable cells was 73% and is therefore twice as high as when FISH counts are compared to AODC (36%). Due to the loss of cells or the effect starvation has on both hybridisation and DAPI staining, FISH detectability can be wrongly interpreted. The comparison, however, shows that FISH counts averaged from all strains investigated are in the same order of magnitude or a factor of 10 lower than the AODC total counts (Fig. 4.49).



**Fig. 4.49: Averaged AODC and FISH counts of starving cells.** Averaged counts of all starving strains are presented. 95% Confidence limits are given. Note that approximately 50% of cells were lost during hybridisation, which has been corrected for.

In the literature there is a number of investigators who do not use a counter-stain after hybridisation. Schippers *et al.* (2005) reported total bacterial CARD-FISH counts whereas Biddle *et al.* (2006) calculated percentages of the two prokaryotic domains from the sum of hybridised cells (FISH). Omoregie *et al.* (2009) used DAPI as a counter-stain but calculated the proportions of different phylogenetic groups based on the AODC total count, which is probably the ideal solution.

The data presented here is an average of all strains investigated under starvation conditions and there is, of course, some variation between different phyla and even genera as indicated by high confidence limits.

However, all strains were detectable with FISH throughout starvation clearly showing the maintenance of viability in a significant proportion of cells over long periods of time. *D. profundus*, *Photobacterium* sp. NA42 and S11, *Shewanella* sp. SCSA3, and *Arcobacter* sp. F17IX are examples where FISH detectability was

lowest ( $\leq 10\%$ ) at the end of their respective starvation periods (between 270 and 548 days). This list contains near-surface as well as deep biosphere strains and spans the three groups of *Proteobacteria* investigated indicating that the reduction of FISH detectable cells might be species or even strain specific.

This also shows that those cells in the deep biosphere that can only be detected by a more sensitive hybridisation method (CARD-FISH) must be extremely starved. During IODP Leg 307 CARD-FISH data was obtained (Webster *et al.*, 2009). Compared to AODC total counts an average of 19% of cells (range: 0.005% - 336%; data not shown) were detected using the CARD-FISH technique. From a starvation perspective it would have been interesting to know which percentage of the AODC total cells would have been detected using FISH in this low nutrient environment.

CARD-FISH was introduced to marine microbial ecology to detect cells that are less active (based on the ribosome content) than cells that can be detected by FISH (Pernthaler *et al.*, 2002). Nowadays, many investigators only use CARD-FISH thereby detecting cells that might also be FISH detectable. The difference between these two *in situ* viability measurements would give an idea of the proportion of potentially active and less active cells. Data from Leg 201, for example, show that separate laboratories can come to very different conclusion. Schippers *et al.* (2005) reported that they could only detect bacterial cells using CARD-FISH while Biddle *et al.* (2006) were able use FISH to estimate both bacterial and archaeal cells in the same sediments. While Schippers *et al.* (2005) concluded that *Bacteria* dominate subsurface sediments (also supported by qPCR data), Biddle *et al.* (2006) found that *Archaea* were more abundant. In both publications, sites 1227 and 1230 were investigated. From the data of Biddle *et al.* (2006) I calculated the number of FISH detectable *Bacteria*. For site 1227 the FISH counts (Biddle *et al.*, 2006) are approximately one order of magnitude below the CARD-FISH counts of Schippers *et al.* (2005),  $\sim 10^4$  compared to  $\sim 10^5$  cells  $\text{cm}^{-3}$  sediment ( $n = 3$ ). The shallowest sediment layer (0.7 mbsf) represents an exception. Biddle *et al.* (2006) detected  $\sim 10^7$  and Schippers *et al.* (2005) only  $\sim 10^5$  cells  $\text{cm}^{-3}$  sediment. For site 1230, however, the CARD-FISH data is consistently one order of magnitude lower than the FISH data,  $\sim 10^5$  compared to  $\sim 10^6$  cells  $\text{cm}^{-3}$  sediment ( $n = 4$ ).

The current debate about which prokaryotic group contributes which proportion of the deep biosphere goes beyond the scope of this thesis. However, the

use of hybridisation techniques with different specificities will lead to contrasting results but could be helpful in understanding differently active subpopulations of the microbial community (Gasol *et al.*, 1999). This is a very simplified view as other factors such as probe specificity (Daims *et al.*, 1999), accessibility of hybridisation sites (Fuchs *et al.*, 1998), and permeabilisation of the cell membrane (Pernthaler *et al.*, 2002) also have an effect on the reliability of these hybridisation techniques.

FISH detectability never ceased during the starvation experiments. It is therefore apparent that the time these prokaryotes were subjected to starvation conditions is nowhere near their capability of surviving substrate deprivation in the environment where more sensitive methods (CARD-FISH) are often required for their detection. The mere detection with FISH or CARD-FISH suggests that cells maintain a minimum level of ribosomes in order to sustain viability. These might be “hibernating ribosomes” (Ortiz *et al.*, 2010) but this base level potentially allows quick response to substrate input as observed in radiotracer experiments.

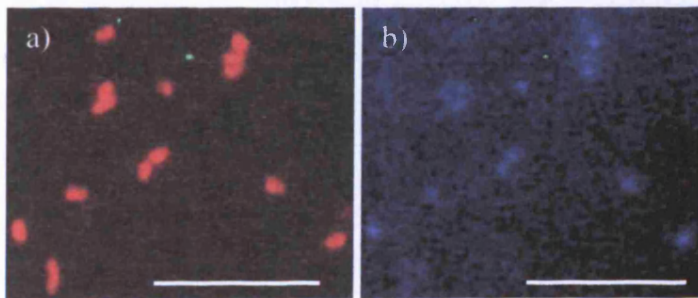
#### c) Loss of signal intensity during starvation and “ghost” cells

Another observation made was that during starvation, signal intensities of AODC and the LIVE/DEAD<sup>®</sup> Kit (mainly concerning the Syto<sup>®</sup>9 dye as indicator for living cells) decreased with time. This was often noticed within the first five to 10 days of starvation. Subsequently the signal intensity remained constant but less bright in comparison with freshly grown cultures. It has previously been reported that metabolically active bacteria emit a brighter signal when stained than inactive or starved cells (Lebaron *et al.*, 1998). This phenomenon can probably be explained by the fact that AO and Syto<sup>®</sup>9 (Invitrogen Manual for Syto<sup>®</sup>9) not only bind to DNA but also to other cell components (e.g. RNA [r-, m- and t-], proteins etc.). When concentrations of these constituents are reduced as part of the starvation response (Kjelleberg *et al.*, 1987), less dye can bind to the cellular content and thus signal intensity decreases.

A similar observation was also made for DAPI, which was used as a counter-stain during the FISH procedure. During the FISH method an unknown number of cells is lost (Glöckner *et al.* [1996] reported <10%; data from this project suggests up to 40%) hence the counter-staining is essential to provide the total number of

potentially hybridisable cells compared to the total cells on the filter. However, for more than 50% of the strains examined during this project, DAPI failed to stain cells effectively after certain periods of starvation (Fig. 4.34). For *Vibrio* strains it was unreliable throughout the experiment whereas it failed in other strains after 45 days of starvation (e.g. *Pseudomonas* strains) or later (after 90 days in *Raoultella* sp. F17VIII and *S. marinus*). In contrast, the hybridisation itself was successful in all of these cases showing that the probes could bind to many ribosomal target sites to give a sufficient signal for counting. For these reasons and for better comparability between strains, in many of the above graphs (see Results) the actual FISH total cell count is shown rather than the proportion of hybridised versus total cells (DAPI) on the filter.

Additionally, morphologies of hybridised cells were fully visible when observed under the microscope suggesting an equal distribution of ribosomes throughout the cytoplasm. In contrast, DAPI stained cells often showed a local, condensed bright spot while the rest of the cell was poorly stained (Fig. 4.50), which suggests staining of the bacterial chromosome (nucleoid-containing cells, Zweifel and Hagstrom, 1995).



**Fig.4.50: Hybridisation versus DAPI staining in starved cells.** Micrograph of *A. malicum* "C" after one year of starvation. a) Cells hybridised with probe EUBI. Entire cell morphologies are visible. b) Same field of view as in a) counter-stained with DAPI. Instead of entire cells only smaller dots are visible, presumably where bacterial chromosome is located. Bars equal 10  $\mu$ m.

Most publications report a loss of signal intensity in regard to the hybridisation rather than the counter-stain DAPI (Tolker-Nielsen *et al.*, 1997; Glöckner *et al.*, 1999; Oda *et al.*, 2000; Baker and Leff, 2006). Some publications, however, describe that DAPI is insufficient in staining cells (Suzuki *et al.*, 1993; McNamara *et al.*, 2003; Queric *et al.*, 2004). McNamara *et al.* (2003) reported an increase of this effect in carbon-starved cells. This finding is of great importance when investigating oligotrophic environments. However, it seems to be largely ignored by FISH studies of

environmental samples (only nine citations for this publication at the time of writing). McNamara *et al.* (2003) suggested that DNA binding proteins block sites where DAPI would usually bind (AT-rich sequences in the minor groove of the B-conformation of dsDNA [Kapuscinski, 1995]) in *Burkholderia cepacia* and conclude that other bacteria might exhibit the same response.

This insufficient staining of carbon-starved bacteria when using DAPI was observed in a range of bacterial groups during this study for members of the gamma- and delta-*Proteobacteria* (but not the epsilon subgroup), *Actinobacteria* (*S. marinus*), and *Firmicutes* (*Acetobacteria* but not *Marinilactibacillus*). All these strains are representative of the deep biosphere bacteria. If they can be detected by FISH but not DAPI the proportion of active cells in the deep biosphere might be overestimated as the total count would be underestimated.

The results of this project and those of McNamara *et al.* (2003) potentially contradict those of Zweifel and Hagstrom (1995), as they argued that cells that are stained with DAPI before, but not after a washing step, were non-nucleoid containing cells and therefore inactive. In this study a brief washing step in ddH<sub>2</sub>O was applied, which was not nearly as thorough as conducted by Zweifel and Hagstrom (1995) but could have potentially been sufficient to remove unspecifically bound DAPI either from cells where DNA binding proteins occupied target sites or from nucleoid devoid cells. Although Zweifel and Hagstrom (1995) argue their case well there are some concerns:

- i) If cells that are not stained by DAPI are ghosts and have lost their DNA, how did this macromolecule leave the cell? The number of cells with a compromised cell membrane (as indicated by the LIVE/DEAD<sup>®</sup> Kit) remained low in most cultures whereas the loss of DAPI signal affected almost all hybridised cells in a culture at once.
- ii) If the DNA was degraded within the cell and the DAPI signal was lost it seems implausible that these cells should still be detectable by FISH. It has been reported that rRNA is much less stable than DNA in starving cells (Davis *et al.*, 1986) thus if fluorescently labelled probes can still bind to their rRNA target sites DNA should still be present.
- iii) AO and Syto<sup>®9</sup> provided a fluorescent signal of starved cells during the long-term experiments. This is true despite a loss of signal intensity (as



mentioned above). Plus total cell numbers estimated with AO (and Syto<sup>®</sup>9) remained unchanged in most cultures indicating the presence of DNA within the starved cells yet staining with DAPI often failed.

Although starved cells became more translucent when observed in phase contrast and therefore appear somewhat “ghost” like (Fig. 4.5, 4.7, 4.13, and 4.16). I remain cautious to accept Zweifel and Hagstrom’s (1995) findings. For the reasons given above, the explanation of McNamara *et al.* (2003) that the inability of DAPI to stain carbon starved cells is caused by DNA-binding proteins or possibly by DNA coiling (Karner and Fuhrman, 1997) and not by the loss of chromosomal DNA seems more likely.

As DAPI appears insufficient to stain starved cells during the FISH technique another counter-stain is needed. AO would be ideal as total counts are routinely determined using this dye. Unfortunately, the background fluorescence especially on white filters is too high. Other DNA-binding dyes such as SybrGreen<sup>®</sup>I or Syto<sup>®</sup>9 are promising. During the course of this project, SybrGreen<sup>®</sup>I was tested but the staining efficiency was poor (unequal staining of the filter and fading). Nonetheless, this is an important matter for future development.

#### d) Culturability compared with cell counts

During starvation culturability decreased in all of the strains. As a consequence cultivation efficiencies also decreased because AODC total counts remained relatively stable during the experiments (Table 4.1; Fig. 4.51).

Prokaryotic cell concentrations decrease on a logarithmic scale in marine sediments from  $\sim 10^9$  cells  $g^{-1}$  sediment at the surface to  $\sim 10^7$  cells  $cm^{-3}$  sediment in  $\sim 500$  mbsf (Parkes *et al.*, 1994). Despite this decrease of total numbers, prokaryotic isolates have been obtained from considerable depths (Bale *et al.*, 1997, 900 m water depth, 500 mbsf; Nogi *et al.*, 1998b, 5110 m water depth; Toffin *et al.*, 2004a, 2005, 4790 m water depth, 4.2 mbsf; Xiao *et al.*, 2011, 5368 m water depth) and the number of culturable prokaryotes in sediment samples is relatively routinely determined (e.g. Cragg *et al.*, 1992; Süß *et al.*, 2004; Biddle *et al.*, 2005; Engelen *et al.*, 2008; Webster *et al.*, 2009). Cultivation efficiencies in regard to total counts, however, are generally low (Table 4.1) but have been reported to be elevated, for

example, in organic matter rich sediment layers (e.g. in sapropels, Süß *et al.*, 2004). Depth itself does not appear to be a proxy for culturability. Probably certain geological factors (D'Hondt *et al.*, 2004; Parkes *et al.*, 2005; Engelen *et al.*, 2008) and geochemical fluxes (e.g. sulphate flux into the sediment column, D'Hondt *et al.*, 2002) have great impact on the *in situ* viability and thus culturability of microorganisms.

Microbiologists face an additional problem to varying *in situ* states of cell viability: the “great plate count anomaly” (Staley and Konopka, 1985). This is the phenomenon that only a minority of microbes can currently be brought into laboratory culture (<99%, Amann *et al.*, 1995). Cultivation techniques and media select for opportunistic microbes (Jorgensen *et al.*, 2006) that are able to grow on the selected substrate(s) and chosen culture conditions (e.g. temperature and pH). Media with lower substrate concentrations or gradient tubes (Sass *et al.*, 2001; Connon and Giovannoni, 2002; Kaeberlein *et al.*, 2002; Zengler *et al.*, 2002; Süß *et al.*, 2004) to avoid substrate accelerated death (Postgate and Hunter, 1964) or the addition of growth factors (Bruns *et al.*, 2002) have been applied to tackle the “uncultivated majority” (Rappe and Giovannoni, 2003; Kuypers, 2007). Nevertheless, accommodating the nutritional needs for all microbes in a given sample will probably remain beyond the microbiological reach not to mention laboratory practicality. This is especially true for deep sediment prokaryotes some of which might grow at rates not fast enough for laboratory cultivation.

When average cultivation efficiencies of starving cells (this project) are compared with environmental studies (Table 4.1), it becomes apparent that relatively high percentages of cells are still culturable after long periods of starvation. During the first 20 days of starvation, average culturable cell numbers exceeded the total count by far (>1.000%), similar to surface tidal flat sediments (Köpke *et al.*, 2005; Table 4.1). Subsequently, cultivation efficiencies decreased by several orders of magnitude but remained between 1% and 10% until 190 days of starvation (Table 4.1; Fig. 4.51). These values are comparable to cultivation efficiencies of organic matter rich sapropel layers in the Mediterranean Sea (Süß *et al.*, 2004). After 190 days of

**Table 4.1: Cultivation efficiencies during starvation and from selected deep biosphere habitats.** Averaged cultivation efficiencies (Cult. Eff.) compared to total counts of all strains during starvation experiments are given and compared to data from selected marine sediments. Some of the latter served as source for isolates used during this study. <sup>1)</sup> Köpke *et al.* (2005); <sup>2)</sup> Webster *et al.* (2009), <sup>‡</sup>Sediment from Carbonate Mound; <sup>3)</sup> Süß *et al.* (2004); <sup>4)</sup> Cragg *et al.* (1992); <sup>5)</sup> Engelen *et al.* (2008).

Starving Cultures		Environmental Studies									
Starvation Time [days]	Cult. Eff. [%]	Tidal Flat <sup>1)</sup>		Challenger Mound <sup>2)</sup>		Mediterranean Sea <sup>3)</sup>		Japan Sea <sup>4)</sup>		Juan de Fuca Ridge <sup>5)</sup>	
		Depth [mbsf]	Cult. Eff. [%]	Depth [mbsf]	Cult. Eff. [%]	Depth [mbsf]	Cult. Eff. [%]	Depth [mbsf]	Cult. Eff. [%]	Depth [mbsf]	Cult. Eff. [%]
0	456	0	1194	39 <sup>‡</sup>	0.006	0	2.9	0.1	0.04	31	0.3
2	1360	1	9.4	96 <sup>‡</sup>	0.009	0.3 (S1)	2.7	0.6	0.6	52	0.01
5	1461	5	0.01	115 <sup>‡</sup>	0.2	4 (S5)	3.3	10	0.03	99	0.6
10	633			197	0.003			51	7×10 <sup>-5</sup>	112	2.7
20	827			254	0.01			133	0.001	122	0.001
25	5							253	5×10 <sup>-4</sup>	169	0.002
90	4							504	0.2		
125	4										
190	1										
260	0.2										
365	0.8										

starvation cultivation efficiencies decreased again by one order of magnitude to below 1% but on average remained above 0.1% (Table 4.1; Fig. 4.51). The latter and even lower values for cultivation efficiencies are frequently reported in cultivation-based studies (e.g. 0.001% - 0.1% for seawater, Amann *et al.*, 1995 and Table 4.1).

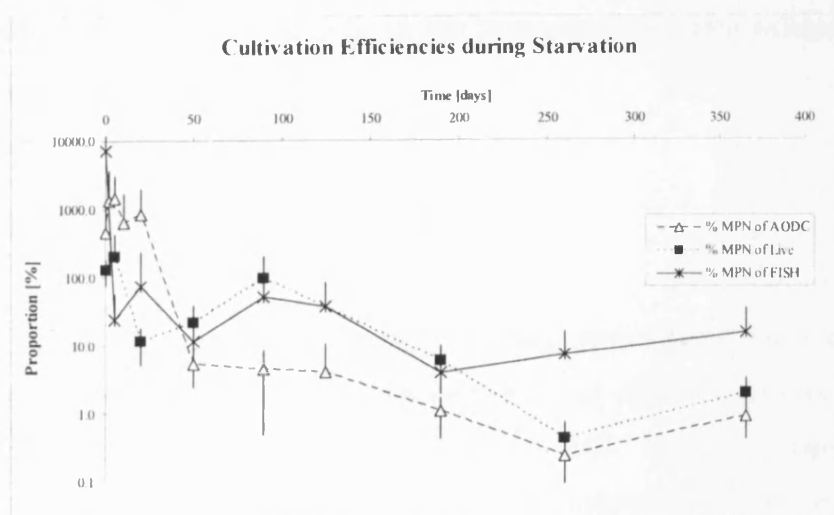
Cultivation efficiencies of cultures that have been starved for one year are still several orders of magnitude higher than the majority of cultivation efficiencies found in the deep biosphere (Table 4.1). This shows not only that microbes can withstand long periods of starvation *in situ* and *in vitro* but also that the conducted starvation-survival experiments have not stressed the microbes beyond their survivability, which is consistent with FISH results (see above) and radiotracer experiments (section 3.).

The above mentioned medium suitability should not have been a major factor during this project as all strains were routinely maintained in the same media that were later used for MPN dilution series. If total counts remain stable and culturability decreases, more and more cells enter the viable but nonculturable state (VBNC, Roszak and Colwell, 1987). This term is often equated with dormancy and is thought to allow non-sporulating bacteria survival of disadvantageous conditions (Mukamolova *et al.*, 2003). This physiological state, however, is still not very well understood, although it has been shown that temperature (e.g. Ravel *et al.*, 1995; Wai *et al.*, 2000) and other stresses like starvation (e.g. Jiang and Chai, 1996; Ramaiah *et al.*, 2002; Archuleta *et al.*, 2005), osmotic shock (Roth *et al.*, 1988), or a combination of them (Mizunoe *et al.*, 2000; Vattakaven *et al.*, 2006) can cause microbes to enter the VBNC state. Recovery or resuscitation from this state has been observed (Ravel *et al.*, 1995; Wai *et al.*, 1996; Whitesides and Oliver, 1997; Ramaiah *et al.*, 2002; Coutard *et al.*, 2007) but some reports suggested that resuscitation is the growth of still culturable cells rather than recovery of VBNC cells (Bogosian *et al.*, 1998). Others speculate that VBNC cells are not a successful phenotype but aid the survival of culturable cells by releasing organic substances upon death, thereby benefitting the population as a whole (Aertsen and Michiels, 2004; Arana *et al.*, 2007).

In this study, starvation is the cause of decreasing culturability. Cells in the VBNC state represent the difference between viable cell count and culturable cells. The total cell count should not be used as a comparison value because dyes such as AO or

DAPI do not directly distinguish between living and dead cells. Instead, total viable counts need to be used to exclude the proportion of dead cells (Colwell, 2000; Arana *et al.*, 2007). Several viability determining methods are available, including but not limited to: LIVE/DEAD<sup>®</sup> Kit (based on cell membrane integrity); 5-cyano-2,3-ditolyl tetrazolium reduction (CTC, based on respiratory activity, Rodriguez *et al.*, 1992; Coallier *et al.*, 1994); rhodamine 123 (based on membrane potential, Kell *et al.*, 1991; Diaper *et al.*, 1992), fluorescein diacetate (based on cellular enzymatic activity and membrane integrity; Rotman and Papermaster, 1966, Prosperi, 1990). These methods, however, might not detect the same proportion of “viable” cells in a sample due to the different aspects of viability tested and subpopulations of the microbial community exhibiting different viabilities.

One characteristic of viability that all cells require to recover from any environmental stress is the presence of ribosomes. These intracellular structures are quickly degraded after cell death (Davis *et al.*, 1986) but are a prerequisite for *de novo* protein synthesis after starvation. Thus a viable cell must maintain a base level of ribosomes. Cells without any ribosomes should be either dead or would be soon. Ribosomes are detected by FISH or the more sensitive CARD-FISH method. Viability counts using general probes that supposedly detect all *Bacteria* (Daims *et al.*, 1999) and *Archaea* (Stahl and Amann, 1991) might therefore be a more suitable standard than other viable counts or total counts to base VBNC or cultivation efficiency calculations on.



**Fig 4.51.: Cultivation efficiencies during starvation.** Cultivation efficiencies are calculated based on ADOC total count, LIVE count, and FISH count. Data of all strains was averaged. 95% Confidence limits are given.

During the starvation experiments quite a number of strains showed low culturability whilst FISH detectability remained high (e.g. *Pseudomonas* sp. F18V, Fig. 4.14c; *Raoultella* sp. F17VIII, Fig.4.15c; *D. desulfuricans*, Fig. 4.23c; *D. acrylicus*, Fig. 4.25c; *Arcobacter* sp. NA105, Fig. 4.30c; *A. malicum*, Fig. 4.32c; *Acetobacterium* sp. R002, Fig. 4.33c). There are, however, also examples where FISH detectability was low whilst culturability remained high (e.g. *Shewanella* sp. SCSA3 and F17V, Fig. 4.17c and 4.20c, respectively).

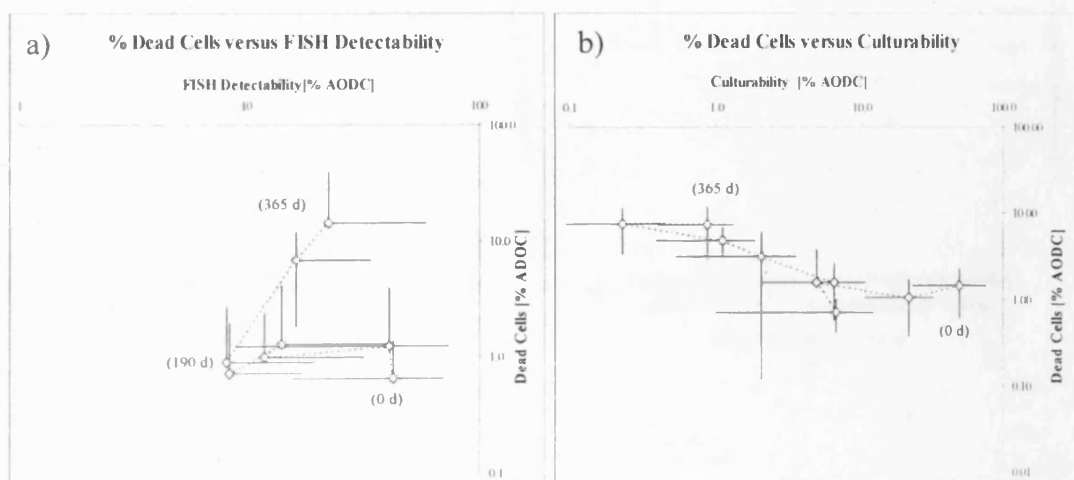
Hence, the FISH detection limit for pure cultures of ~370 rRNA molecules per cell (Hoshino *et al.*, 2008) is probably too high to be a proxy for viability. FISH detectability generally decreased with starvation (Fig. 4.48) and therefore cultivation efficiencies based on FISH counts are on average one order of magnitude higher (22%) than those based on the AODC total counts (2%) between 90 and 365 days of starvation (Fig. 4.49). According to this, between 78% and 98% of cells entered the VBNC state after 90 days of starvation. Theoretically, cultivation efficiencies based on the live count should be somewhere between the other two measures as there should be less live than total cells and more live than FISH detectable cells in a sample. This, however, is not the case (Fig. 4.51). The detection limit for CARD-FISH is between 21 and 46 times lower than that of FISH (Hoshino *et al.*, 2008) and therefore the discrepancy between the above calculated values of VBNC cells should decrease using the more sensitive method. However, calculating cultivation efficiencies from CARD-FISH counts would most likely be closer to the true viable but nonculturable number than when calculating it from any other cell count (e.g. AODC), which can include dead cells or cell fragments with DNA remains (Gasol *et al.*, 1999).

#### 4.1.2 Correlation between measures of viability

Total cell counts remained relatively stable during starvation in most cultures and treatments. Thus, starvation time did not (yet) have an effect on total cell numbers. With starvation, however, other measures of cell viability changed. FISH detectability and culturability decreased while the proportion of dead cells slightly increased. These measures of viability are now compared using their proportions based on the AODC total count.

a) Percentages of dead cells versus FISH detectability and culturability

It could be expected that with a decreasing number of hybridisable cells in starving cultures the number of dead cells would increase (inversely correlated). To investigate this relationship, averaged data of all strains were grouped according to starvation time and these two measures of cell viability were compared (Fig. 4.52a; total n = 71). For the first 190 days of starvation FISH detectability decreased (from 43% to 8%) while the proportion of dead cells remained relatively constant (between 0.7% and 1.3%). For the final two samplings, however, the proportion of dead cells significantly increased as did FISH detectability (Fig. 4.52a). Therefore, FISH detectability cannot be correlated with the percentage of dead cells ( $R^2$  value of linear regression based on log-transformed data = 0.02). That these two measures of viability do not correlate is maybe not surprising as, for example, in *Marinilactibacillus* sp. G8a3 94% of dead cells and 40% FISH detectable cells were detected at the same time (365 days; Table 4.2; Fig. 4.35).



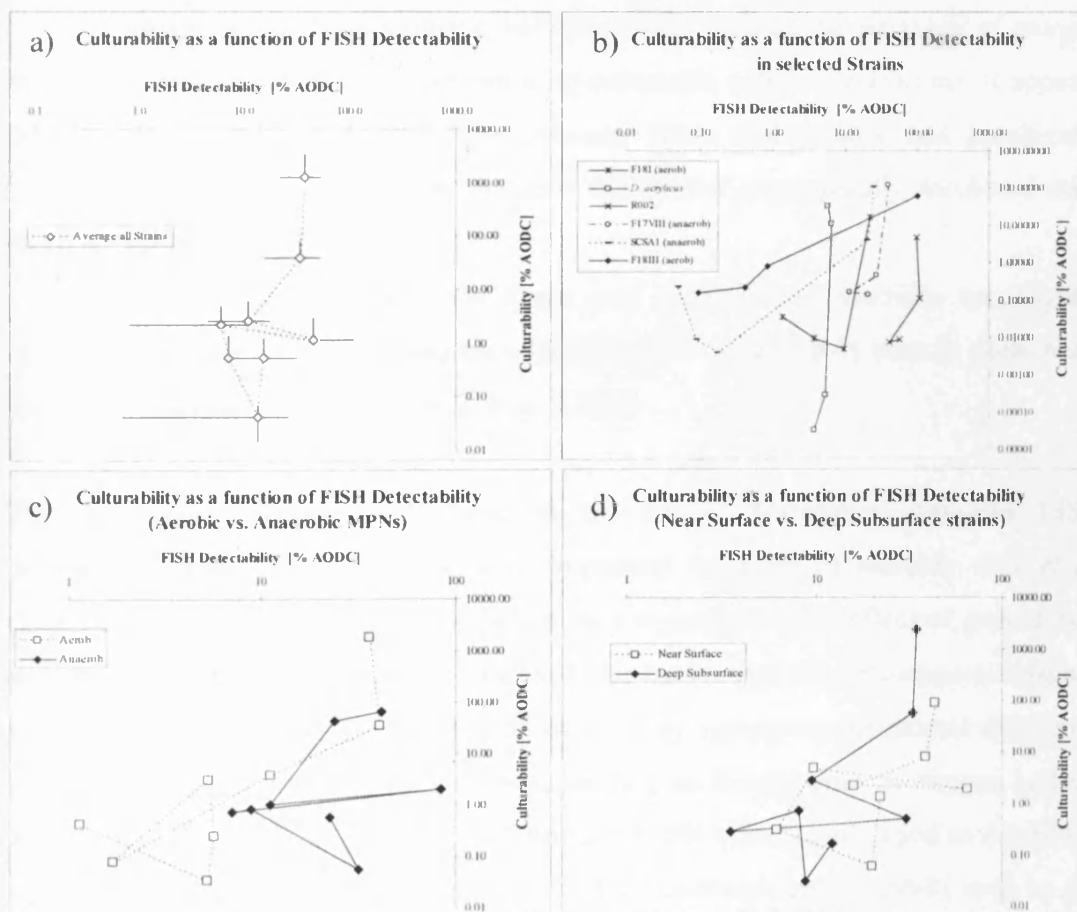
**Fig. 4.52: Correlation between percentages of dead cells versus FISH detectability and culturability.** Percentages of dead cells compared to FISH detectable and culturable cells based AODC total counts are presented in dependence of starvation time (average data of all strains, grouped for 0, 5, 20, 50, 90, 190, 260, and 365 days). a) % Dead cells compared to FISH detectability. b) % Dead cells compared to culturability (for description refer to text). 95% Confidence limits are given.

It could also be expected that with a decreasing number of culturable cells in starving cultures the number of dead cells should increase (inversely correlated). This is supported by average data of cultures grouped after starvation time (Fig. 4.52b; n = 398). Although culturability decreased over two orders of magnitude (from 49% to

0.9%) the fraction of dead cells only increased slightly (from 1.4% to 7.4%). A linear relationship was found when the data was log-transformed ( $R^2 = 0.7$ , not significant).

b) Culturability as a function of FISH detectability

The presence of ribosomes is an indicator for potentially active cells. Therefore, if hybridisation efficiencies decrease with starvation, so should cultivation efficiencies. It was already discussed above that the detection limit of FISH (~370 rRNA molecules per *E. coli* cell, Hoshino *et al.*, 2008) might be too high to draw definite conclusions. Nonetheless, culturability as a function of FISH detectability was investigated (Fig. 4.53 a-d).



**Fig. 4.53: Culturability as a function of FISH detectability.** Percentages of FISH detectable and culturable cells based AODC total counts are presented in dependence of starvation time (average data of all strains, grouped for 0, 5, 20, 50, 90, 190, 260, and 365 days). a) Average data of all strains. b) Selection of strains (for description refer to text). c) Aerobic versus anaerobic incubation of MPNs. d) Near surface versus deep biosphere strains. 95% Confidence limits are only given in a) for clarity reasons.



Averaged data of all strains grouped according to starvation time showed a correlation between these two measures of viability, although a weak one ( $R^2$  value of log-transformed data = 0.27, not significant; Fig. 4.53a).

The only significant correlation between FISH detectability and culturability was found in *Shewanella* sp. F18III (Fig. 4.53b;  $R^2 = 0.98$ ). When looking at individual strains, the variation in their response to starvation becomes apparent. In *Shewanella* sp. F18III, for example, FISH detectability decreased from 100% to 0.1% and culturability from 62% to 0.2%. In contrast, in *Acetobacterium* sp. R002 FISH detectability only halved during starvation whilst culturability decreased by two orders of magnitude (Fig. 4.53b). In other strains such as *Photobacterium* sp. F18I and *Vibrio* sp. SCSA1 both culturability and FISH detectability decreased rapidly, subsequently the latter recovered slightly in both strains.

Cultivation conditions during MPN analysis such as the presence of oxygen might have had an effect when determining culturable cell concentrations. It appears that there is a stronger relationship between FISH detectability and aerobically incubated MPN dilution series ( $R^2$  value = 0.63) than anaerobically incubated ones (with  $R^2$  value = 0.07; Fig. 4.53c).

Furthermore, it appears that these two measures of viability are slightly stronger correlated in deep subsurface isolates ( $R^2$  value = 0.4) than in their near-surface counterparts ( $R^2$  value = 0.2; Fig. 4.53d).

These analyses indicate that there is no simple correlation between FISH detectability and culturability, two very important measures of viability. Oda *et al.* (2000) came to a similar conclusion when they investigated the effect of growth rate and starvation on hybridisation success with the model organism *Rhodospseudomonas palustris*, with starvation of less than 58 days. They reported a substantial difference in FISH detectability during starvation depending on the presence of oxygen and the growth rate during initial cultivation. Although FISH is frequently used to determine viability in oligotrophic environments (e.g. Llobet-Brossa *et al.*, 1998) such as the deep biosphere (Biddle *et al.*, 2006) “the method is of limited value for quantitative detection or for obtaining information on bacterial activity if used in complex natural systems with an unknown distribution of growing, non-growing and starving bacteria” (Oda *et al.*, 2000). The research presented here extends the report by Oda *et al.* (2000) considerably by increasing the number of prokaryotes studied, comparing

near-surface and subsurface sediment isolates, extending starvation times up to three years and by measuring metabolic activities directly on starved cultures.

## 4.2 Potential metabolic activities after starvation

The metabolic activities determined after extensive periods of starvation showed that many of the strains readily if not immediately used the available substrate (*Raoultella* sp. F17VIII, *Shewanella* sp. F17V, both *Vibrio* strains, *D. acrylicus*, *Acetobacterium* sp. R002, and *Marinilactibacillus* sp. G8a3; see section 3. of this chapter and Table 4.2). This clearly shows the presence of viable and active cells, which must have maintained their effective intracellular enzyme systems, as well as probably ribosomes, in order to allow rapid uptake and conversion of the added radioactive glucose or lactate.

### 4.2.1 Potential metabolic activities compared to measures of viability

In some strains a lag phase occurred prior to substrate utilisation (e.g. *Ornithinimicrobium* sp. F18IV [ $>721$  hours], *A. malicum* [ $>721$  hours]; *D. desulfuricans* [240 hours]; *Photobacterium* sp. SAMA2 [240 hours], S10 [10 hours]; *Shewanella* sp. SCSA3 [ $>48$  hours]). After the lag phase, these strains exhibited much lower rates of activity ( $0.001 - 0.06 \text{ pmol ml}^{-1} \text{ d}^{-1}$ ; Table 4.2) than strains without a lag phase ( $0.4 - \geq 33 \text{ pmol ml}^{-1} \text{ d}^{-1}$ ; e.g. *Vibrio* sp. F17IV; *Acetobacterium* sp. R002; Table 4.2). It would seem logical that strains where a lag phase occurred might have had a lower proportion of viable or culturable cells in the culture. This, however, was not the case for the comparison strains of the *Acetobacteria*, *Photobacteria*, *Shewanella*, or *Vibrio* (Table 4.2). Therefore other variables need to be responsible. Maybe lag-phase strains had reduced their intracellular protein content more than non-lag phase strain and therefore had to slowly synthesise enzymes for metabolic activity. This strategy though, would be disadvantageous in the environment if competitors were present that quickly metabolise available substrate and would therefore outcompete lag-phase strains.

Calculated rates for substrate utilisation are compared to other measures of viability (Table 4.2). No correlation was found between metabolic activity and starvation time, FISH detectability, or culturability.

In some strains, such as the strictly aerobic *Pseudomonas* strains but also in microaerophilic *Arcobacter* strains, glucose was not metabolised (up to 116 days). *Arcobacter* sp. NA105, however, showed growth or at least activity (decolourisation of resazurine) in some of the anaerobically incubated MPNs. Additionally, it was isolated anaerobically and is or was known to metabolise glucose without oxygen (H. Sass, personal communication). During the radiotracer experiment, however, no activity was detected. The deep biosphere strain *Arcobacter* sp. F17IX also did not consume the available glucose but this was expected as this strain was previously tested negative for glucose utilisation (Chapter III).

Additionally, no activity was detected in the deep biosphere strains *D. profundus* and *Desulfovibrio* sp. F16I. The former had lost culturability and FISH detectability during starvation (Fig. 4.29c), the latter showed low culturable cell numbers throughout the experiment but remained FISH detectable close to 100% of the total count (Fig. 4.26).

Interestingly, *Photobacterium* sp. SAMA2, which had never grown in anaerobic MPNs (incubation periods up to 1 year) despite having been isolated anaerobically, showed activity after 10 days in the radiotracer experiment. Subsequently, glucose was metabolised at a very low rate ( $0.003 \text{ pmol ml}^{-1} \text{ d}^{-1}$ ) and even after 81 days 64% of the radioactively labelled glucose was still present (Fig. 4.37a).

There appeared to be no difference in metabolic activity in regard to the substrate used. The highest rate was determined with lactate in *A. malicum* ( $33.3 \text{ pmol ml}^{-1} \text{ d}^{-1}$ ), which is approximately 10 times higher than the quickest metabolism of glucose by *Raoultella* sp. F17VIII ( $3.6 \text{ pmol ml}^{-1} \text{ d}^{-1}$ ). This could be linked to the fact that lactate was added in 10 times higher concentrations than glucose (3.8 nM compared to 0.4 nM). Wirsen and Jansch (1986) reported that a tenfold increase in substrate concentrations also lead to an increase in metabolic rates by a factor of 10. Exactly this might have occurred here. That aside, lactate utilising strains had similar rates as glucose metabolising ones (Table 4.2).

**Table 4.2: Potential metabolic rates of starving cultures compared to measures of viability.**

Strain	Starvation Time <sup>a)</sup> [days]	Metabolic Activity [pmol ml <sup>-1</sup> d <sup>-1</sup> ]	Lag phase <sup>b)</sup>	AODC <sup>c)</sup> [cells ml <sup>-1</sup> ]	% Dead <sup>c)</sup> (of AODC)	% FISH <sup>c)</sup> (of AODC)	MPN (aerob) <sup>c)</sup> (of AODC) [cells ml <sup>-1</sup> ]	MPN (anaerob) <sup>c)</sup> (of AODC) [cells ml <sup>-1</sup> ]
<i>Actinobacteria</i>								
<i>S. marinus</i>	256	0.002	Y	6.5×10 <sup>8</sup>	79	3.5	0.007	-
<i>Ornithinimicrobium</i> sp. F181V	315	0.001	Y	2.7×10 <sup>7</sup>	17	5.2	0.2	0.0009
<i>Firmicutes</i>								
<i>A. malicum</i>	675	0.01	Y	8.4×10 <sup>7</sup>	98	78	-	3×10 <sup>-5</sup>
<i>Acetobacterium</i> sp. R002	675	≥ 33.3	N	2.2×10 <sup>8</sup>	98	55	-	0.01
<i>Marinilactibacillus</i> sp. G8a3	519	1.3	N	6.8×10 <sup>7</sup>	94	40	0.004	0.4
<i>Gammaproteobacteria</i>								
<i>Photobacterium</i> sp. SAMA2	970	0.003	Y	1.7×10 <sup>8</sup>	29	25	0.3	-
<i>Photobacterium</i> sp. S10	754	0.06	Y	3.5×10 <sup>7</sup>	1.6	0.2	0.7	0.004
<i>Photobacterium</i> sp. F181	469	0.7	N	4.3×10 <sup>7</sup>	0	1.3	0.03	0.6
<i>Raoultella</i> sp. F17VIII	330	3.6	N	7.9×10 <sup>7</sup>	0.4	20	0.06	0.14
<i>Shewanella</i> sp. SCSA3	339	0.004	Y	3.0×10 <sup>7</sup>	0.3	3.0	1.6	3.7
<i>Shewanella</i> sp. F17V	343	3.2	N	5.9×10 <sup>7</sup>	0.9	0.05	0.05	0.04
<i>Vibrio</i> sp. SCSA1	385	0.7	N	1.1×10 <sup>8</sup>	0	0.05	0.02	0.2
<i>Vibrio</i> sp. F17IV	385	0.4	N	1.0×10 <sup>8</sup>	0.4	0.2	0.04	0.01
<i>Deltaproteobacteria</i>								
<i>D. desulfuricans</i>	506	0.01	Y	1.3×10 <sup>8</sup>	-	9	-	2×10 <sup>-6</sup>
<i>D. acrylicus</i>	568	6.0	N	6.3×10 <sup>7</sup>	2.2	4	-	3×10 <sup>-5</sup>

a) Starvation time: Time from onset of starvation until start of radiotracer experiment

b) Presence of lag phase prior to activity indicated by Y for yes, N for no or absent

c) Data from final sampling, which may be up to a few month prior to radiotracer experiment. For *Acetobacterium* the final sampling was conducted 10 month prior to radiotracer experiment, for *Photobacterium* sp. SAMA2 it was 125 days after the start of radiotracer experiments.

During the radiotracer experiments, radioactively labelled substrate was added to carbon substrate depleted cultures. Therefore, all rates calculated are potential rates (Jannasch, 1967) as the addition of even these tiny amounts (glucose:  $0.37 \text{ pmol ml}^{-1}$ ; lactate:  $3.8 \text{ pmol ml}^{-1}$ ) significantly changed the available substrate concentration. If the concentrations of added radiotracer only represent a fraction of the *in situ* substrate concentrations and do not change it significantly, then they probably would not affect the *in situ* prokaryotic activity. This situation, however, is not likely to occur when investigating oligotrophic environments and is definitely not the case for the experiments conducted here.

Nonetheless, the potential rates determined must be interpreted as minimum rates for two reasons. Firstly, for some strains the rates were calculated from only two timepoints because the respective strains had consumed the available substrate very quickly (e.g. *Acetobacterium* sp. R002; *Raoultella* sp. F17VIII) and might have done so quicker than observed. Secondly, Wirsen and Jannasch (1986) reported from a study with oxic marine surface sediments that higher substrate concentrations also led to higher metabolic rates. The aims of the radiotracer experiments conducted here were to investigate whether cells could metabolise available substrate at all after starvation and if so whether they could utilise the small amounts likely to be present *in situ* (e.g. release of substrates when a neighbouring cell lyses). The use of small substrate concentrations was also essential to not cause significant changes in cell viability. Thus substrate concentrations in these experiments were sometimes immediately rate limiting.

Rates of metabolic activity were not calculated on a per cell basis here. As with the calculation of VBNC cells (see above) it would be difficult to decide, which viability measurement to choose from (total count, live count, MPN count, or FISH count). Instead it would have been of great use to investigate which proportion of cells metabolised the added substrate. Do all cells, despite decreasing culturability and FISH detectability, still metabolise or is it just a certain subpopulation, which has maintained its metabolic activity? Micro-autoradiography (MAR) could have answered this question (Brock and Brock, 1966). Even further, combined with FISH phylogenetic identity can be linked to metabolically active microorganisms in the environment (Nielsen *et al.*, 1999). When working with pure cultures FISH is obviously not needed to identify the prokaryotes in question but it would have been

intriguing to see whether cells that are actively utilising the substrate are also FISH detectable. Samples for MAR or MAR-FISH were taken and frozen but were not processed due to the lack of time. Hopefully this can be rectified at a later time.

#### 4.2.2 Metabolic rates of starving cultures compared to *in situ* studies

In order to compare the determined minimum, potential metabolic rates of starving cultures to *in situ* measurements the strains were divided into two groups (Table 4.3 and 4.4). One group consists of strains that did not show a lag phase and immediately metabolised the available substrate and the other group contains those strains that exhibited a lag phase prior to substrate utilisation. Average metabolic rates are  $6.2 \text{ pmol ml}^{-1} \text{ d}^{-1}$  for non-lag-phase cultures and  $0.02 \text{ pmol ml}^{-1} \text{ d}^{-1}$  for lag-phase cultures (Table 4.3). *Acetobacterium* sp. R002, however, has a high impact on the first group. Without this strain the rate for non-lag-phase cultures is slightly lower ( $2.3 \text{ pmol ml}^{-1} \text{ d}^{-1}$ ) but still remains two orders of magnitude above the minimum activity of lag phase strains.

Grouping strains by their origin, near surface versus deep biosphere strains, resulted in very similar means for metabolic rates if the terrestrial, deep subsurface strain *Acetobacterium* sp. R002 was omitted (Table 4.3). A similar situation to that described above occurs if strains are grouped by the substrate or furthermore, by substrate and the presence of a lag phase (Table 4.3).

**Table 4.3: Metabolic activity rates for differently grouped substrate starved pure cultures.**

Group	MR <sup>1)</sup> [pmol ml <sup>-1</sup> d <sup>-1</sup> ]	n	Group	MR <sup>1)</sup> [pmol ml <sup>-1</sup> d <sup>-1</sup> ]	n
Lag	0.02 (0.03)	5	Glucose	1.1 (1.1)	9
Non-lag	6.2 (9.3)	8	Lactate	9.8 (25.3)	4
Non-lag (-R002)	2.3 (1.9)	7	Lactate (-R002)	2.0 (8.5)	3
Near Surface	1.1 (2.5)	6	Glucose lag	0.02 (0.08)	3
Deep subsurface	6.1 (11.2)	7	Glucose non-lag	1.7 (1.5)	6
Deep subsurface (-R002)	1.5 (1.6)	6	Lactate lag	0.001 (0)	2
			Lactate non-lag	19.7 (173)	2
			Lactate non-lag (-R002)	6 (n.a.)	1

<sup>1)</sup> MR: Metabolic rate; 95% Confidence limits are given in brackets.

Glucose consumption rates of starving cultures are in the same order of magnitude as rates measured in anoxic Mediterranean Sea sediments (non-sapropel layer Z6,

28 mbsf; Coolen *et al.*, 2002; Table 4.4). In organic matter-rich sapropel layers activities are elevated (Coolen *et al.*, 2002) and one order of magnitude above the glucose consumption rates of starving cultures but comparable to the rate of *Acetobacterium* sp. R002 (33 pmol ml<sup>-1</sup> d<sup>-1</sup> for lactate).

Novitsky and Kapkey (1981) measured metabolic rates of Halifax marine harbour sediments, which were oxic above 0.4 mbsf and anoxic below. The determined rates however are on a mmol scale rather than in pmol and therefore at least 10<sup>9</sup> times higher compared to rates in Mediterranean sediments or starving cultures. The assumption is that Halifax Harbour is a relative nutrient rich environment, which would explain the high metabolic activities.

Wirsen and Jannasch (1986) compared metabolic activities in oxic deep sea sediments (Atlantic Ocean). They found decreasing activities with increasing water (12 m to 4175 m) and sediment depths (<10 cmbsf) probably accompanied by decreasing organic matter availability (Table 4.4). Compared with the pure culture results, all rates determined by Wirsen and Jannasch (1986) were at least one order of magnitude below rates of non-lag-phase strains. Rates of lag-phase cultures are one order of magnitude below rates from the near-shore Buzzards Bay site and the top three cm of continental slope and abyssal plain sites. In the open ocean, however, rates determined below 0.03 mbsf were in one instance comparable to rates of lag-phase cultures but otherwise one order of magnitude lower (Table 4.4).

It seems that the potential metabolic rates of long-term starved cultures fit somewhere between those occurring in relatively organic matter poor, anoxic non-sapropel subsurface sediments and open ocean sites (oxic sediments). This also suggests that research on long-term starved cultures is a valuable approach to investigating prokaryotic viability in low energy flux habitats.

#### a) Incorporation versus energy metabolism

Not many studies on the deep biosphere use glucose or lactate when investigating metabolic activities despite their metabolic importance. Glucose is a versatile, central metabolite (Novitsky and Kapkey, 1981) and lactate can be used by a variety of microorganisms such as heterotrophs, sulphate reducers, and acetogens. Instead many studies use acetate (e.g. Wellsbury *et al.*, 2002), as it is an important metabolic intermediate in anaerobic organic matter degradation (Parkes *et al.*, 1989), and used

by terminal electron accepting prokaryotes including, sulphate reducers, and methanogens. Acetate was furthermore shown to be produced *in situ* under certain conditions (Wellsbury *et al.*, 1997), and therefore is a key metabolite for deep biosphere prokaryotes. However, for the strains investigated here, acetate is a metabolic product and could thus not be used as a substrate. Another approach often used is to measure the incorporation of radioactively labelled amino acids (Jeffrey *et al.*, 1996; Meyer-Reil, 1987) or nucleotides (e.g. Cragg *et al.*, 1992; Parkes *et al.*, 2005; Wellsbury *et al.*, 1996) into cell biomolecules, which provides an index of productivity and growth. However, the aim here was to test for metabolic activity and substrate conversion rates not so much for incorporation into cellular constituents.

In experiments with starving cultures only a small fraction of glucose or lactate (or breakdown products thereof) was incorporated into cells. Approximately 20% of the radioactivity from glucose was present in the cells during incubation (e.g. in *Shewanella* or *Vibrio* strains) but subsequently this value decreased to below 10%. Less than 1% of the radioactivity was incorporated into cells of sulphate reducers or acetogens at any time. Instead the substrate was metabolised to VFAs or CO<sub>2</sub> for glucose and exclusively to CO<sub>2</sub> for lactate (no acetate detected). These results broadly agree with those of Novitsky and Kapkey (1981) who measured a vertical profile of heterotrophic activity in Halifax harbour sediment (1 mbsf) and reported incorporation of < 5% of lactate and an average of 30% for glucose. In contrast, Wirsen and Jannasch (1986) reported generally higher incorporation than respiration rates for the four substrates tested (acetate, glucose, glutamate, trimethylamine) in Atlantic Ocean sediments with increasing water depths. This was also reported by Meyer-Riel (1978) who observed that respiration accounted for only 5% to 11% of the radioactively labelled glucose in Baltic Sea beach sediments. Unlike the time-course experiments with starved cultures, the reported *in situ* incubations were stopped after certain periods (e.g. two hours, Novitsky and Kapkey, 1981) and therefore only represent a snapshot in time. These studies might have come to different conclusions if different incubation times had been chosen.

Metabolising rather than incorporating seems logical after long periods of starvation. The first thing a cell requires is energy in form of ATP. This energy could then be



**Table 4.4: Metabolic rates of pure cultures compared to in situ studies.**

Pure Culture Averages		Mediterranean Sea					Atlantic Ocean <sup>3)</sup>			
		Sediments <sup>1)</sup>		Halifax Harbour Sediments <sup>2)</sup>			Buzzards Bay	Cont. Slope	Abyssal Plain	
Group	MR <sup>4)</sup> [pmol ml <sup>-1</sup> d <sup>-1</sup> ]	<sup>14</sup> C-Glucose		<sup>14</sup> C-Glucose		<sup>14</sup> C-Lactate	Depth [mbsf]	<sup>14</sup> C-Glucose MR <sup>4)</sup> [pmol ml <sup>-1</sup> d <sup>-1</sup> ]	<sup>14</sup> C-Glucose MR <sup>4)</sup> [p mol ml <sup>-1</sup> d <sup>-1</sup> ]	<sup>14</sup> C-Glucose MR <sup>4)</sup> [pmol ml <sup>-1</sup> d <sup>-1</sup> ]
		Depth [mbsf]	MR <sup>4)</sup> [pmol ml <sup>-1</sup> d <sup>-1</sup> ]	Depth [mbsf]	MR <sup>4)</sup> [mmol ml <sup>-1</sup> d <sup>-1</sup> ]	MR <sup>4)</sup> [mmol ml <sup>-1</sup> d <sup>-1</sup> ]				
Lag phase strains	0.02	0	132	0	26.6	53.3	0.03	0.3	0.13	0.13
Non-lag phase (-R002)	6.2	0.1 (S1)	43	0.2	66.6	40.0	0.06	0.6	0.03	0.008
Non-lag phase (-R002)	2.3	0.6 (Z1)	0.1	0.4	213.1	119.9	0.09	0.3	0.007	undetected
		2.6 (S6)	26	0.6	6.7	40.0				
Glucose strains	1.1	2.8 (Z6)	5	0.8	0	13.3				
Lactate strains (+R002)	9.8	3.2 (S7)	26	1.0	6.7	13.3				
Lactate strains (-R002)	2.0									

<sup>1)</sup> Coolen *et al.* 2002

<sup>2)</sup> Novitsky and Kapkey 1981, note the units are in mmol ml<sup>-1</sup> d<sup>-1</sup> and rates are therefore ~10<sup>7</sup> times higher in these marine surface sediments than in deeper (water and sediment depth) Mediterranean sediments and starving cultures. 0 - 0.2 mbsf, oxic sediment; 0.4 mbsf interface; 0.6 - 1.0 mbsf anoxic sediment.

<sup>3)</sup> Wirsen and Jannasch 1986; water depth of Buzzards Bay: 12 m, continental slope: 2825 m, abyssal plain: 4175m.

<sup>4)</sup> MR: Metabolic rate

used for proton motive force or protein synthesis to, for example, repair macromolecules that might have been damaged during starvation. If more substrate is available greater incorporation would probably occur next, leading to growth and replication. However, in the environment it is likely that any substrate input is sudden (e.g. neighbouring cell lyses) and concentrations are low and decrease further due to microbial consumption. Hence, cells need to be able to use even the smallest amounts available to maximise energy gain and must therefore produce extracellular enzymes and transport mechanisms with great substrate affinity. This would provide them and potentially others with monomers or oligomers that can be transported across the cell membrane. This leads to the problem of the threshold levels for substrate uptake.

#### b) Threshold levels for substrate uptake

Input of organic matter into marine (surface) sediments is carried out via sinking particles (marine snow, photo-detritus) but is already utilised during sinking (Karl *et al.*, 1988; Banse, 1992). Therefore, the input of organic matter into marine sediments is low for most of the seafloor ( $1 \text{ g of carbon m}^{-2} \text{ a}^{-1}$ , Jahnke and Jackson, 1992). Wirsen and Jannasch (1986) argued that a heterogeneous distribution is essential for efficient utilisation of the substrates. They added that if soluble substrates were homogeneously distributed, concentrations might well be below threshold levels (Jannasch, 1967). If this was the case however, one would expect concentrations to increase with continued input, yet they remain low. This indicates prokaryotic consumption at a very low level with high substrate affinity. Law and Button (1977) reported that “glucose limitation was characterised by a threshold  $0.21 \text{ mg l}^{-1}$  for growth”. This equates to a glucose concentration of  $1.2 \text{ } \mu\text{mol l}^{-1}$ . Although this amount could be lowered to  $0.04 \text{ } \mu\text{mol l}^{-1}$  by the addition of arginine, it appears somewhat high as a concentration minimum for bacterial uptake. According to Meyer-Reil (1978) the concentration of dissolved glucose in sandy beach sediment pore water is only between  $0.6 \text{ } \mu\text{mol l}^{-1}$  and  $1.6 \text{ } \mu\text{mol l}^{-1}$ .

During radiotracer experiments on average  $7.4 \text{ pmol l}^{-1}$  of glucose were not utilised by non-lag-phase strains. This apparent minimum concentration is four to six orders of magnitude lower than the threshold values of Law and Button (1977) and the *in situ* pore water concentrations quoted by Meyer-Reil (1978). In

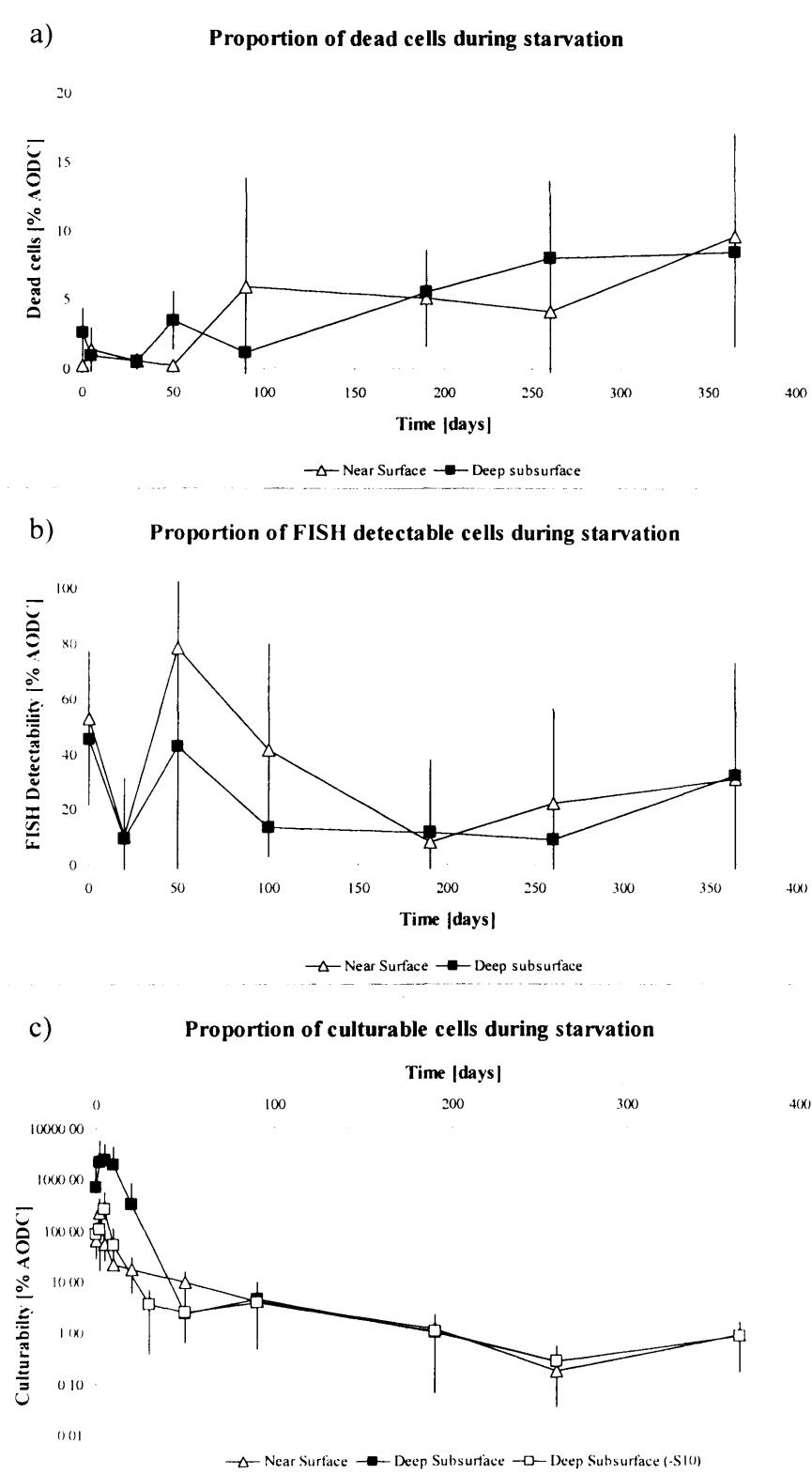
*Acetobacterium* sp. R002 and *D. acrylicus* (non-lag-phase strains using lactate), no radioactively labelled substrate could be detected after rapid substrate removal, indicating that all substrate was consumed or fell below the detection limit. For lactate experiments, 1 DPM  $\mu\text{l}^{-1}$  equalled 8.2 pmol  $\text{l}^{-1}$ , which is in the same order of magnitude as minimum glucose concentrations. At the beginning of the radiotracer experiments, approximately  $10^3$  to  $10^4$  molecules of substrate were available per cell (assumed cell concentration of  $1 \times 10^8$  cells  $\text{ml}^{-1}$ ). After metabolic activity only 45 glucose molecules and less than 49 lactate molecules were present per cell thus proving the effective and generally quick removal of added substrate by long-term starved prokaryotes.

### 4.3 Comparison of near-surface to deep biosphere isolates

So far changes of viability measures were mainly discussed as averages of the investigated strains. This is justifiable as microbial communities consist of many different phylotypes with variable physiologies. Thus, when investigating sediments from the deep biosphere in regard to FISH detectability, metabolic activity or culturability, average responses from mixed microbial communities are also determined. In the following section the attention is now turned to the differences between near-surface and deep subsurface strains.

#### 4.3.1 Proportion of dead cells in near-surface and deep subsurface strains during starvation

The percentages of dead cells in cultures of near-surface and deep subsurface strains slowly and slightly increased during starvation (Fig. 4.54a). Generally confidence limits are quite high for this measure of viability and significant differences between the two groups did not occur. Differences did occur between Gram-positive strains in the proportion and appearance of dead cells. In the pelagic *S. marinus* (Fig. 4.1) dead cells appeared earlier and in higher percentages than in *Ornithinimicrobium* sp.



**Fig. 4.54: Comparison of near-surface to deep subsurface strains.** Viability measures are presented according to origin of isolates and as proportion of AODC total count during starvation. 95% Confidence limits are given. a) Proportion of dead cells. b) Proportion of FISH detectable cells. c) Proportion of culturable cells. Note: different scales on y-axis.

F18IV, after 90 days on average 88% dead cells compared to 15% after 180 days, respectively (Fig. 4.1 and 4.3). For *Acetobacteria*, however, the deep biosphere strain R002, (Fig. 4.33) showed elevated numbers of dead cells compared to *A. malicum* (Fig. 4.32) on average 49% and 32% between 180 and 365 days of starvation, respectively.

In general significantly higher percentages of dead cells were present in Gram-positive than in Gram-negative bacteria ( $p < 0.05$ ). For the latter no differences between near-surface and deep subsurface strains were observed.

#### 4.3.2 Proportion of FISH detectable cells in near-surface and deep subsurface strains during starvation

In regard to FISH detectability no significant differences between near-surface and deep biosphere strains were observed (Fig. 4.54b). The two curves follow a similar trend throughout the experiments. Although confidence limits are quite high and overlapping, FISH detectability was elevated in near surface strains at 50, 100, and 270 days of starvation. Comparing pairs of one genus indicates that FISH detectability was higher in the near-surface strains for *Arcobacter* (Fig. 4.30 and 4.31), whereas, it was higher in deep subsurface strains for *Shewanella* (Fig. 4.17, 4.19 and 4.20). For other genera FISH detectability in near-surface and deep biosphere strains was relatively equal.

#### 4.3.3 Culturability in near-surface and deep subsurface strains during starvation

It appears that the culturability is the only measure of viability where a difference between near-surface and deep-subsurface strains did occur (Fig. 4.54c). However, *Photobacterium* sp. S10, a deep subsurface strain, exhibited cultivation efficiencies of up to 46,000% of the total count. Growth of this strain was highly variable throughout starvation experiments (Fig. 4.9). If S10 is omitted from the grouped cultivation efficiencies for deep subsurface strains the graph for culturability of the latter becomes indistinguishable from the one of near-surface strains (Fig. 4.54c).

Thus it can be concluded that there is most likely no difference in culturability during starvation between deep biosphere strains and their near-surface counterparts.

When looking at comparison pairs individually, culturability in near-surface isolates was higher for *Shewanella* (one to two orders of magnitude, Fig. 4.17, 4.19, and 4.20) but lower for *Acetobacteria* (one order of magnitude, Fig. 4.32 and 4.33). Culturability in the remaining comparison strains was relatively similar as indicated above (Fig. 4.54c).

In conclusion, significant differences in viability and culturability of near-surface and deep subsurface microorganisms were not observed when cells were starved. During radiotracer experiments, however, it was noted that more near-surface strains ( $n = 4$ ) showed a lag phase prior to substrate utilisation than deep biosphere strains ( $n = 1$ ; *Photobacterium* sp. S10).

#### 4.4 Comparison of survivability between phylogenetic groups

It was shown above that there are no differences in the ability of near-surface and deep subsurface strains to survive starvation. What factors other than origin could affect survivability? Phylogenetic grouping may have a great impact on how different phylotypes cope with substrate limitation and this is considered in the following section.

##### 4.4.1 Proportion of dead cells according to phylogenetic groups

Earlier it was observed that percentages of dead cells in starving cultures generally increased with time (section 4.1.1a; Fig. 4.47). It appears, however, that this is strongly influenced by data from strains belonging to the *Actinobacteria* (Family: *Intrasporangiaceae*, high GC) and *Firmicutes* (Families: *Carnobacteriaceae* and *Eubacteriaceae*, low GC; Fig. 4.55a). Starvation seems to affect *Proteobacteria* less regarding the number of dead cells, which might explain why this group represents a high proportion of the deep biosphere (Fry *et al.*, 2008). However, confidence limits especially for *Actinobacteria* are high due to great variances between the two strains investigated (43% in *S. marinus*, 15% in *Ornithinimicrobium* sp. F18IV).

#### 4.4.2 FISH Detectability depending on phylogenetic groups

For FISH detectability of averaged data an initial decrease within the first 20 days of starvation occurred (Section 4.4.1b; Fig. 4.48b). This decrease occurs especially in members of the gamma and epsilon subgroup of the *Proteobacteria* and less pronounced in the delta subgroup (Fig. 4.55b). FISH detectability based on AODC total counts was less than 3% for *Actinobacteria* throughout the experiment but very high in *Firmicutes* where it actually increased during starvation accompanied by an increase in dead cells. Once more, this is accompanied by high confidence limits. In this case it is caused by high FISH detectability in *A. malicum* (up to 380% of total count). Thus average FISH detectability was much higher for *Actinobacteria* than for any other group.

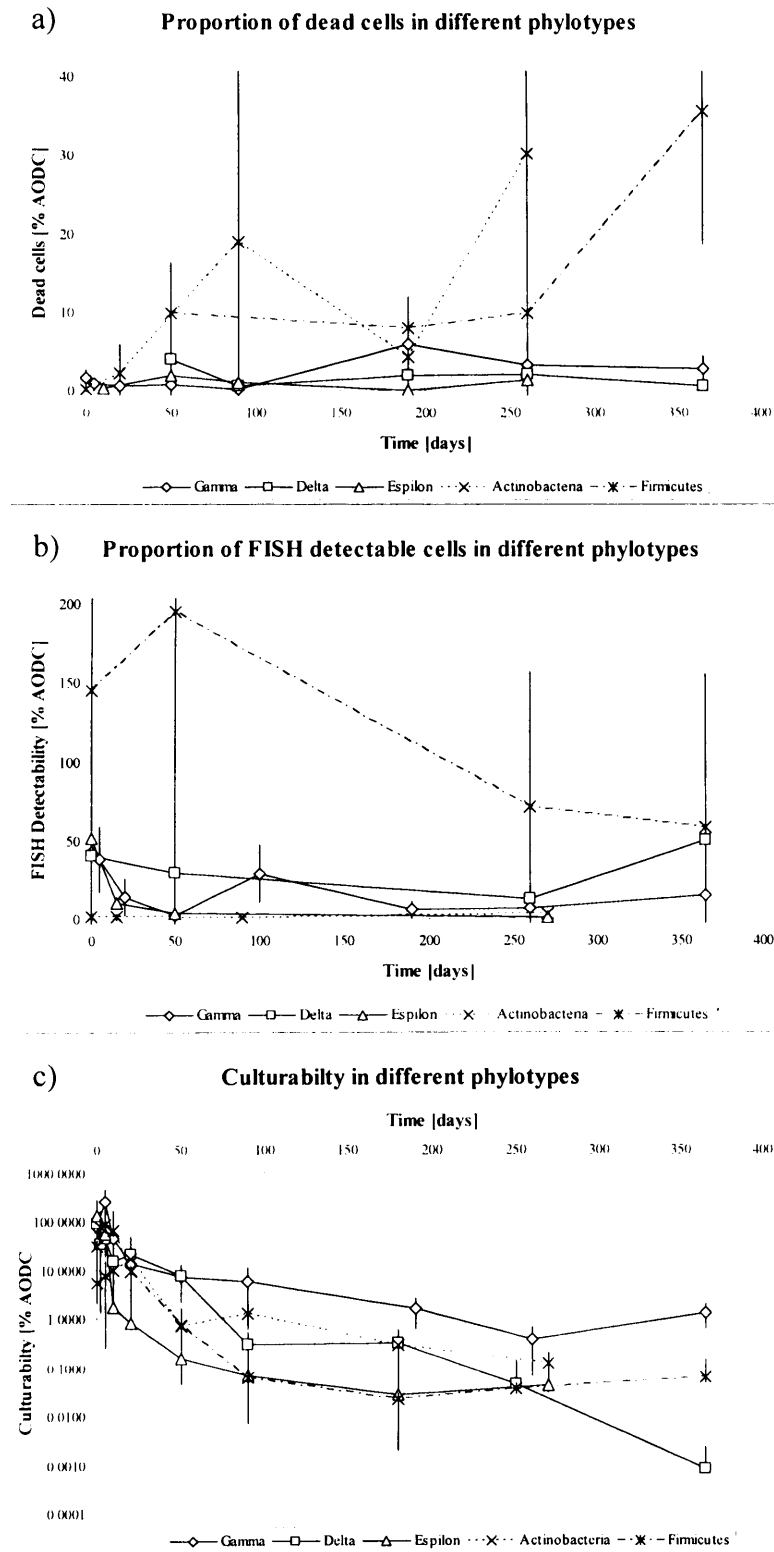
#### 4.4.3 Culturability depending on phylogeny

A general decrease of culturability occurred during starvation (Section 4.4.1 d; Fig. 4.51). The same is true for individual phylogenetic groups (Fig. 4.55c). Culturability decreased most rapidly in members of the *Epsilonproteobacteria* and least in *Gammaproteobacteria*. The latter showed highest cultivation efficiencies from 50 days of starvation onwards. The lowest culturability occurred for sulphate reducers, after one year of starvation and was equally low in all these *Deltaproteobacteria* strains (Fig. 4.23, 4.25, 4.26, 4.29).

To summarise the responses towards starvation of different phylogenetic groups it has to be taken into account that mainly *Gammaproteobacteria* (14 strains) were investigated and that other phyla were only represented by two to four strains. Therefore, drawing conclusions from such a limited number of representatives for vast and diverse phyla is probably not possible.

However, lowest percentages of dead cells accompanied by the highest cultivation efficiencies occurred in *Gammaproteobacteria*, one of the most frequently detected and cultivated phylum in the deep biosphere (Fry *et al.*, 2008). Gram-positive bacteria showed elevated numbers of dead cells compared to Gram-negative bacteria. In addition, the highest and lowest proportions of FISH detectable

cells during starvation were detected in Gram-positives showing the variability of FISH detection even in pure culture experiments (Fig. 4.55b).



**Fig. 4.55: Comparison of viability measures according to phylogenetic groups.** Viability measures are presented according to phylogeny and as proportion of AODC total count during starvation. 95% Confidence limits are given. a) Proportion of dead cells. b) Proportion of FISH detectable cells. c) Proportion of culturable cells. Note: different scales on y-axis.



## 4.5 Effect of starvation treatment on survivability

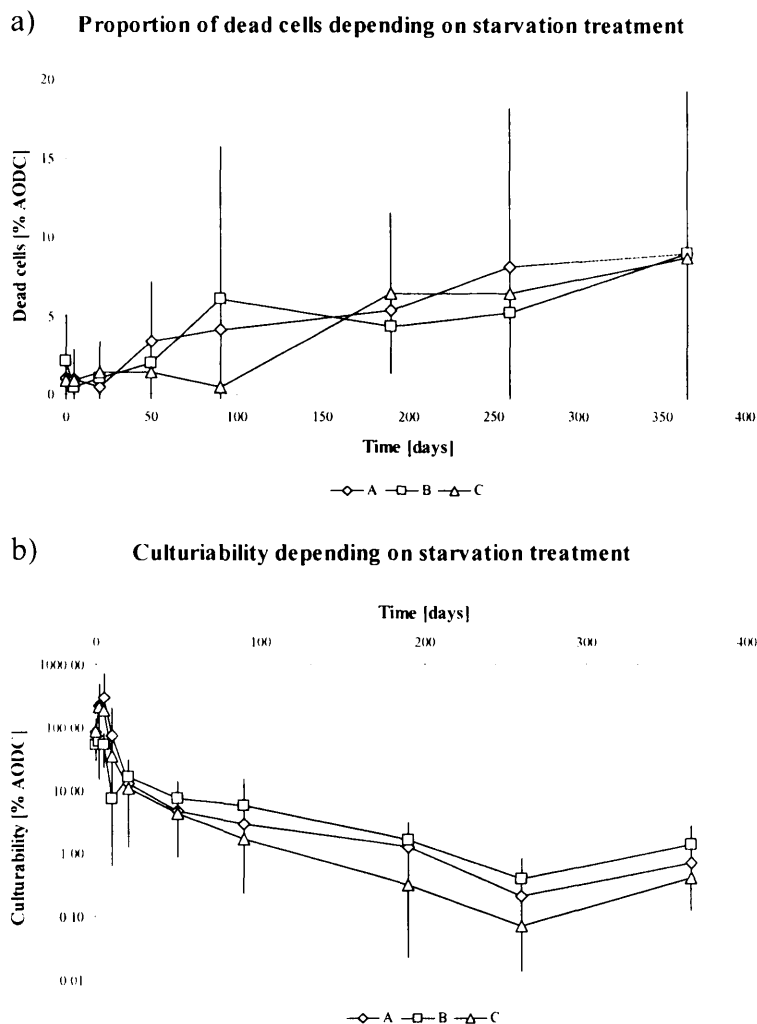
The effect of starvation on deep biosphere and near surface isolates was tested in three different ways. For treatment “A” and “B” cells were harvested after growth and resuspended in ASW. Treatment “A” represents a substrate-free incubation while “B” was supplemented with 15  $\mu$ M substrate. Treatment “C” represents the continued incubation of the original culture, which contained metabolic endproducts (e.g. VFAs, H<sub>2</sub>S) and generally exhibited a lower pH than “A” and “B” (often two pH units). In some respect, the spent medium in “C” represents the natural environment better than “A” or “B” as organic carbon is still present but inaccessible for the respective strain, however, at much higher concentrations than normally present *in situ*.

### 4.5.1 Effect of starvation treatment on percentages of dead cells

During starvation the percentages of dead cells were lower in treatment “C” than in the other two treatments. This occurred for eight strains (e.g. *Photobacterium* sp. F18I, Fig. 4.8; *Arcobacter* sp. NA105, Fig. 4.30) while the opposite only occurred for one, *Marinilactibacillus* sp. G8a3 (Fig. 4.35a). For the remaining strains and for averages of all strains (Fig. 4.56a) the chosen starvation treatments did not seem to affect the percentage of dead cells in the cultures. The variation between individual strains must, therefore, be higher than the differences caused by the three starvation treatments. A reason for this phenomenon might be that centrifugation was used for harvesting cells for treatments “A” and “B”. Maybe some strains are more susceptible to this procedure than others and therefore showed higher numbers of dead cells in “A” and “B” than in “C” but only during starvation.

### 4.5.2 Effect of starvation treatment on culturability

It was observed that starvation treatment might have an effect on culturability. After certain starvation times, some strains had higher MPNs in treatment “A” or “B” than



**Fig. 4.56: Comparison of viability measures according to starvation treatment.** Viability measures are presented according to starvation treatment and as proportion of AODC total count during starvation. 95% Confidence limits are given. a) Proportion of dead cells. b) Proportion of culturable cells. Note: different scales on y-axis.

in “C” (e.g. *Photobacterium* sp. 67TD [Fig 4.11]; *D. profundus* sp. [Fig. 4.29]; *A. malicum* [Fig. 4.32]) but the opposite also occurred (e.g. *Arcobacter* sp. F17IX [Fig. 4.31]).

When comparing average culturabilities of all strains (Fig. 4.56b) it is apparent that MPNs in treatment “B” were initially lower than in “A” and “C” but higher from 20 days onwards. Culturability in “C” was and remained lowest after 20 days until the end of the experiments. Although confidence limits are relatively high, this finding seems logical as cultures of treatment “B” were supplemented with substrate and potentially inhibitory (negative feedback effect on energetics and possible kinetics) or toxic ( $H_2S$ ) metabolic products were removed by washing in “A” and “B”. Additionally, the pH of the ASW used for resuspension was between

pH 7.2 and pH 7.4. Culturability in treatment “A” was lower than in “B” most likely due to the lack of substrate but it was higher than in “C” probably caused by the benefit of washing and optimal pH. The added substrate in “B” (~1% of glucose compared to normal concentrations for growth and ~0.1% for lactate) could have provided energy for cells to “prepare” for starvation, hence in “B”, cells began using endogenous substrates later compared to “A” and “C”, which maybe conferred higher culturability during starvation. Cells in “C” were not washed and thus the pH of the medium was lower than in “A” and “B” due to the production of VFAs during growth (sometimes below pH 7). This might have lead to additional stress, which then caused lower culturability.

#### 4.6 Effect of oxygen on starving *Photobacterium* strains

The effect of oxygen on survivability of *Photobacteria* is discussed in the Appendix.

### 5. Summary

This is the first comprehensive study of (facultatively and obligate) anaerobic deep biosphere bacteria subjected to long-term starvation. The pure-culture experiments revealed that all strains investigated showed great resilience towards substrate limitation. Changes in total cell counts occurred rarely during starvation. However, the number of dead cells slowly increased, while FISH detectability and culturability decreased with time. No significant differences were found between starvation treatments or between deep biosphere and near-surface isolates. However, some differences in LIVE/DEAD<sup>®</sup> staining and FISH occurred between certain phylogenetic groups. The majority of strains maintained viability and metabolic activity as shown in radiotracer studies even after long-term starvation.

This study highlights the survivability of environmental strains during starvation as it commonly occurs in oligotrophic environments. This leads to the conclusion that bacteria are well adapted to substrate limitation and it is thus not surprising that vast numbers of prokaryotes exist and continue to persist with increasing depth in marine deep subsurface sediments despite low organic matter availability.

## **Chapter V:**

# **Sequential heating experiments with estuarine surface sediment to investigate the effects of mimicked burial and temperature stress on prokaryotic viability**

### **1. Introduction**

Vast numbers of microorganisms are present in deep-sea sediments (Whitman *et al.*, 1998) reaching down to at least 1600 metres below the seafloor where *in situ* temperatures are between 60°C and 100°C (Roussel *et al.*, 2008). These microbes have shown to be active (Schippers *et al.*, 2005) but metabolic rates are generally low (D'Hondt *et al.*, 2002) and estimated division times exceed thousands of years (Schippers *et al.*, 2005; Coolen *et al.*, 2002). In the previous chapter the resilience of typical deep biosphere bacteria towards starvation was investigated in pure culture experiments to illuminate their survivability in deeply, buried sediments, which are mainly characterised by low energy fluxes (D'Hondt *et al.*, 2004) and low substrate availability. However, with increasing depths recalcitrant organic matter can potentially become available for microbial degradation caused by the increased temperature (Wellsbury *et al.*, 1997) and furthermore, thermogenic processes at even higher temperatures (>100 °C) might provide substrates for the deep biosphere (Parkes *et al.*, 1994; Roussel *et al.*, 2008).

In this chapter the focus lies on how microbial communities respond to increasing temperatures and what effects this stress has on their viability and culturability. To investigate this, intertidal surface sediments (top 12 cm) from the Tamar estuary (St. John's Lake, Cornwall, UK) were used in anaerobic sequential heating experiments (from 15°C to 90°C; 3°C every 2 weeks). This mimics the temperature increase during sediment burial as it occurs in deep-sea sediments. Assuming a temperature gradient of 30°C per km (Parkes *et al.*, 1994), the temperature increase during this experiment represents a burial of 2.5 km. According to sedimentation rates published by Müller and Suess (1979) the temperature increases in the sediment slurry reflect burial times between 2.8 Ma for rapidly

accumulating hemipelagic sediments (e.g. off southwest America [Peru]) and 833 Ma for slowly accumulating pelagic sediments (e.g. Central Pacific). Thus effects that temperature might have on recalcitrant organic matter and on the sedimentary microbial community over geological timescales are tremendously shortened in these experiments (343 days during this project).

Similar experiments have been conducted before but these focussed mainly on the biogeochemistry and the effect of temperature and prokaryotes on organic matter availability (Wellsbury *et al.*, 1997; Parkes *et al.*, 2007) or the effect of added minerals on microbially mediated hydrogen production (Parkes *et al.*, 2007 and 2011). The applicability and transferability of such sequential heating experiments to marine sediments has, however, been demonstrated. Parkes *et al.* (2007), for example, were able to show prokaryotic processes *in vitro* that occur *in situ* as a depth and temperature dependent succession in marine sediments (e.g. Nankai Trough sediments). These processes are sulphate reduction, shallow and deep methanogenesis, and acetate and hydrogen production (Parkes *et al.*, 2007). The production of acetate at high temperatures was also observed by Wellsbury *et al.* (1997) in sequential heating experiments and *in situ*. They concluded that the generation of acetate from heated organic matter supports the significant and widespread prokaryotic presence in hot and deep sediment layers.

This project now aimed to investigate how increasing temperatures during mimicked burial affect the viability and culturability of the microbial community from a marine near-surface sediment with the help of sequential heating experiments and how results relate to *in situ* processes in the deep biosphere.

## **2. Biogeochemistry of Tamar estuary sediments used for sequential heating experiments**

Sediment cores obtained from the Tamar estuary were stored in an upright position at 4°C until processing. For the following analyses and experiments only undisturbed cores that exhibited a clear black zonation (reduced conditions) within the sediment were selected. For details of the core sampling and how the sediment slurry was set up, please refer to Chapter II.

## 2.1 Geochemistry of the sediment

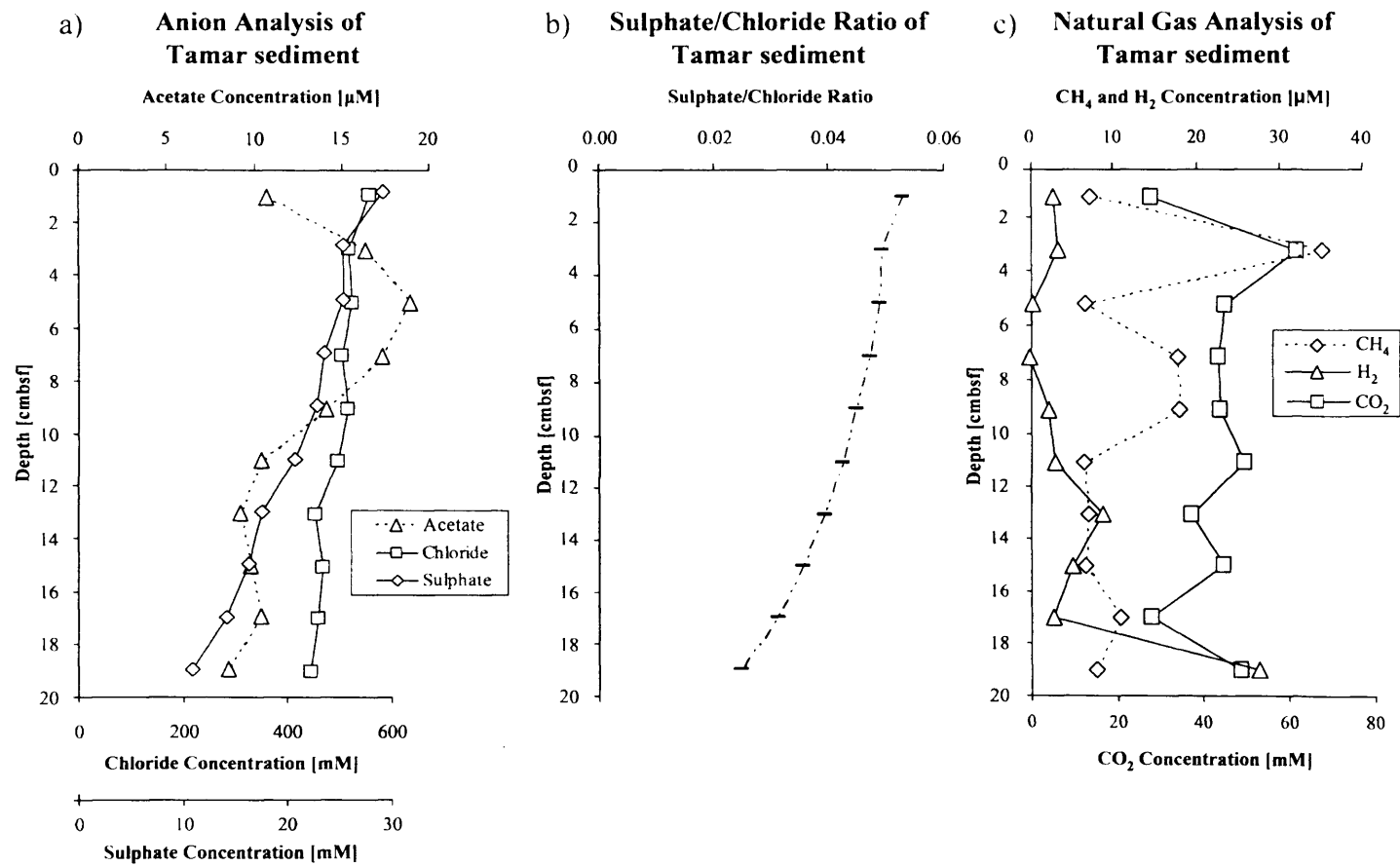
The upper 20 cm of a Tamar sediment core were investigated in regard to the geochemistry (Fig. 5.1 a-c). Chloride and sulphate concentrations decreased with depth. No zone of elevated sulphate reduction occurred but concentrations continuously decreased from 29.5 mM near the surface to 11 mM at 19 cmbsf (Fig. 5.1a). The ratio of sulphate versus chloride concentration was 0.053 near the surface and continuously decreased to 0.025 at 19 cmbsf (Fig 5.1 b). This indicates a slow but steady reduction of sulphate in the sediment column. No nitrate was detected in any of the sediment layer investigated. Acetate concentrations increased within the top five cm of the sediment from 11  $\mu$ M to 19  $\mu$ M before declining towards the bottom of the sampled sediment column to 19  $\mu$ M (Fig. 5.1a). Formate and lactate were only detected in a few layers and if present were below 10  $\mu$ M (data not shown).

Natural gas analysis revealed two local maxima of methane, one at 3 cmbsf (35  $\mu$ M) and a second one between 7 and 9 cmbsf (18  $\mu$ M; Fig. 5.1c). In other layers methane concentrations were between  $\sim$ 7 and 11  $\mu$ M. Hydrogen concentrations were also low (up to  $\sim$ 5  $\mu$ M) but elevated concentrations occurred at 13 and 19 cmbsf with 9 and 28  $\mu$ M, respectively. Carbon dioxide concentrations increased from 28 mM at the sediment surface to 62 mM at 3 cmbsf. Subsequently, they decreased to an average value of 43 mM between 5 cmbsf and the bottom of the sediment column.

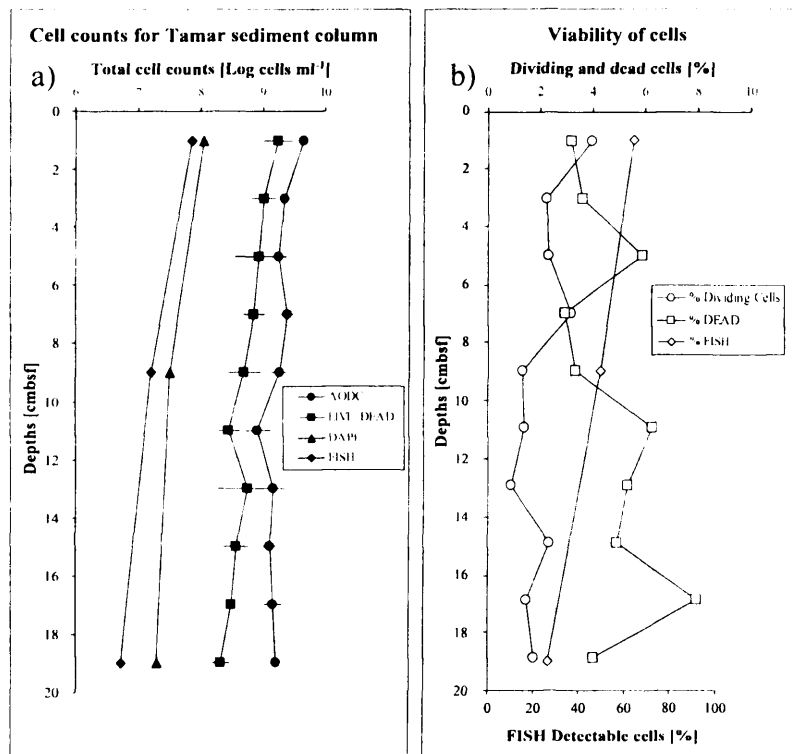
## 2.2 Cell counts of the sediment column

AODC total counts generally decreased from  $4 \times 10^9$  cells  $g^{-1}$  sediment at the surface to  $9 \times 10^8$  cells  $g^{-1}$  at 11 cmbsf. Subsequently, a slight increase towards the bottom of the analysed sediment occurred reaching  $2 \times 10^9$  cells  $g^{-1}$  (Fig. 5.2a). The percentage of dividing cells was highest near the sediment surface (4%). On average higher percentages of dividing cells were detected in the top 7 cm (3%) than below (1.6%). Generally a decrease with depths occurred (Fig. 5.2b).

Fig. 5.1: Geochemical data of Tamar sediment. Depth profiles of a) Anions concentrations; b) Sulphate/Chloride ratio; c) Natural gas concentrations.



Total cell numbers using the LIVE/DEAD<sup>®</sup> Kit showed an almost identical trend compared with AODC total counts but were consistently lower. On average  $2 \times 10^9$  cells  $g^{-1}$  were detected with AODC but only  $7 \times 10^8$  cells  $g^{-1}$  with the LIVE/DEAD<sup>®</sup> Kit. However, below 13 cmbsf, LIVE/DEAD<sup>®</sup> cell counts decreased rather than increased as occurred with AODC (Fig. 5.2a). The proportion of dead cells was relatively variable but a general increase of dead cells with depths occurred from 3% near the surface to 8% at 17 cmbsf (Fig. 5.2b).



**Fig. 5.2: Cell counts and cell viability of Tamar sediment.** Depth profiles of a) Total cell counts; b) Viability assessment of cells. Confidence limits are given for total cell counts; if not visible they fall within the width of the individual data points.

FISH was performed for three sediment layers. Total cell numbers for FISH and DAPI decreased with depth (Fig. 5.2a) as did the percentages of FISH detectable cells compared to DAPI from 64% near the surface to 27% at 19 cmbsf (Fig. 5.2b). FISH total cell counts were lower than DAPI counts and both of them were up to two orders of magnitude below AODC (Fig. 5.2a).

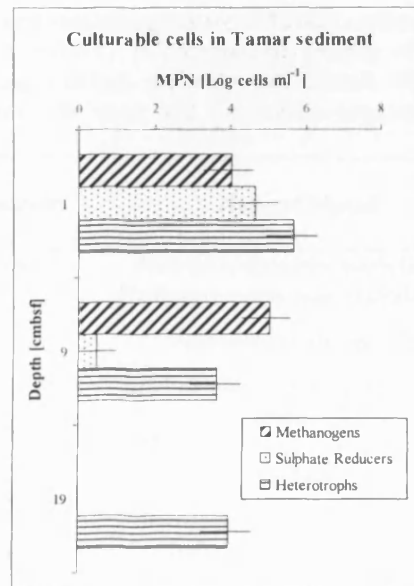
Interestingly, below 13 cmbsf AODC total counts and LIVE/DEAD<sup>®</sup> total counts showed opposing trends, while the percentages of dead cells increased in these sediment layers. This could indicate that Syto<sup>®</sup>9 does not stain all cells detected with AO. PI can not be the cause of missing cells as all cells stained with PI should



also be stained with Syto<sup>®</sup>9 and both dyes result in the red colour interpreted as dead cells. There seems to be a good correlation between proportions of dead and FISH detectable cells. While the former increased, the latter decreased.

### 2.3 Culturability of prokaryotes in Tamar sediment

The number of culturable anaerobic prokaryotes in Tamar sediment was determined for three sediment layers using the MPN dilution series method (Fig. 5.3). Three different media were used for methanogenic, sulphate-reducing (SRP), and heterotrophic prokaryotes. However, it is quite likely that other physiological groups (e.g. acetogens) were also stimulated to grow in these media and thus contribute to the determined MPNs as only growth rather than product formation was assessed. With this implication in mind subsequently, it will only be referred to these three groups in the assessment of culturability. The incubation temperature for MPNs was 15°C, which was slightly higher than on site determined *in situ* temperatures (~12°C).



**Fig. 5.3: Culturable prokaryotes in Tamar sediment.** Depth profile of culturable cell numbers for anaerobic enrichment of methanogenic, sulphate-reducing (SRP), and heterotrophic prokaryotes.

Culturable cell numbers using methanogen-medium increased between the surface ( $1.1 \times 10^4$  cells ml<sup>-1</sup>) and 9 cmbsf ( $1.1 \times 10^5$  cells ml<sup>-1</sup>) but surprisingly no growth occurred in the deepest sample (Fig. 5.3). Elevated numbers of methanogens at 9 cmbsf agree with the local maximum of methane found in this layer (Fig. 5.1c),

Growth of sulphate reducers was only present in the shallowest sample as indicated by FeS precipitation. However, growth in SRP medium also occurred in samples from 9 cmbsf but without FeS formation indicating the lack of sulphate reducers. Heterotrophs were enriched from all three layers with highest numbers in the 1 cmbsf sample ( $2 \times 10^5$  cells  $g^{-1}$ ). The cultivation efficiencies based on AODC total cell counts ranged between  $1.6 \times 10^{-7}$  % and 0.01% (Table 5.1).

**Table 5.1: Cultivation efficiencies of Tamar sediment based on AODC and FISH counts.**

Depth [cmbsf]	Medium for	Cultivation efficiency based on AODC [%]	Cultivation efficiency based on FISH [%]	FISH Detectability [%]
1	Methanogens	$2.5 \times 10^{-4}$	n.a.	64
	SRPs	$1.0 \times 10^{-3}$	0.06	
	Heterotrophs	0.01	0.6	
9	Methanogens	$5.9 \times 10^{-3}$	n.a.	50
	SRPs	$1.6 \times 10^{-7}$	$1.9 \times 10^{-5}$	
	Heterotrophs	$2.5 \times 10^{-4}$	0.03	
19	Methanogens	-	n.a.	27
	SRPs	-	-	
	Heterotrophs	$5.5 \times 10^{-4}$	0.2	

Legend: n.a.: not applicable, cultivation efficiencies of methanogenic *Archaea* based on FISH data of the eubacterial probe (EUB I) were not calculated

**Table 5.2: Isolated and enriched methanogens from Tamar sediment.** Data provided by Andrew J. Watkins (PhD Thesis, Cardiff University, in preparation), Identity of enrichment cultures determined by PCR and direct sequencing, cultures probably still contain *Bacteria*; when no other culture condition given ASW medium was used and incubation temperature was 25°C; Legend: †pure cultures.

Depth	Substrate and Culture Condition	Strain	Nearest Match	%	NCBI Accession No.
3 cm	Methylamine	TM2†	<i>Methanococcoides methylutens</i>	99%	<a href="#">FR733669.1</a>
	H <sub>2</sub> /CO <sub>2</sub>		<i>Methanosarcina mazei</i> strain LM5	95%	<a href="#">DQ987528.1</a>
	Formate		<i>Methanoculleus</i> sp. T10	99%	<a href="#">AB436897.1</a>
	Freshwater		<i>Methanobacterium subterraneum</i> strain 9-7	99%	<a href="#">DQ649330.2</a>
	Formate (38°C)		<i>Methanoplanus limicola</i> strain Mic8c07	98%	<a href="#">AB546259.1</a>
19 cm	Methylamine	TM1†	<i>Methanococcoides</i> strain Na1	99%	<a href="#">Y16946.1</a>
	H <sub>2</sub> /CO <sub>2</sub>		<i>Methanospirillum hungatei</i>	95%	<a href="#">AB517987.1</a>
	Formate		<i>Methanospirillum hungatei</i>	95%	<a href="#">AB517987.1</a>
	Freshwater		<i>Methanosarcina semesiae</i> MD1	96%	<a href="#">NR_028182.1</a>
	Acetate		<i>Methanosarcina semesiae</i> MD1	98%	<a href="#">NR_028182.1</a>
Acetate					
Freshwater					

Sediment from the Tamar estuary was also used for direct enrichment and isolation of methanogens as part of Andrew J. Watkins' PhD Thesis (Cardiff University, in preparation). Two strains of the genus *Methanococcoides* were isolated using

methylamine as a substrate. Sequences related to this species were also detected in some of the sediment layers investigated with PCR-DGGE (see below). Furthermore, a number of genera were successfully enriched using a variety of substrates and culture conditions (Table 5.2). In addition to the listed strains, a member of the genus *Methanosaeta* (based on morphology, Andrew J. Watkins, personal communication) was also obtained but this needs to be confirmed by molecular identification.

## 2.4 Molecular biological analysis of Tamar sediment

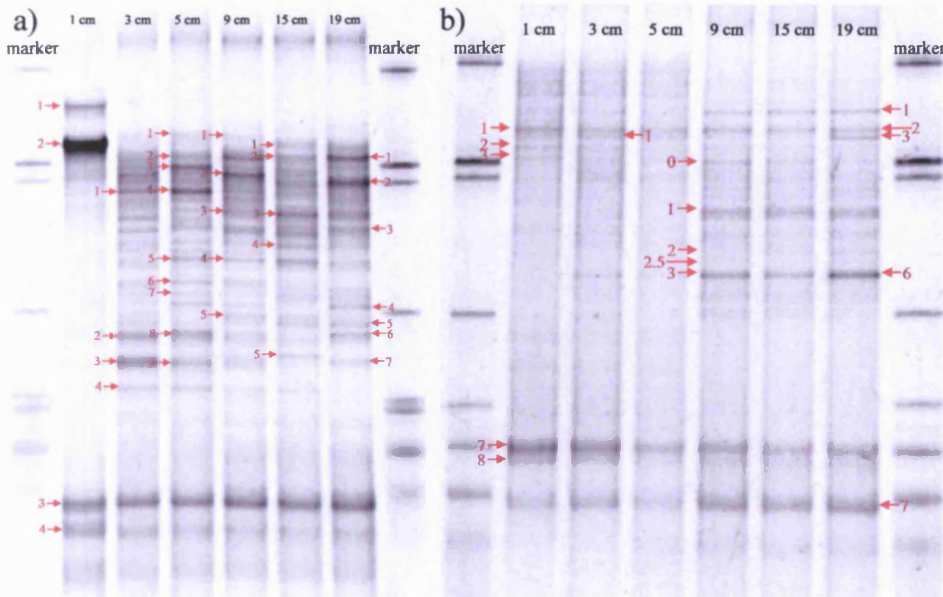
The molecular biological assessment of the microbial diversity in Tamar sediments revealed a higher diversity for *Archaea* than for *Bacteria* (Fig. 5.4).

The archaeal community composition changed between the uppermost sediment layer (1 cmbsf) and deeper ones where diversity increased (Fig. 5.4a). Many of the bands were closely related to clone sequences obtained from the marine environment (91% - 100%; Table 5.3) and cultivated relatives (82% - 100%; Table 5.3). An example for this is the genus *Methanococcoides*, strains of which were not only isolated from three and 19 cmbsf by Andrew J. Watkins (Table 5.2) but closely related sequences were also detected at three and five cmbsf (Table 5.3).

One band (A1-3) at 1cm that remained prominent throughout the sediment column potentially belongs to the recently discovered genus *Nitrosopumilus* (95%), which is an autotrophic ammonia oxidiser (Könneke *et al.*, 2005) so far only found in the marine water column. Interestingly, bands A1-1 and A1-2 also relate to this microorganism (95%) with the latter band being the most prominent in the entire sediment column. Detecting different bands of the same organism with DGGE usually indicates different sequences/alleles of the same gene (differing melting pattern due to sequence dissimilarities). It was surprising to find this for the genus *Nitrosopumilus* as for *N. maritimus* only one 16S rRNA operon per genome was reported (Walker *et al.*, 2010).

Two archaeal sequences from five and nine cmbsf matched sequences detected in a previous study by Parkes *et al.* (2011) using Tamar Estuary sediment for sequential heating experiments to investigate hydrogen production from added minerals (Bands A5-2 [92%] and A9-1 [95%]; Fig. 4.4 a and Table 4.2). This could

indicate the *in situ* activity of these microorganisms as they must have persisted in Tamar sediment for several years.



**Fig. 5.4:** DGGE profiles of Tamar sediment. a) DGGE for *Archaea*, data and profile provided by Andrew J. Watkins (PhD Thesis); b) DGGE for *Bacteria*.

The bacterial community composition remained very similar in the top five cm of the sediment but changed in deeper layers with the appearance of a number of bands (Fig. 5.4b). The upper five cm of the sediment were dominated by members of the *Cytophaga-Flavobacterium* group as well as members of the gamma- and delta-subclasses of the *Proteobacteria*. In deeper layers, however, members of the *Chloroflexi* were dominant (Fig. 4.4 and Table 4.3). Only two prominent bands, one belonging to the *Gammaproteobacteria* (B1-7, *Natronocella* sp., 93%) the other to the *Chloroflexi* (19-7, *Dehalogenimonas* sp., 92%), were present throughout the entire column.

## 2.5 Summary

The chosen sampling site in the Tamar estuary presents a typical intertidal mud flat that is relatively free from anthropogenic influences. The geochemical analyses revealed the absence of an intense sulphate reduction zone. Instead, sulphate was slowly and consistently removed. Low concentrations of methane occurred

**Table 5.3: Phylogenetic affiliation of sequenced archaeal DGGE bands.** Primers 109F and 958R were used followed by a nested amplification with primers SaFGC and PARCH519R. Bands are labelled according to sediment horizon (first digit) and number of band in that horizon (second digit; Fig. 5.4)

Depth [cmbsf]	Band	Closest affiliation: origin (NCBI accession no.)	Sequence similarity	Closest cultivated relative; origin (NCBI accession no.)	Sequence similarity
1	A1-1	Unc. Arch. Clone MF30a14; Fjord water, Canada (HQ230100)	100%	Candidatus <i>Nitrosopumilus</i> sp. NM25; coastal sand, Japan (AB546961)	95%
	A1-2	Unc. Arch. Clone MF30a14; Fjord water, Canada (HQ230100)	100%	Candidatus <i>Nitrosopumilus</i> sp. NM25; coastal sand, Japan (AB546961)	95%
	A1-3	Unc. Arch. Clone SHFC733; Antarctic seawater (GU234499)	100%	Candidatus <i>Nitrosopumilus</i> sp. NM25; coastal sand, Japan (AB546961)	95%
	A1-4	Unc. Arch. Clone livecontrolA102; Methane seep sed., Eel River Basin, USA (FJ264792)	91%	Euryarchaeote DJ3.25-13; anoxic hypolimnion, Solar Lake, Egypt (AF199376)	83%
3	A3-1	Unc. Arch. Clone HSZ-Q73; Yellow River sed., China (HQ267267)	95%	Euryarchaeote DJ3.25-13; anoxic hypolimnion, Solar Lake, Egypt (AF199376)	87%
	A3-2	Unc. Arch. Clone AMSMV-5-A48; Amsterdam mud volcano, Eastern Mediterranean Sea (HQ588654)	100%	<i>Methanococcoides methylutens</i> <sup>T</sup> ; marine Sed., USA (FR733669)	100%
	A3-3	Unc. Arch. Clone AMSMV-5-A48; Amsterdam mud volcano, Eastern Mediterranean Sea (HQ588654)	100%	<i>Methanococcoides methylutens</i> <sup>T</sup> ; marine Sed., USA (FR733669)	100%
	A3-4	Unc. Arch. Clone AMSMV-5-A48; Amsterdam mud volcano, Eastern Mediterranean Sea (HQ588654)	97%	<i>Methanococcoides methylutens</i> <sup>T</sup> ; marine Sed., USA (FR733669)	97%
5	A5-1	Unc. Arch. Clone HSZ-Q2; Yellow River sed., China (HQ267254)	97%	Euryarchaeote DJ3.25-13; anoxic hypolimnion, Solar Lake, Egypt (AF199376)	88%
	A5-2	Unc. Arch. Clone Basalt-Arch-26; Tamar sediment slurry amended with basalt and 1% Guaymas Basin sed. (FR692167)	92%	Euryarchaeote J4.75-15; anoxic hypolimnion, Solar Lake, Egypt (AF199379)	83%
	A5-3	Unc. Arch. Clone BR-TN612; Lake Pontchartrain basin, USA (EF639442)	83%	Euryarchaeote J4.75-15; anoxic hypolimnion, Solar Lake, Egypt (AF199379)	78%
	A5-4	Unc. Arch. Clone 0D_A34; subseafloor sed., Japan (AB598177)	86%	Euryarchaeote J4.75-15; anoxic hypolimnion, Solar Lake, Egypt (AF199379)	82%
	A5-5	Unc. Arch. Clone ArchSMTZ_11D; Aarhus Bay SMTZ sed. (FR695318)	99%	<i>Thermocladium modestius</i> <sup>T</sup> ; acidic hot spring, Japan (AB005296)	85%
	A5-6	Unc. Crenarch. Clone ANT84-BP; marine sed., King George Island, Antarctic Peninsula (GU969450)	100%	<i>Thermofilum</i> sp. 1505; Kamchatka hot springs, Russia (GU187356)	88%
	A5-7	Unc. Arch. Clone LKS12; profundal sed. Lake Kinneret, Israel (AJ310861)	93%	<i>Methanogenium frigidum</i> <sup>T</sup> ; water column, Ace Lake, Antarctica (FR749908)	87%
	A5-8	Unc. Arch. Clone AMSMV-5-A48; Amsterdam mud volcano, Eastern Mediterranean Sea (HQ588654)	93%	<i>Methanococcoides methylutens</i> <sup>T</sup> ; marine Sed., USA (FR733669)	93%
	A5-9	Unc. Arch. Clone AMSMV-5-A48; Amsterdam mud volcano, Eastern Mediterranean Sea (HQ588654)	100%	<i>Methanococcoides methylutens</i> <sup>T</sup> ; marine Sed., USA (FR733669)	100%

**Table 5.3 (cont.): Phylogenetic affiliation of sequenced archaeal DGGE bands.**

Depth [cmbsf]	Band	Closest affiliation; origin (NCBI accession no.)	Sequence similarity	Closest cultivated relative; origin (NCBI accession no.)	Sequence similarity
9	A9-1	Unc. Arch. Clone Basalt-Arch-38; Tamar sediment slurry amended with basalt and 1% Guaymas Basin sed. (FR692175)	95%	Euryarchaeote DJ3.25-13; anoxic hypolimnion, Solar Lake, Egypt (AF199376)	88%
	A9-2	Unc. Arch. Clone HSZ-Q2; Yellow River sed., China (HQ267254)	99%	Euryarchaeote DJ3.25-13; anoxic hypolimnion, Solar Lake, Egypt (AF199376)	89%
	A9-3	Unc. Arch. Clone MC118_29D16; marine sed., Gulf of Mexico (HM601090)	98%	Euryarchaeote J4.75-15 anoxic hypolimnion, Solar Lake, Egypt (AF199379)	89%
	A9-4	Unc. Arch. Clone ArchSMTZ_11D; Aarhus Bay SMTZ sed. (FR695318)	100%	<i>Thermocladum modestus</i> <sup>T</sup> ; acidic hot spring, Japan (AB005296)	84%
	A9-5	Unc. Arch. Clone BD72AR33; marine sed. South China Sea (GU363084)	95%	<i>Staphylothermus</i> sp. 1633; Kamchatka hot spring, Russia (GQ292555)	84%
15	A15-1	Unc. Arch. Clone V.8.ArD1; pockmark sed. Cascadia Margin (AY367341)	98%	Euryarchaeote J4.75-15 anoxic hypolimnion, Solar Lake, Egypt (AF199379)	90%
	A15-2	Unc. Arch. Clone SBAK-shallow-23; marine sed., Skan Bay, Alaska (DQ522939)	98%	Euryarchaeote DJ3.25-13; anoxic hypolimnion, Solar Lake, Egypt (AF199376)	91%
	A15-3	Unc. Arch. Clone livecontrolA102; Methane seep Sed., Eel River Basin, USA (FJ264792)	100%	Euryarchaeote J4.75-15 anoxic hypolimnion, Solar Lake, Egypt (AF199379)	89%
	A15-4	Unc. Arch. Clone SBAK-mid-45; marine sed., Skan Bay, Alaska (DQ640162)	97%	Euryarchaeote DJ3.25-13; anoxic hypolimnion, Solar Lake, Egypt (AF199376)	89%
19	A19-1	Unc. Arch. Clone SBAK-shallow-23; marine sed., Skan Bay, Alaska (DQ522939)	100%	Euryarchaeote J4.75-12; anoxic hypolimnion, Solar Lake, Egypt (AF199378)	89%
	A19-2	Unc. Arch. Clone MC118_29D16; marine sed., Gulf of Mexico (HM601090)	100%	Euryarchaeote J4.75-12; anoxic hypolimnion, Solar Lake, Egypt (AF199378)	91%
	A19-3	Unc Arch. Clone BY5_1h3b_A025; marine sed., South China Sea (HQ606169)	91%	Euryarchaeote J4.75-15 anoxic hypolimnion, Solar Lake, Egypt (AF199379)	84%
	A19-4	Unc. Arch. Clone LV-Arc055; carbonaceous sed., hypersaline lagoon, Brazil (AM943623)	98%	<i>Methanobacterium</i> sp. CM1; water samples produced in the process of oil exploration, China (HM228399)	82%

**Table 5.4: Phylogenetic affiliation of sequenced bacterial DGGE bands.** Primers 357FGC and 903R were used. Bands are labelled according to sediment horizon (first digit) and number of band in that horizon (second digit; Fig. 5.4)

Depth [cmbfs]	Band	Closest affiliation; origin (NCBI accession no.)	Sequence similarity	Closest cultivated relative; origin (NCBI accession no.)	Sequence similarity
1	B1-1	Unc. Gamma proteobact. Clone ANOX-062; marine sed., Spain (JF344624)	96%	<i>Alkalispirillum</i> sp. ACO5; Soda Lake (FJ976681)	94%
	B1-2	Unc. Delta proteobact. Clone. R103-B13; associated with vent worm (AF449229)	86%	<i>Desulfurivibrio alkaliphilus</i> AHT2; sediments from hypersaline Soda Lake (CP001940)	94%
	B1-3	Unc. Bact. Clone SW-Apr-83; seawater (HQ203953)	82%	<i>Stenothermobacter spongiae</i> UST030701; marine sponge Bahamas (DQ064789)	82%
	B1-7	Unc. Bact. Clone Bal; hydrothermal vent chimney, Juan de Fuca Ridge (FJ640808)	94%	<i>Natronocella acetimitrilica</i> ANL 6-2; Kulunda Steppe, Altai, Russia (EF103128)	93%
	B1-8	Unc. Bacteroidetes Clone E08; marine sed. Bay of Cadiz, Spain (GQ249610)	93%	<i>Cytophaga</i> sp. Km; seawater, Japan (AB540007)	92%
3	B3-1	Unc. Bact. Clone CT1; oil production water (AY266270)	87%	Epsilon proteobact. strain AN-BI3A; interface between Bannock basin hypersaline brine and seawater, Mediterranean (AM157656)	87%
9	B9-0	Unc. Bact. Clone CK_2C3_50; siliciclastic sediment from Thalassia sea grass bed, USA (EU488188)	96%	<i>Dehalogenimonas lykanthroporepellens</i> , groundwater from a waste recovery well, USA (CP002084)	94%
	B9-1	Unc. Bact. Clone 3051bac4-27; marine sed. Western Pacific (GU982824)	85%	<i>Dehalogenimonas lykanthroporepellens</i> , groundwater from a waste recovery well, USA (CP002084)	82%
	B9-2	Unc. Bact. Clone Napoli-3B-21; Napoli mud volcano, Eastern Mediterranean (AY592697)	94%	<i>Dehalococcoides</i> sp. BH180-15; enrichment cultures with <i>Alvinella pompejana</i> white tubes collected on East Pacific Rise (AJ431246)	89%
	B9-2.5	Unc. Bact. Clone Er-LAYS-47; sediment and soil slurry, Taiwan (GU180181)	93%	<i>Desulfotomaculum thermocisternum</i> ; water samples produced in the process of oil exploration, China (HM228398)	86%
	B9-3	Unc. Chloroflexi Bact. Clone 289BS; sponge (EU819028)	82%	PCB-dechlorinating bact. OTU-10; Baltimore Harbour and Elizabeth River sediments (AY559073)	79%
19	B19-1	Unc. Bact. Clone AMSMV-30-B5; Amsterdam mud volcano, Eastern Mediterranean (HQ588613)	71%	<i>Geobacter</i> sp. Ply4; subsurface environment (EF527234)	67%
	B19-3	Unc. Bact. Clone E5_10.3_2; marine sed., UK (FJ717153)	82%	<i>Sulfurovum</i> sp. 50cm25-F1; deep-sea hydrothermal field, Mid-Okinawa Trough (AB197160)	82%
	B19-6	Unc. Bact. Clone 3051bac4-27; marine sed., Western Pacific (GU982824)	98%	<i>Dehalogenimonas lykanthroporepellens</i> , groundwater from a waste recovery well, USA (CP002084)	92%
	B19-7	Unc. Bact. Clone CSBC4B06; anoxic sediment hydroelectric power plant reservoir (GU127020)	95%	<i>Dehalogenimonas lykanthroporepellens</i> , groundwater from a waste recovery well, USA (CP002084)	92%

throughout the sediment, unusually decreasing in subsurface layers. This is consistent with the potential presence of low levels of non-competitive substrates for methanogenesis (Oremland and Polcin, 1982). Indeed methylotrophic methanogens were isolated from the sediment e.g. *Methanosarcina* sp. (Table 5.2), which have been implicated with the anaerobic oxidation of methane connected to sulphate reduction (Boetius *et al.*, 2000), along with other methanogens (Table 5.2). Viable and culturable prokaryotes were present in the sediment as shown by FISH (27% - 67%) and MPN analysis ( $1.6 \times 10^{-7}$ % - 0.01% cultivation efficiencies). Although FISH was only performed using the bacterial probe EUB338I the successful enrichment and isolation of a number of methanogens in addition to the occurrence of methane shows that archaeal methanogens were present and active. Molecular analyses showed changes in the prokaryotic community composition with depths thus the following sequential heating experiments used sediment from the top 12 cmbsf spanning these different communities.

### **3. Results of sequential heating experiments**

#### **3.1 Geochemistry of the sediment slurries**

Analysis of the headspace gas revealed continuously increasing concentrations of carbon dioxide from  $1.2 \times 10^5$  ppm at the beginning to  $5.7 \times 10^5$  ppm after 434 days of incubation (Fig. 5.5 a-b). This was either caused by microbial activity as CO<sub>2</sub> is one of the main products of many metabolic pathways or physico-chemical reasons. With increasing temperature, less CO<sub>2</sub> is dissolved in the medium and more is released into the headspace (Weiss, 1974). Other parameters such as salinity (here constant) and pressure have an effect on the solubility of gases (Millero, 2007). Pressure was variable throughout the experiment, generally increasing with temperature. Sampling lead to the decrease of pressure and additionally it was purposely released to prevent breakage of the experimental glass vessel (which had happened prior to the experiments when testing vessels for air-tightness). Due to these constantly changing parameters concentrations of gases were not transformed into  $\mu\text{mol}$  or  $\text{mmol}$  but are given as ppm of the headspace.



The concentrations of C<sub>5+</sub> compounds and hydrogen also increased with time and temperature (from 2 to 75 ppm for C<sub>5+</sub> and from 6 to 243 ppm for H<sub>2</sub>). In slurry I, a local maximum of hydrogen (~1300 ppm; Fig. 5.5a) occurred at around 370 days of incubation (75°C - 78°C). One source of hydrogen is the aromatisation of organic compounds which leads to its release especially when temperatures are above 70°C (Parkes *et al.*, 2009). However, hydrogen is also an excellent electron donor and some hydrogen could have been utilised by anaerobic prokaryotes including methanogens, sulphate reducers or autotrophic acetogens.

The initial increase of methane (Fig. 5.5 a-b) was followed by an almost linear decrease towards the end of the experiment. This can probably be attributed to the sampling of the experimental vessels and a concomitantly slow dilution of the methane. However, both methanogenesis and methanotrophy were either absent or happened at similar rates thereby not causing significant changes in headspace methane concentrations. The initial increase of methane can be explained by its presence in Tamar sediment (see Fig. 5.1c) and its release after initial sampling of the slurry. Theoretically, approximately  $3.7 \times 10^3$  ppm methane could have been released from the Tamar sediment while  $4.2 \times 10^2$  to  $1.2 \times 10^3$  ppm methane were initially detected in the headspace indicating a loss of methane during transfer of sediment into the vessels.

The concentrations of VFAs remained relatively low at lower temperatures (< ~60°C; Fig. 5.5 c-d). Occasionally local maxima of individual organic acids were detected (e.g. lactate after 224 days [45°C] of incubation). Above 60°C, however, increasing concentrations of acetate and formate occurred, which has been reported by Wellsbury *et al.* (1997) and Parkes and Sass (2009). Acetate, however, could have also been produced by autotrophic acetogens using the hydrogen released by aromatisation or cell lysis at higher temperatures.

Sulphate was present in pore waters throughout the experiment and only decreased slowly by approximately 7 mM. This is consistent with the slow sulphate removal with sediment depth in the original Tamar sediment (Fig. 5.1a). No enhanced sulphate reduction and depletion occurred as previously reported by Parkes *et al.* (2007 and 2011). This may have been due to the absence of added thermo- and hyperthermophiles (Parkes *et al.*, 2011). The original mesophilic population was

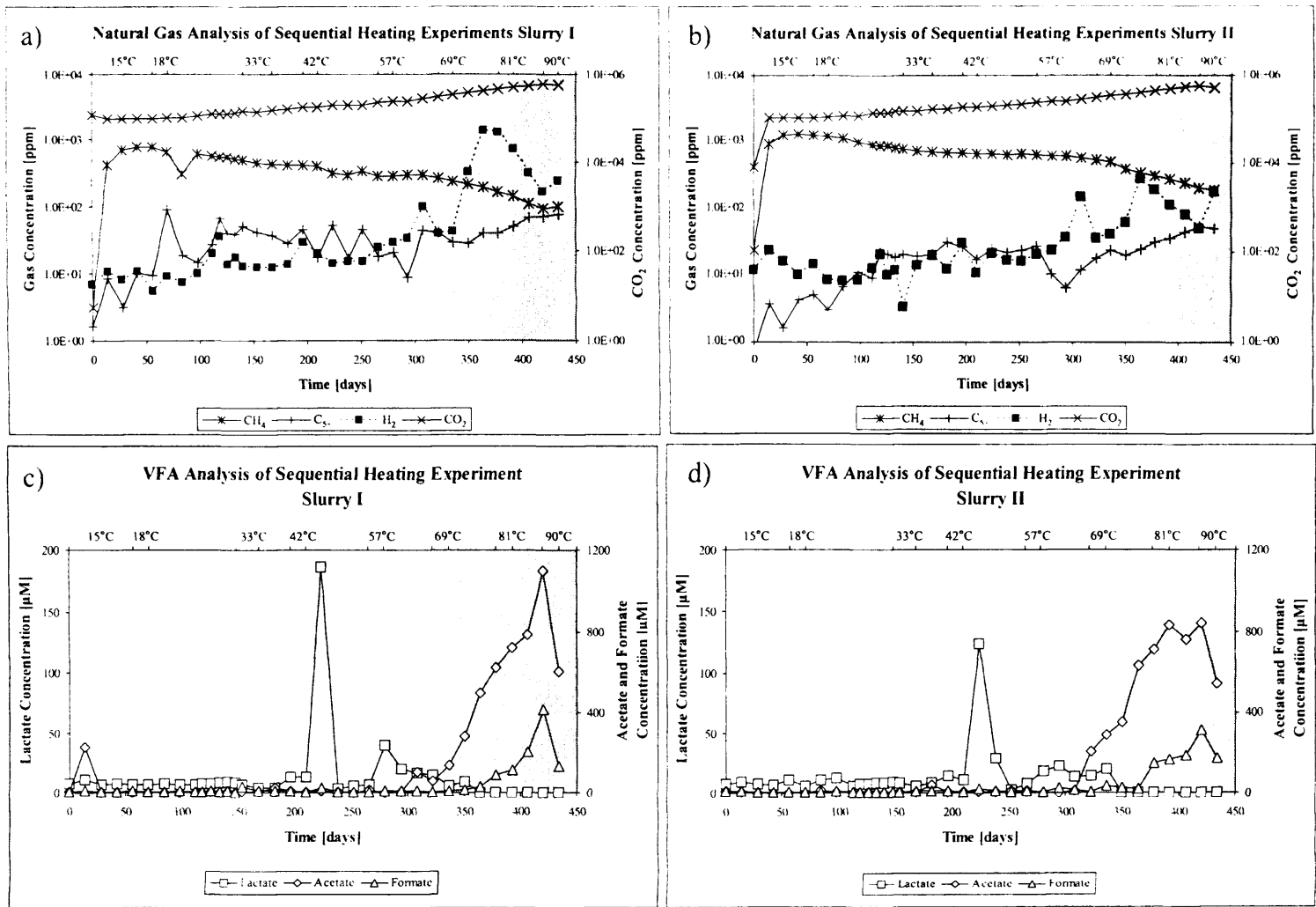


Fig. 5.5: Geochemical data of sequential heating experiments. a) NGA analysis of slurry I; b) NGA analysis of slurry II; c) VFA analysis of slurry I; d) VFA analysis of slurry II

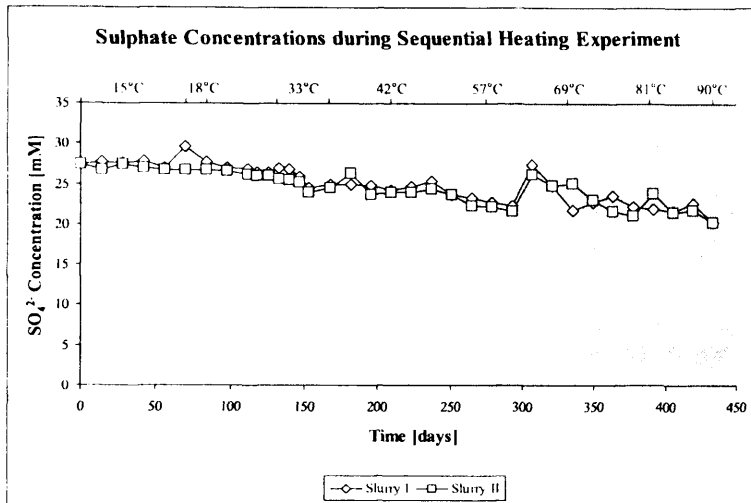


Fig. 5.6: Sulphate concentrations during sequential heating.

probably initially substrate-limited and subsequently stressed at higher temperatures, which is reflected in changes in viability (see below).

The geochemistry in the two parallel sequential heating experiments is very comparable. This reproducibility indicates that the microbial communities in both slurries are exposed to very similar conditions and that subsequent results from one parallel incubation can be assumed for the other.

### 3.2 Cell counts during sequential heating experiment

AODC total counts increased from  $1.2 \times 10^8$  cells  $\text{ml}^{-1}$  at the beginning to 212% after 210 days of incubation (42°C; Fig. 5.7). This was followed by a slow, linear decrease until 81°C (37% of initial count after 392 days) and a steep decrease afterwards (2% of initial count after 434 days). The estimation of total cell numbers using the LIVE/DEAD<sup>®</sup> Kit was only possible for the first two samplings (both 15°C). A decrease in LIVE/DEAD<sup>®</sup> total cell numbers (from  $9.4 \times 10^7$  to  $7.2 \times 10^7$  cells  $\text{ml}^{-1}$ ) and in the percentage of dead cells (from 10% to 5%) was observed for these two timepoints. The former opposes the trend observed for AODC total counts while the latter opposes the trend of FISH detectability (see below). Subsequently, the LIVE/DEAD<sup>®</sup> procedure did not result in any analysable stainings (Fig. 5.8b). The problem was that neither cells nor sediment particles were visible potentially due to

unspecific binding of the dyes possibly to reactive organic matter (aromatic compounds) in the sediment slurry making it impossible to see or count any cells.

The percentage of FISH detectable cells was relatively high (79%) at the onset of the experiment, exceeding the detectability in the original sediment column (27% - 64%, Fig. 5.2b). Within the first 56 days of incubation during which no increase in temperature was applied the FISH detectability decreased dramatically to only 32% opposing the significant increase of total cell counts during this time. Subsequently, FISH detectability decreased more slowly until 69°C (26%) and then more rapidly at higher temperatures down to 7% in the final sample (90°C; Fig. 5.7). Total cell counts for LIVE/DEAD<sup>®</sup>, DAPI and FISH remained below those obtained with AODC except for DAPI at the last sampling. However, FISH and DAPI cell counts generally followed the trend observed with AODC.

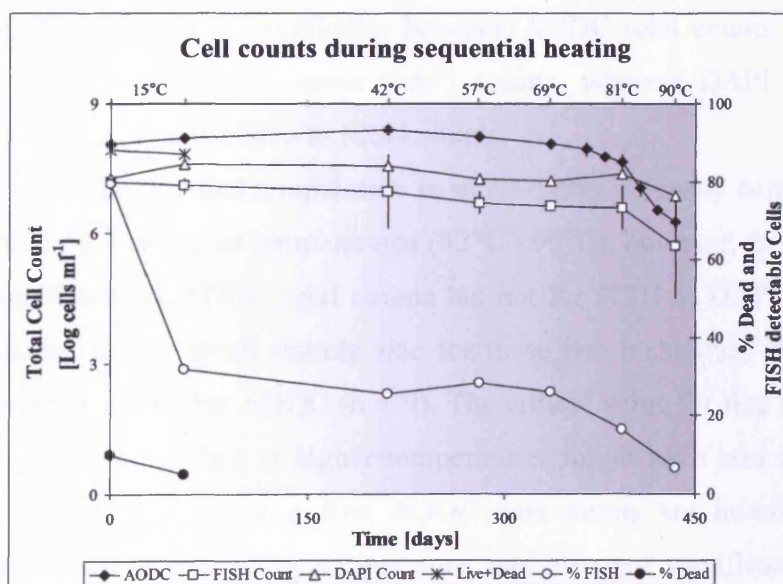


Fig. 5.7: Cell counts during sequential heating.

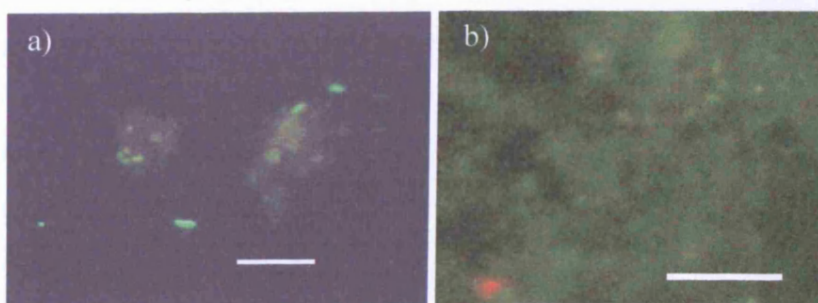


Fig. 5.8: LIVE/DEAD<sup>®</sup> staining of sedimentary prokaryotes. a) Tamar core, 19 cmbsf; b) Slurry experiment after 339 days (69°C). While cells and particles are visible in a) no cells or particles could be identified at higher temperatures in the heating experiment b).

Pearson's correlation coefficient was used to correlate increasing temperatures with cell counts (log data used) and cell counts with one another (Table 5.5). If the entire temperature range was taken into account a significant, inverse correlation was found between increasing temperatures versus AODC total counts ( $n = 11$ ; critical value 0.6) and versus FISH counts ( $n = 7$ ; critical value = 0.7) but not versus DAPI counts. Furthermore, significant positive correlations were present between AODC total count versus FISH and DAPI counts and between FISH versus DAPI ( $n = 7$ ; critical value = 0.7). If only data at higher temperatures (42°C - 90°C; Table 5.5) was investigated a significant, inverse correlation between temperature and cell counts was only found for AODC ( $n = 9$ ; critical value = 0.67). FISH and DAPI counts were not significantly correlated but calculated values were close to the critical value in these cases ( $n = 5$ , critical value = 0.88; Table 5.5).

Again a significant, positive correlation between AODC total counts versus FISH counts was observed, but not versus DAPI counts, whereas DAPI counts were significantly, positively correlated to FISH counts.

It is not surprising that temperature is significantly inversely correlated to cell counts (Table 5.5). For higher temperatures (42°C - 90°C), however, this relationship was only significant for AODC total counts but not for FISH or DAPI counts. This can be explained by the small sample size for these two techniques ( $n = 5$ ) whilst more data were available for AODC ( $n = 9$ ). The critical value for five data points is relatively high and more data at higher temperatures might have lead to a different conclusion. If the corresponding five AODC data points are investigated, their inverse relationship to increasing temperature was also not significant (calculated value 0.85; critical value 0.88), which supports the need for more FISH and DAPI data to draw definite conclusions.

Observed significant, positive correlations between cell counts were to be expected. It seems logical that with more cells present (AODC) more cells can be detected with other methods (FISH and DAPI). The same applies to DAPI versus FISH. If more cells are present on the hybridisation filter (DAPI), more cells will be detected using FISH.

When plotting FISH and DAPI counts versus AODC total counts the relationships can be visualised (Fig. 5.9). While AODC total counts increased for the first 210

days (by 212% until 42°C), DAPI only increased until 56 days (also by 212%; 15°C) and decreased afterwards (Fig. 5.9). From 210 days (42°C) onwards both counts continuously decreased. Only DAPI showed a local maximum at 392 days (81°C,  $2.4 \times 10^7$  cells ml<sup>-1</sup>). Despite these fluctuations, DAPI counts are significantly correlated with AODC counts (entire temperature range tested; Table 5.5).

**Table 5.5: Pearson's correlations coefficients for cell counts and temperature.** Significance of correlations were calculated for two different temperature ranges using Pearson's correlation coefficient ( $p < 0.05$ ). Significant positive (+) or inverse (i) correlations are highlighted in bold. Note that for comparison of temperature versus AODC more data were available (11 datapoints for entire range and 9 for 42°C - 90°C) than for other comparisons.

15°C – 90°C	Temperature	AODC	FISH	DAPI
Temperature	–	<b>0.68 (i)</b>	<b>0.87 (i)</b>	0.66 (i)
AODC		–	<b>0.91 (+)</b>	<b>0.82 (+)</b>
FISH			–	<b>0.84 (+)</b>
DAPI				–
42°C – 90°C	Temperature	AODC	FISH	DAPI
Temperature	–	<b>0.83 (i)</b>	0.84 (i)	0.77 (i)
AODC		–	<b>0.98 (+)</b>	0.85 (+)
FISH			–	<b>0.94 (+)</b>
DAPI				–

FISH counts showed less variability than DAPI counts and generally decreased throughout the experiment (Fig. 5.9). Therefore, they are significantly, inversely correlated with temperature (Table 5.5) and significantly, positively correlated with AODC total counts (Table 5.5).

It is noteworthy that between the beginning of the experiment and the first sampling (both timepoints at 15°C) a slight decrease in FISH counts (from  $1.5 \times 10^7$  to  $1.3 \times 10^7$  cells ml<sup>-1</sup>) but a big increase in the DAPI counts (from  $1.9 \times 10^7$  to  $4.1 \times 10^7$  cells ml<sup>-1</sup>) occurred (Fig. 5.9). This leads to the apparent loss of FISH detectability (from 80% to 32%; Fig. 5.6). If FISH detectability is calculated based on AODC total counts, it decreases from 13% at the beginning of the experiment to 8% after 56 days (both 15°C; Fig. 5.10).

Interestingly, when calculating the proportions of FISH and DAPI counts from AODC total counts, the detectabilities increase towards the end of the heating experiment. This is caused by a bigger loss of AODC total counts than observed with either FISH or DAPI (Fig. 5.7 and 5.10). DAPI even exceeds the AODC total count at the final sampling (almost 400% of AODC). FISH detectability based on DAPI, however, follows the expected pattern and shows a loss of viability with increasing temperature as the ADOC total counts themselves suggest (Fig. 5.6).

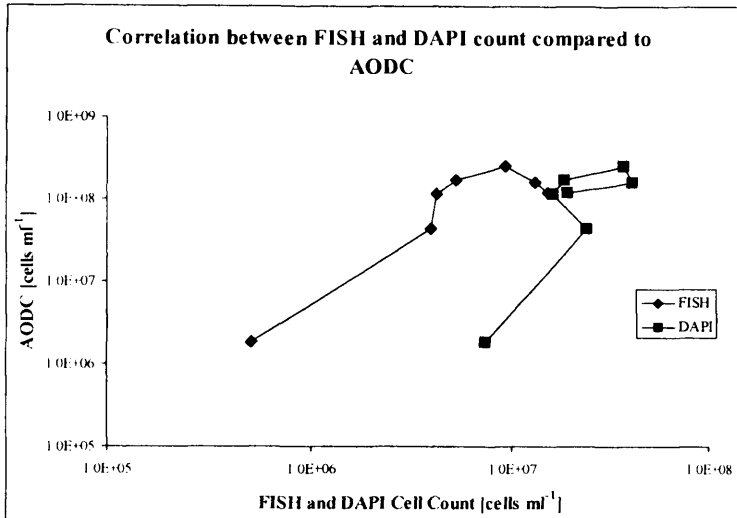


Fig. 5.9: Correlation between FISH and DAPI counts with AODC.

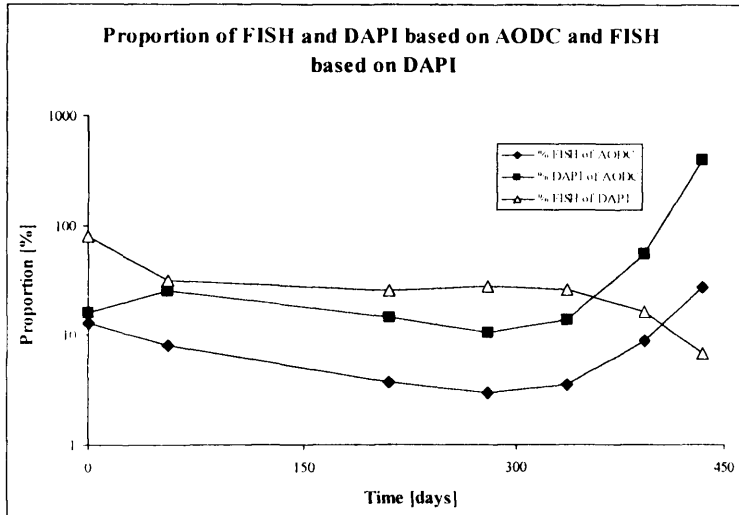


Fig. 5.10: Proportions of FISH and DAPI detectable cells based on AODC total counts and proportion of FISH detectable cells based on DAPI counts.

### 3.3 Culturability during sequential heating

The culturability of certain physiological groups was investigated over the increasing temperature range. As for the original Tamar sediment, three different media were used to stimulate growth in methanogenic, sulphate-reducing (SRP), and heterotrophic prokaryotes. Additionally, with increasing temperature, parallel MPN plates were incubated at not only the current *in situ* temperature of the slurry but also previous sampling temperatures resulting in culturability matrices for the three groups (Fig. 5.15 a-c). First, however, culturability profiles of specific temperatures and/or of interesting incubation timepoints were selected and are presented.

### 3.3.1 Culturability at selected temperatures

#### a) Culturability at respective *in situ* slurry temperature

Culturability of all three metabolic groups increased between the first and second sampling (both 15°C but 8 weeks apart, Fig. 5.13 a) by almost one order of magnitude for heterotrophs (~730%), more than one order for SRP (~2,200%), and three orders for methanogens (~200,000%). This increase was potentially caused by the homogenisation of the sediment during the setup of the slurry. Micro-environments were removed and electron donors and/or acceptors might have suddenly become available for microbes, which were limited in the respective layers of the original sediment column. However, during the same time a loss of FISH detectable cells (Fig. 5.6) occurred contradicting this increase in culturability.

Subsequently, with increasing temperature culturability decreased. Between 15°C and 42°C culturable cell numbers for heterotrophs decreased by 90%, for SRPs by 37%, and for methanogens by 94%. This directly opposes the trend observed for AODC total counts but agrees with slightly decreasing FISH detectability between these two temperatures (Fig. 5.6). It indicates, however, that only a small proportion of the population targeted with these three media is adapted to higher temperatures.

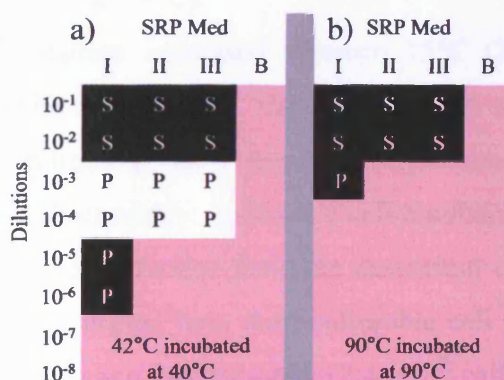
Unfortunately, the next three MPN incubations (at 57°C 69°C, and 81°C) oxidised during incubation. However, culturable cells were still detected in MPN plates from the highest temperature (90°C) incubated at 90°C (Fig. 5.13a) albeit since 42°C a further loss of culturability occurred. Merely 0.2% of heterotrophic, 0.3% of SRP, and 1.7% of methanogenic culturable cell numbers were detected at 90°C compared to 42°C incubations. It is left to speculation whether these cultured cells were survivors or maybe germinated spores of hyperthermophiles. The decrease in culturability fits to the decreasing AODC total counts and the further reduced FISH detectability (Fig. 5.6) at higher temperatures.

The media used target certain physiological groups but it is likely that others were also stimulated to grow. This was frequently observed for SRP medium. In the two incubations at 15°C, for example, the total MPN counts were  $1.1 \times 10^4$  (at  $t_0$ ) and  $2.4 \times 10^5$  cells ml<sup>-1</sup> (at 56 days) but H<sub>2</sub>S formation indicated by FeS precipitation only occurred in a few wells. Basing MPN calculations on this information leads to “true”



culturable cell numbers for SRPs. These are slightly lower with  $4.6 \times 10^3$  and  $1.1 \times 10^5$  cells  $\text{ml}^{-1}$ , respectively. For  $42^\circ\text{C}$  no FeS formation occurred whereas for  $90^\circ\text{C}$  the “true” SRP cell number is equal to the total number determined (data not shown).

There is however, a slight problem with the interpretation of the data in this way. Due to the high content of reduced, black sediment in the first two dilution steps it could not be distinguished between newly formed FeS and the background colour. Thus if higher dilutions (e.g. the  $10^{-3}$  dilution) showed FeS precipitation it was assumed that this also occurred in the less diluted, sediment-containing wells ( $10^{-1}$  and  $10^{-2}$ ; Fig. 5.11b) and culturable cell numbers were calculated accordingly. This is justifiable as a certain number of the same cell type (strain) must have been present in the original sample for it to be able to grow in the higher dilutions. This approach, however, could also lead to an overestimation of actual SRP numbers if, for example, FeS precipitations only occurred in higher dilutions while intermittent wells that are positive for growth are FeS-negative (Fig. 5.11a). A possible explanation for this is that some cells (maybe attached to sediment particles) could have just been transferred from one dilution step to the next despite using new pipette tips each time and thus respective wells might represent outliers.



**Fig. 5.11: Schematic representation of two MPN results for SRP medium.** One MPN dilution series consists of three parallels (I-III) and the blank (B). Legend: “S” on black background: sediment-containing well; “P” on white background: cell pellet without FeS precipitation; “P” on a black background: cell pellet with FeS formation; grey wells: no activity or growth observed. a) MPN result of  $42^\circ\text{C}$  sample incubated at  $40^\circ\text{C}$ . b) MPN result of  $90^\circ\text{C}$  incubated at  $90^\circ\text{C}$ .

From the final MPN plate (samples from  $90^\circ\text{C}$  incubated at  $90^\circ\text{C}$ ) subsamples were taken from the most dilute positive MPN wells of the SRP and heterotrophic dilution series and transferred into fresh medium and incubated at  $90^\circ\text{C}$ . After four months of incubation growth only occurred in the heterotrophic incubation and was analysed

microscopically. This revealed a relatively homogenous culture of straight, long rods (Fig 5.12).



**Fig. 5.12:** Cells grown in a subculture of a heterotrophic MPN series after sequential heating to 90°C. All incubation temperatures were 90°C.

b) Culturability in a temperature range after final heating to 90°C

Samples from the final timepoint of the sediment slurry heating experiment (two weeks at 90°C) were used for the determination of culturability and incubated over a temperature range (Fig. 5.13b). Growth occurred at all temperatures except at 81°C due to oxidation of the MPN plate. Generally with increasing temperature (up to 69°C) increasing numbers of culturable cells occurred for methanogens (from 43 cells ml<sup>-1</sup> at 15°C to 2.4×10<sup>3</sup> cells ml<sup>-1</sup> at 69°C) and heterotrophs (from 93 cells ml<sup>-1</sup> at 15°C to 4.6×10<sup>3</sup> cells ml<sup>-1</sup> at 69°C).

MPNs in SRP medium increased between 15°C (2.4×10<sup>2</sup> cells ml<sup>-1</sup>) to a maximum of 2.4×10<sup>5</sup> cells ml<sup>-1</sup> at 40°C. However, no FeS precipitation occurred in these two incubations indicating that other microorganisms than sulphate-reducers must have grown here. Subsequently, culturable cell numbers in SRP medium slowly decreased with increasing temperatures from the maximum value at 40°C to 4.6 ×10<sup>2</sup> cells ml<sup>-1</sup> at 90°C but were higher here than culturable cell numbers determined for methanogens (~8 cells ml<sup>-1</sup>) and heterotrophs (2.4 ×10<sup>2</sup> cells ml<sup>-1</sup>; Fig. 5.13b). FeS precipitates, however, were only present at 57°C and 90°C. If these are used for MPN calculations the “true” number of culturable sulphate reducers is one order of magnitude lower than the total number of cells stimulated to grow in SRP medium at 57°C (2.3×10<sup>3</sup> versus 1.5×10<sup>4</sup> cells ml<sup>-1</sup>) but the same at 90°C. The same data are presented twice for samples from 90°C incubated at 90°C in Fig. 5.13a and b.

Cell numbers for methanogens and heterotrophs were highest in incubations at 69°C with 2.4×10<sup>3</sup> and 4.6×10<sup>3</sup> cells ml<sup>-1</sup>, respectively (Fig. 5.13b). MPN plates incubated at 90°C also showed growth and calculated MPNs were comparable to

other temperatures (e.g. 15°C for heterotrophs; 15°C and 69°C for SRPs). Methanogen MPNs, however, were lowest at 90°C.

It is worth noting that after the sedimentary microbial community had been stressed by increasing temperatures up to 90°C culturable cells still occurred at psychrophilic and mesophilic temperatures. In addition, culturable cell numbers in SRP medium (although not necessarily sulphate reducers) were higher than in any other medium at all temperatures (except at 69°C). Once more the question arises whether cultivated cells at 40°C were able to withstand the high temperatures or if spores of psychrophiles and mesophiles were revived.

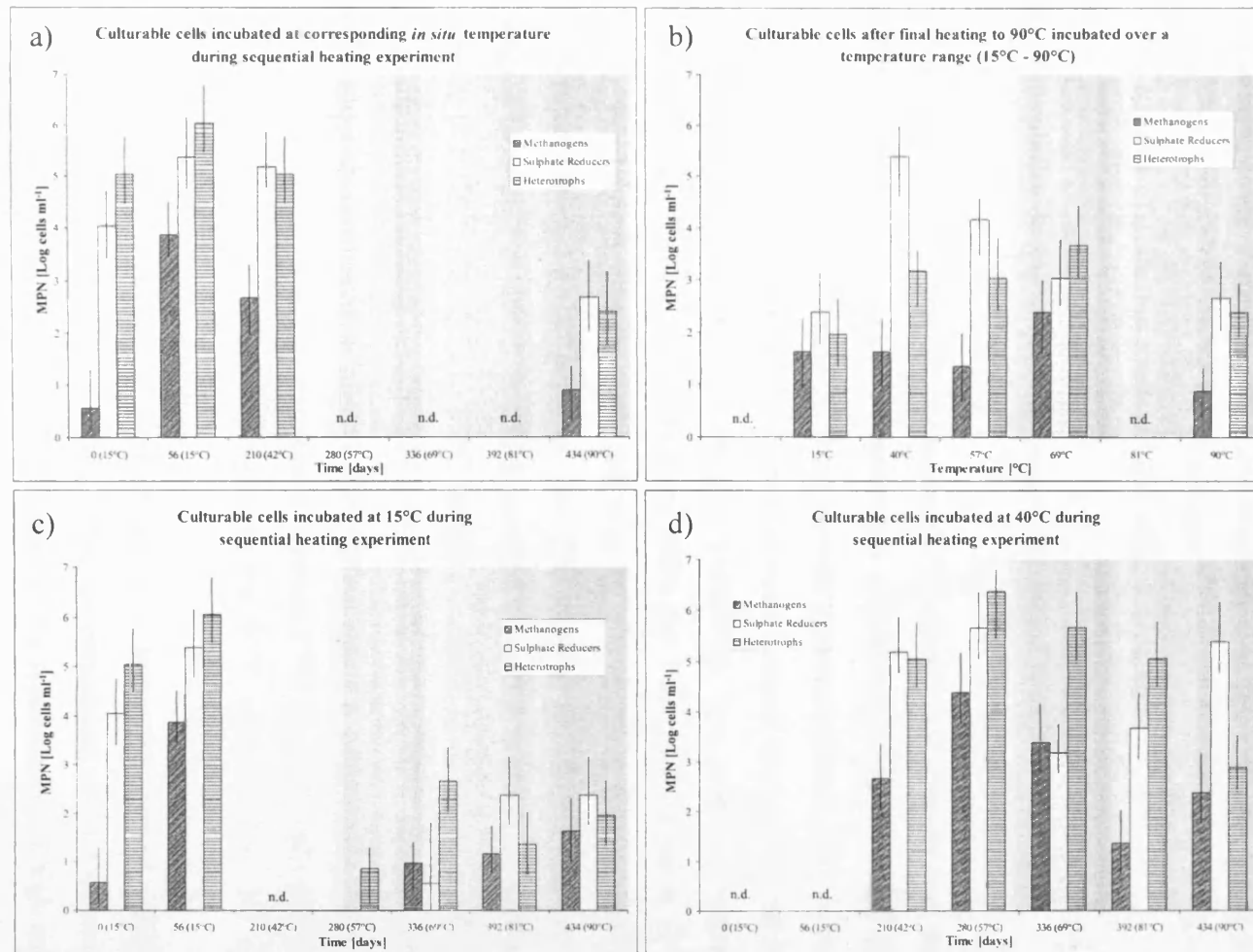
### c) Culturability during sequential heating incubated at 15°C

The temperature of the originally sampled Tamar sediment was measured at around 12°C, thus MPN incubations at 15°C were near to *in situ* conditions (Fig. 5.13c). Culturability of prokaryotes at 15°C increased within the first 56 days of incubation without heating (for the first two timepoints same data as for respective *in situ* temperatures [Fig. 5.13a] is presented and thus not described again).

Unfortunately, the MPN plate for 42°C samples incubated at 15°C oxidised, thus no data are available here. Between 56 days (15°C) and 280 days (57°C) a complete loss of culturability occurred for methanogens and SRPs. Culturability in heterotrophic MPNs incubated at 15°C decreased from  $1.1 \times 10^6$  cells ml<sup>-1</sup> to only 7 cells ml<sup>-1</sup> during this time and temperature span.

Subsequently culturability recovered. The number of culturable methanogens increased from 9 cells ml<sup>-1</sup> in samples from 69°C to 43 cells ml<sup>-1</sup> in samples from 90°C slurry temperature. The number of SRPs increased from 4 cells ml<sup>-1</sup> in 69°C samples to  $2.4 \times 10^2$  cells ml<sup>-1</sup> in 81°C samples and remained at this level for 90°C samples (no FeS precipitates in either). In contrast, culturability in heterotrophs varied for these three samples between  $2.3 \times 10^1$  cells ml<sup>-1</sup> and  $4.6 \times 10^2$  cells ml<sup>-1</sup>. The MPNs of the final 90°C sample are the same data as shown in Fig. 5.13b where 90°C samples were incubated over the temperature range.

The decrease of culturability in the three metabolic groups between 15°C and 57°C shows either the disappearance of psychrophilic microorganisms or their loss of viability and culturability. However, culturability in samples from 69°C to 90°C



**Fig. 5.13: Prokaryotic culturability during sequential heating.** a) Culturability during sequential heating at respective *in situ* sampling temperatures; b) Culturability from final sampling (90°C) over the temperature range; c) Culturability during sequential heating incubated at 15°C; d) Culturability during sequential heating incubated at 40°C. n.d.: no data, due to oxidation of the MPN plate during incubation. Note that some of the data are presented more than once, in a) and c) 15°C at 0 and 56 days and in a) and b) 90°C at 434 days

incubated at 15°C was still relatively high indicating the potential presence of prokaryotes able to withstand thermophilic temperatures whilst being able to grow at psychrophilic temperatures (15°C). An explanation for this phenomenon could be that spores of psychrophiles were revived whilst non-spore-forming psychrophiles died at higher temperatures and a change of the original population occurred (similar to pasteurisation). Furthermore, it is intriguing that for the three target groups the number of culturable cells in 90°C samples incubated at 15°C (Fig. 5.12c) is similar to the number of cells that grew at 90°C (Fig. 5.13 a-b).

d) Culturability during sequential heating incubated at 40°C

Culturability in MPN plates incubated at 40°C was generally higher than those found at 15°C (up to four orders of magnitude in certain cases; Fig. 4.9 c-d). The data of 42°C samples incubated at 40°C were already presented (Fig. 5.12a). In incubations at 40°C an increase in MPNs occurred between samples from 42°C and 57°C (two orders of magnitude for methanogens [ $\sim 5200\%$ ], one order of magnitude for heterotrophs [ $\sim 2200\%$ ], and  $\sim 300\%$  for SRPs [no FeS precipitation at 57°C]) (Fig. 5.12d). For heterotrophs this was followed by a continuous decline with increasing temperatures from  $2.4 \times 10^6$  cells ml<sup>-1</sup> in 57°C samples to  $7.5 \times 10^2$  cells ml<sup>-1</sup> in 90°C samples incubated at 40°C. For SRP medium a sudden decrease of MPNs in 57°C samples ( $4.6 \times 10^5$  cells ml<sup>-1</sup>) to 69°C samples occurred ( $1.5 \times 10^3$  cells ml<sup>-1</sup>) followed by a continuous increase resulting in  $2.4 \times 10^5$  cells ml<sup>-1</sup> in 90°C samples incubated at 40°C (no FeS formation in either). For methanogens a comparable trend occurred. First culturable cell numbers decreased between 57°C ( $2.4 \times 10^4$  cells ml<sup>-1</sup>) and 81°C (23 cells ml<sup>-1</sup>) before increasing again in the 90°C samples ( $2.4 \times 10^2$  cells ml<sup>-1</sup>) incubated at 40°C.

Again MPNs from samples of the 90°C timepoint showed relatively high numbers of culturable cells indicating either the persistence of microorganisms from a cold environment at elevated temperatures or the resuscitation of high-temperature spores. However, MPNs from 90°C samples incubated at 40°C were higher than those incubated at 15°C and 90°C, particularly for SRP medium despite the apparent absence of sulphate-reducing activity (Table 5.6).

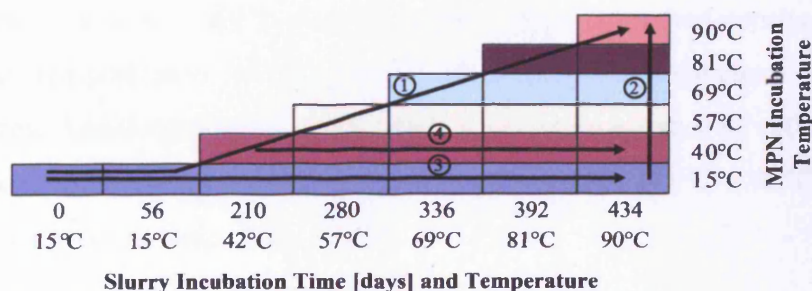


**Table 5.6: Comparison of culturable cells of 90°C samples incubated at different temperatures.**

90°C samples at selected temperatures	Methanogens [cells ml <sup>-1</sup> ]	SRP [cells ml <sup>-1</sup> ]	Heterotrophs [cells ml <sup>-1</sup> ]
15 °C	43	240	93
40 °C	240	240,000	750
90 °C	78	460	240

### 3.3.2 Culturability matrices of sequential heating experiments

The results presented above represent the majority of the culturability data gathered during the sequential heating experiment. In this section, however, all the data for target groups will be shown individually in culturability matrices for a coherent overview. These matrices consist of an x-axis showing the slurry incubation time and temperature. The y-axis shows the MPN incubation temperature while the z-axis represents the number of cultured cells. A schematic diagram is shown first (Fig. 5.13) to help understand what the theoretical matrix looks like (z-axis is omitted). Unfortunately, due to the oxidation of several MPN plates (e.g. at 81°C) during incubation the actual matrices are incomplete. Nonetheless, general trends are described.



**Fig. 5.14: Schematic diagram of culturability matrix.** For description refer to text. Arrows indicate previously shown data for culturability 1) at corresponding *in situ* temperature (Fig. 2.13a); 2) after final heating incubated over temperature range (Fig. 5.13b); 3) at 15°C (Fig. 5.13c); 4) at 40°C (Fig. 5.13d).

#### a) Culturability matrix for methanogens

Culturability of methanogens ranged from  $< 3$  cells ml<sup>-1</sup> (detection limit according to tables of de Man (1983) if no growth occurs in any of the three parallels) to  $2.4 \times 10^4$  cells ml<sup>-1</sup> (Fig. 5.15a) throughout sequential heating. The only incubation where no growth ( $< 3$  cells ml<sup>-1</sup>) occurred was in 57°C samples incubated at 15°C. The highest number of culturable cells was found in 57°C samples incubated at 40°C. However, relatively high numbers also occurred for 15°C (56 days) samples incubated at 15 °C

( $7.5 \times 10^3$  cells ml<sup>-1</sup>), for 42°C samples incubated at 40°C ( $4.6 \times 10^2$  cells ml<sup>-1</sup>), and for 69°C samples incubated at 40°C and 57°C (each  $2.4 \times 10^3$  cells ml<sup>-1</sup>). Thus, a range of psychrophilic to thermophilic prokaryotes were stimulated to grow in methanogen medium. However, predominantly mesophiles were present, which survived thermophilic temperatures up to 69°C. Interestingly, at hyperthermophilic temperatures (81°C-90°C) growth occurred in psychrophilic, mesophilic and hyperthermophilic MPN incubations (between 8 and 240 cells ml<sup>-1</sup>; Fig. 5.15a).

#### b) Culturability matrix for SRPs

Culturability of sulphate reducers ranged from  $< 3$  cells ml<sup>-1</sup> to  $4.6 \times 10^5$  cells ml<sup>-1</sup> (Fig. 5.15b) throughout the experiment. As for methanogens, no growth occurred for 57°C samples incubated at 15°C while highest culturability was detected for 57°C samples incubated at 40°C. Relatively high culturabilities ( $\sim 2 \times 10^5$  cells ml<sup>-1</sup>) occurred for 15°C (56 days) samples incubated at 15 °C and for 42°C samples incubated at 40°C (pattern similar to methanogen results, although values are two to three orders of magnitude higher in SRP medium) but also for 90°C incubated at 40°C. These data suggest that a great proportion of the microbial community grew at mesophilic temperatures (40°C) while being able to withstand thermophilic temperatures. Additionally, it is remarkable that a large number of cells ( $2.4 \times 10^2$  to  $2.4 \times 10^5$  cells ml<sup>-1</sup>) from hyperthermophilic temperatures (81°C - 90°C) grew over the entire temperature range (Fig. 5.15b).

#### c) Culturability matrix for heterotrophs

Culturability of heterotrophs ranged from 7 cells ml<sup>-1</sup> to  $2.4 \times 10^6$  cells ml<sup>-1</sup> (Fig. 5.15c) throughout the experiment. These values stem from the same MPN plate as the minimum and maximum values observed with the other two media (57°C samples incubated at 15°C and at 40°C, respectively). Similar to results obtained for methanogens and SRPs, culturability was also high ( $1.1 \times 10^5$  and  $1.1 \times 10^6$  cells ml<sup>-1</sup>) for 15°C (0 and 56 days) samples incubated at 15°C and for 42°C samples incubated at 40°C. Additionally, high culturability also occurred for 69°C and 81°C samples incubated at 40°C. Once more, relatively high numbers of culturable cells (23 to

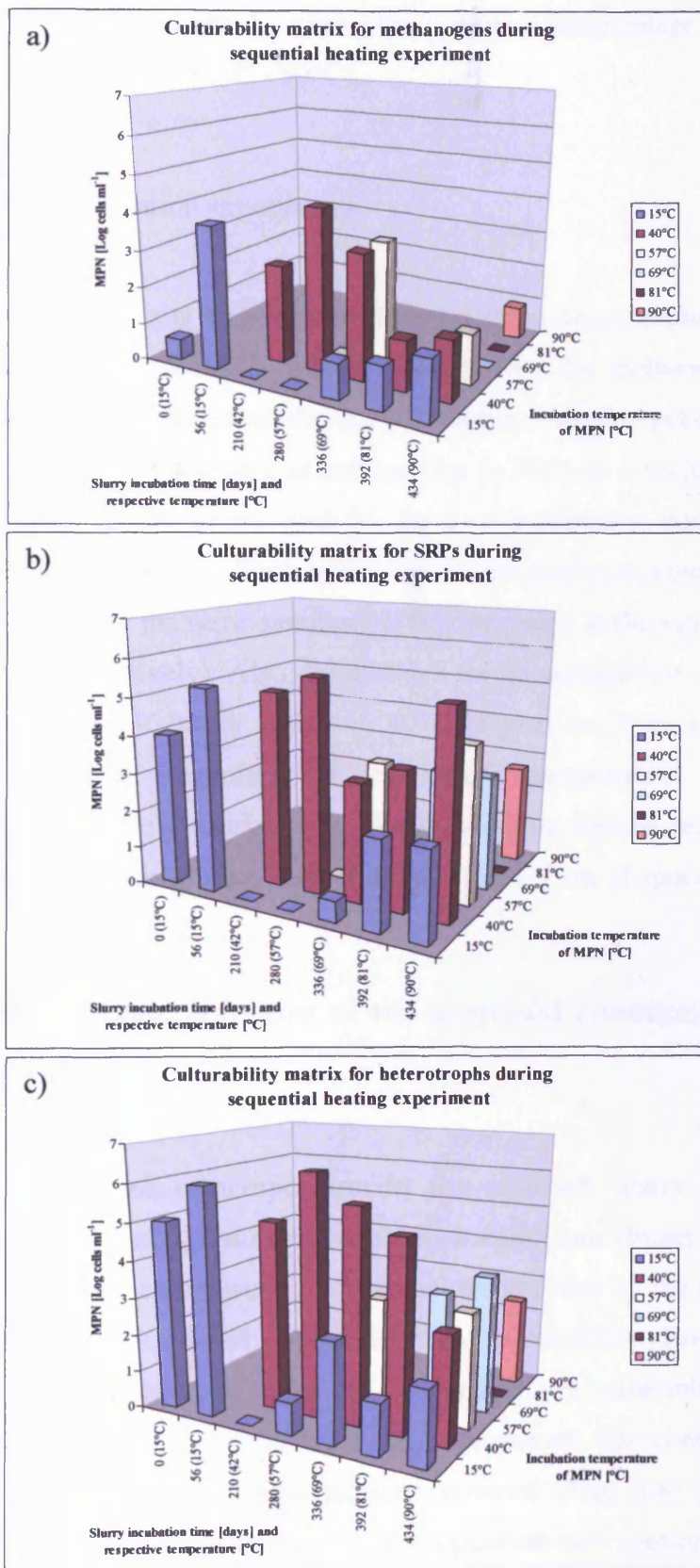


Fig. 5.15: Culturability matrices of prokaryotes during sequential heating. a) Methanogens; b) SRPs; c) Heterotrophs. Confidence limits are not shown as this feature is not available for this particular type of graph (Excel, Microsoft Office). However, confidence limits ranged between 0.6 and 0.7 units [Log cells ml<sup>-1</sup>]



$1.1 \times 10^5$  cells  $\text{ml}^{-1}$ ) occurred over the entire temperature range from hyperthermophilic samples ( $81^\circ\text{C}$  -  $90^\circ\text{C}$ ).

### 3.3.3 Summary of cultivation experiments

On average culturability was highest in medium targeting heterotrophs ( $2.7 \times 10^5$  cells  $\text{ml}^{-1}$ ) followed by SRPs ( $6.6 \times 10^4$  cells  $\text{ml}^{-1}$ ) and lowest for methanogens ( $2.5 \times 10^3$  cells  $\text{ml}^{-1}$ ). Despite differences in the actual numbers, similar patterns of growth occurred. For example, an increase of culturability ( $\sim 700\%$  to  $\sim 200,000\%$ ) occurred between the setup of the slurry and 56 days of incubation during which the temperature remained constant. Furthermore, minimum and maximum culturable cell numbers were found for the same samples ( $57^\circ\text{C}$ ) incubated at the same temperatures ( $15^\circ\text{C}$  and  $42^\circ\text{C}$ , respectively). Also, for all three media culturability was on average between 292% and  $\sim 250,000\%$  higher in  $40^\circ\text{C}$  incubations than any other MPN incubation temperature (regardless of the sample temperature) indicating the dominance of mesophilic prokaryotes in Tamar estuarine surface sediments able to withstand hyperthermophilic temperatures, maybe in the form of spores.

### 3.4 Potential metabolic activities of the microbial community in “aged” sediment slurries

The continuous increase of temperature in the sediment slurry which mimics temperature increases during sediment burial had a significant impact on the viability of the *in situ* prokaryotic community. This was documented by the decline in total cell numbers above  $70^\circ\text{C}$ , FISH detectability and culturability. However, even at  $90^\circ\text{C}$  intact cells were observed under the microscope and culturable cells of three different physiological groups, including SRP, were present. The continued presence of SRP was consistent with active sulphate removal (Fig. 5.6) throughout the experiment and shows that a proportion of the population was continuously active in the slurry.

In order to investigate the metabolic activities of the prokaryotic community further, radiotracer experiments were performed at 90°C using three different substrates (glucose, acetate, and lactate).

In all experiments the added substrate was utilised and radioactivity was present in metabolic products (Fig. 5.16 a-c). However, in autoclaved controls, which were analysed after 720 hours of incubation, substrate concentrations were also lower compared to  $t_0$  and radioactivity was also present in metabolic endproducts. This was much more pronounced for lactate, followed by acetate but negligible for glucose. It is unclear whether this effect was due to microbial activity of cells that survived autoclaving especially as hyperthermophiles were present in heated slurry (as shown by culturability) or due to physico-chemical reactions taking place including mineral catalysis at high temperatures (90°C, radiotracer added after autoclaving). However, the effect on glucose was minimal (80% of the glucose remained in the control; Fig. 5.16a), which suggests limited microbial activity.

### 3.4.1 Glucose metabolism in “aged sediment slurry

Glucose was metabolised within 24 hours without a lag phase and the majority of the radioactivity was then present in the acetate, lactate, and formate fraction (ALF 48%) and also after 240 hours (ALF 64%; glucose 2%; cells 6%; B/S 7%; CO<sub>2</sub> 23%; Fig. 5.16a). Subsequently, the ALF fraction itself was metabolised by the microbial community after a lag phase of at least 9 days and after 30 days of incubation the original glucose signal was fully converted to bicarbonate/succinate and CO<sub>2</sub> (44% and 48%, respectively). A small, increasing proportion of the <sup>14</sup>C-label in the cell fraction (6% at 24h and 8% at 720h) indicates cellular synthesis and potentially growth.

The results from the glucose experiment and the subsequent consumption of the produced ALF fraction suggest that microbial activity caused the disappearance of the substrates in the acetate and lactate supplemented slurry experiments as well, regardless of the potential physico-chemical reactions in the respective negative controls.

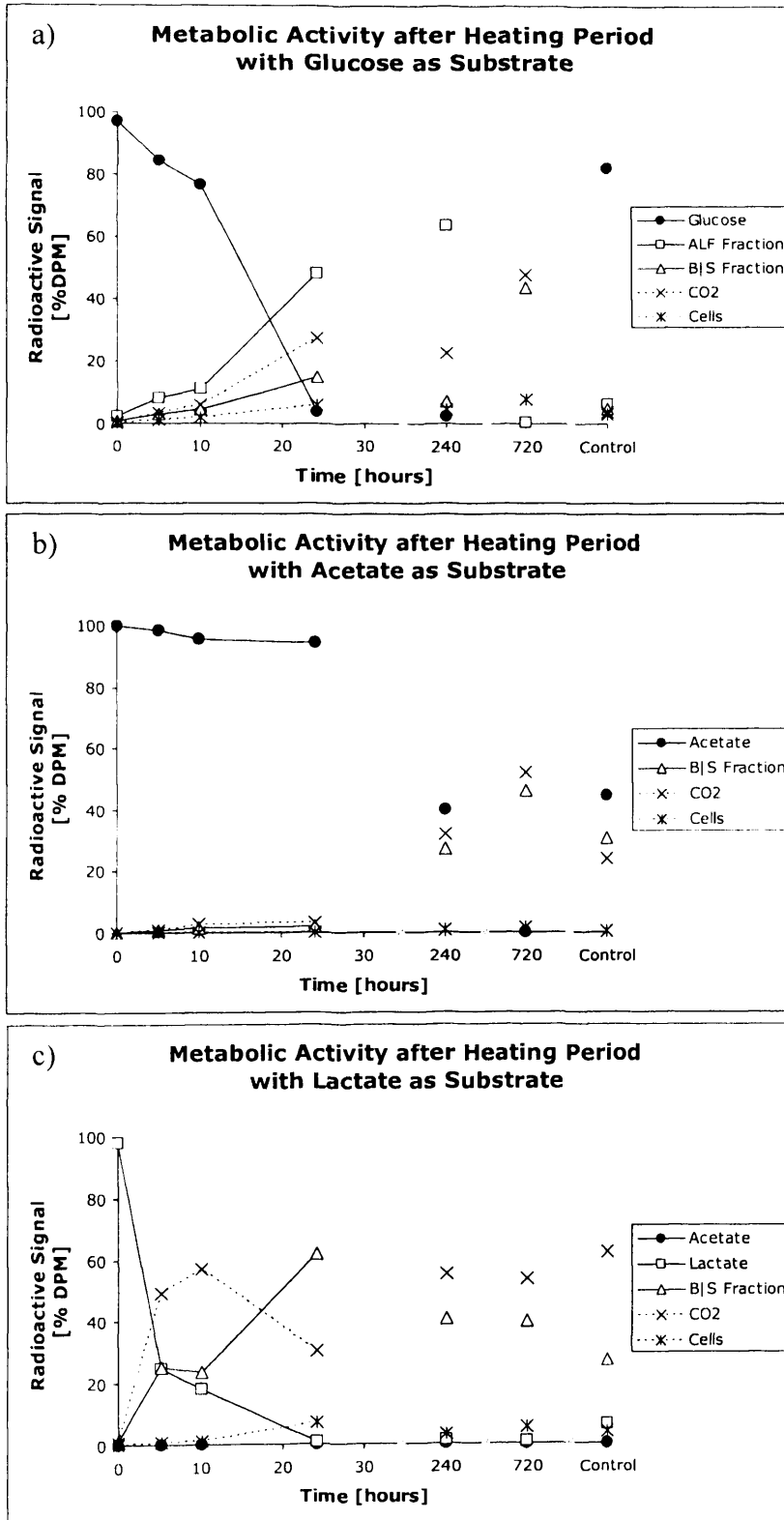


Fig. 5.16: Metabolic activity of microbial community at 90°C after sequential heating. Different substrates were used: a) glucose; b) acetate; c) lactate.

### 3.4.2 Acetate metabolism in “aged” sediment slurry

Acetate concentrations were only slightly reduced after 24 hours of incubation (95%) and approximately 60% were metabolised after 240 hours (Fig. 5.16b), which indicates a lag phase for this substrate of more than one but less than 10 days, similar to the observed lag phase of the ALF fraction in the glucose slurry. The remaining signal was present in the bicarbonate/succinate fraction (27%) and in the CO<sub>2</sub> (32%), just as occurred when the glucose derived ALF fraction was degraded (Fig. 5.16a, after 720 hours). After 720 hours all acetate was removed and the <sup>14</sup>C-label was present in the bicarbonate/succinate fraction (46%) and the CO<sub>2</sub> (52%). Again a slow increase of radioactivity within cells (2% at 720h) occurred although this only represents a fourth of the value observed during the glucose experiment at this time.

### 3.4.3 Lactate metabolism in “aged” sediment slurry

Lactate concentrations were reduced by 75% after only five hours of incubation and completely depleted after 24 hours. However, no detectable acetate was produced (Fig. 5.16c) as might be expected from acetogenic or sulphate-reducing activity. But as the entire produced ALF fraction was converted to bicarbonate/succinate and CO<sub>2</sub> in the glucose experiment this was not too surprising. However, potentially any produced acetate could have been immediately utilised by certain members of the microbial community (e.g. sulphate reducers) and converted to CO<sub>2</sub>. This, however, is contradicted by the initial slow consumption of acetate (Fig. 5.16b) and thus lactate was probably directly converted to CO<sub>2</sub>. This gas and the bicarbonate/succinate fraction contained most of the radioactive signal (~94%) as was observed for the degradation of the ALF fraction in the glucose amended slurry (Fig. 5.16a, after 240 hours) and also in the acetate slurry (Fig. 5.16b). Some signal was present in the filtrate (6%), indicating cellular incorporation or binding to sediment particles.

### 3.4.4 Metabolic activity and turnover rates

Metabolic consumption rates were highest for lactate ( $3.3 \text{ pmol ml}^{-1} \text{ d}^{-1}$ ) followed by glucose ( $2 \text{ pmol ml}^{-1} \text{ d}^{-1}$ ) and lowest for acetate ( $0.7 \text{ pmol ml}^{-1} \text{ d}^{-1}$ ).

Furthermore, the removal of the produced ALF fraction in the glucose slurry can be calculated. Although VFAs were not separated, it is likely that mainly acetate was produced from glucose. This assumption is based on the observed lag phase of at least 10 days between ALF production and its subsequent removal in the glucose slurry (Fig. 5.16a). A lag phase also occurred in the acetate slurry where the consumption of the substrate started between 1 and 10 days of incubation. Lactate did not show any lag phase. Thus, if lactate had been the major product of glucose fermentation it would have been quickly converted to succinate/bicarbonate and  $\text{CO}_2$  as observed in the lactate slurry (Fig. 5.16c) and not been detectable in the glucose slurry after 1 day and 10 days of incubation (Fig. 5.16a).

If acetate was the sole product of glucose fermentation this molecule itself was consumed at a rate of  $0.3 \text{ pmol ml}^{-1} \text{ d}^{-1}$ . This slow consumption also supports the identity of the molecule as this rate is similar to the one observed in the acetate slurry.

### 3.4.5 Bicarbonate/succinate fraction versus $\text{CO}_2$

In the above analyses of metabolic activities in heated sediment slurries the amount of produced  $\text{CO}_2$  was determined in two ways. Firstly, bicarbonate was measured via ion chromatographic separation and radioactivity was determined with subsequent scintillation counting. Secondly, all  $\text{CO}_2$  (present in gas and liquid phase) was chemically bound to phenethylamine (7% solution in liquid scintillation fluid) and its radioactivity was also determined (for detailed method description refer to Chapter II 7.2). The two separately determined signals of essentially one compound ( $\text{CO}_2$ ) were added alongside those of other components (e.g. cells, VFAs) to determine the total amount of radioactivity in the subsamples and the percentages of each radioactive compound at individual timepoints. However, by doing so, an unknown proportion of radioactive  $\text{CO}_2$  was accounted for twice. This proportion is unknown because the eluent gradient used during chromatographic separation caused bicarbonate and

succinate to have very similar retention times and were thus virtually impossible to separate.

a) Contribution of succinate to the bicarbonate/succinate fraction

If succinate was produced it most likely occurred as a metabolic intermediate of the mixed-acid fermentation of glucose in the glucose slurry. And indeed after 24 hours not only the ALF fraction was present (48%) but also the bicarbonate/succinate fraction made up 15% of the radioactivity in the glucose slurry (Fig. 5.16a and Table 5.7). However, this value does not exceed the total CO<sub>2</sub> (28%) at this timepoint and it can possibly be concluded that either no succinate was produced or that it was rapidly mineralised to CO<sub>2</sub>. For the final timepoint of the glucose slurry the radioactivity determined for the bicarbonate/succinate fraction and the total CO<sub>2</sub> are almost identical indicating that no succinate was present and all CO<sub>2</sub> was completely dissolved and present (e.g. as bicarbonate) in the liquid phase.

It seems unlikely that the C<sub>4</sub>-compound succinate was produced in acetate (C<sub>2</sub>) or lactate (C<sub>3</sub>) amended slurries. These substrates were probably directly mineralised to CO<sub>2</sub>. This is supported by relatively equal proportions of the B/S fraction and the total CO<sub>2</sub> in the acetate slurry (Table 5.7). In the lactate slurry, however, for most timepoints more radioactivity was detected in the CO<sub>2</sub> than in the B/S fraction except for t<sub>0</sub> and after 24 hours, which also suggests that no succinate was produced.

b) Total CO<sub>2</sub> versus dissolved bicarbonate in artificial seawater

The solubility of CO<sub>2</sub> depends on pressure and salinity, temperature, and pH of the liquid (ASW). The only constant during slurry incubation and subsequent analysis was the salinity. Pressure and temperature slowly decreased between taken the slurry out if the incubator (90°C) and processing one slurry after the other at room temperature (starting with glucose, followed by acetate and then lactate). Especially the decreasing temperature will have increased CO<sub>2</sub> solubility. At higher temperatures (e.g. slurry incubation temperature of 90°C) only little gas is dissolved and the majority is present in the headspace. Samples for ion chromatographic separation of the glucose slurry were taken first whilst it was still relatively hot hence

less CO<sub>2</sub> was probably present as bicarbonate than as gas. This, however, only appears to be true for the 24 and the 240 hour samplings (Table 5.7). The acetate slurry was sampled approximately 10 to 15 minutes after removing it from the incubator thus more CO<sub>2</sub> should be present in the form of bicarbonate. Indeed almost equal proportions of the B/S fraction and total CO<sub>2</sub> suggest that all CO<sub>2</sub> was in the liquid phase at the time of analysis. The lactate slurry was sampled last, approximately 20 to 30 minutes after removing it from the incubator hence also here all CO<sub>2</sub> should be in the liquid phase. This, however, is not the case (Table 5.7) and for most timepoints more CO<sub>2</sub> was present in the gas than in the liquid phase.

**Table 5.7: Percentages of bicarbonate/succinate and CO<sub>2</sub> fractions in sediment slurries.**

Time [hours]	Glucose Slurry		Acetate Slurry		Lactate Slurry	
	B/S [%]	CO <sub>2</sub> [%]	B/S [%]	CO <sub>2</sub> [%]	B/S [%]	CO <sub>2</sub> [%]
0	0.8	0.2	0	0	1.3	0.6
5	3	3	1	1	25	49
10	5	6	2	3	23	57
24	15	28	27	32	62	30
240	7	23	46	52	41	55
720	44	48	31	24	40	54

It appears that for most cases the dissolved bicarbonate resulted in similar radioactivities as the total CO<sub>2</sub>. Thus the overestimation of the produced CO<sub>2</sub> lies approximately by a factor of 2. This means that other compounds of the slurry experiments were probably underestimated in Fig. 5.16 a-c and that their proportions might be up to two times higher than shown. This is mainly the case for the radioactivity in filtered sediment slurry representing microbial cells or substrate adsorbed to sediment particles. The corrections for the overestimation of the CO<sub>2</sub> were undertaken for individual timepoints and the outcome supported the above conclusions.

#### 4. Discussion

The sampling site in the Tamar Estuary was located on an intertidal mudflat, as they commonly occur in the UK and other countries worldwide but especially those that have a shoreline with the North Sea (e.g. The Netherlands or Germany). Extensive microbiological and biogeochemical studies have been conducted on these highly productive coastal marine ecosystems (e.g. Alongi, 1998; Böttcher *et al.*, 2000; Gittel

*et al.*, 2008; Webster *et al.*, 2010). Diverse microbial communities were detected in these sediments (Webster *et al.*, 2007; Wilms *et al.*, 2007) and it has been suggested that they represent a model system for the deep biosphere (Engelen and Cypionka, 2009). The presence of high microbial numbers, primary production and heterotrophic activities (Dittman, 1999; Poremba *et al.*, 1999; Kim *et al.*, 2005) often result in steep geochemical gradients and distinct biogeochemical zones (Webster *et al.*, 2010), which is supported by organic matter input from both land and sea (Engelen and Cypionka, 2009). Oxygen is rapidly consumed by aerobic (micro-) organisms and only penetrates the top few millimetres to centimetres of the sediment (Böttcher *et al.*, 2000; Ziebis *et al.*, 1996; Billerbeck *et al.*, 2006). Deeper layers are thus exclusively dominated by anaerobic metabolic processes (e.g. fermentation, sulphate reduction, methanogenesis; Webster *et al.*, 2010).

## 4.1 The Tamar Estuary as a representative intertidal mudflat environment

### 4.1.1 Biogeochemistry of Tamar sediments

The biogeochemical characterisation of the Tamar Estuary sampling site revealed the presence of both sulphate and methane throughout the sediment column (upper 20 cm investigated). Such sulphate-methane transition zones are not uncommon and are often found in upper layers of intertidal mudflats (e.g. Wadden Sea sites NN and JS; Wilms *et al.*, 2006b; Gittel *et al.*, 2008; Table 5.8). However, they also exist in deeper sediments (0.3 to 0.6 mbsf, Webster *et al.*, 2011; 2 to 3 mbsf, Wilms *et al.*, 2006b), and especially in deep marine sediments (between >10 and >100 mbsf e.g. Webster *et al.*, 2006a; Engelen *et al.*, 2008). Although sulphate reduction occurred in Tamar sediments, it was not depleted and concentrations (11 to 30 mM) were in a similar range compared to other sites (Table 5.8).

Acetate was also detected throughout the sediment column (9 to 19  $\mu\text{M}$ ). This organic acid is an important substrate for methanogens and sulphate reducers but concentrations in marine sediments are usually low (< 15  $\mu\text{M}$ ) due to prokaryotic consumption (Jorgensen, 1982; Wellsbury and Parkes, 1995). Wellsbury *et al.* (1997)



highlighted the importance of this substrate in potentially sustaining the vast biosphere buried in marine sediments as it can be produced from organic matter at higher temperatures.

In conclusion, the Tamar Estuary biogeochemistry is similar to and thus representative for other intertidal mudflats.

**Table 5.8 Sulphate and methane concentrations of selected intertidal mudflat sediments.** Data from 1) this study, site St. John's Lake, Plymouth, UK; 2) Webster *et al.* (2010), site Woodhill Bay, Portishead, UK; 3) Wilms *et al.* (2006b), site Neuharlingersiel Nacken, Neuharlingersiel, Germany; 4) Gittel *et al.* (2008), site Janssand, Neuharlingersiel, Germany; 5) Böttcher *et al.* (2000), site Dangast, Dangast, Germany.

Depth [cmbsf]	Tamar Estuary <sup>1)</sup>		Severn Estuary <sup>2)</sup>		Wadden Sea (NN) <sup>3)</sup>		Wadden Sea (JS) <sup>4)</sup>		Wadden Sea (D) <sup>5)</sup>	
	SO <sub>4</sub> <sup>2-</sup> [mM]	CH <sub>4</sub> [μM]	SO <sub>4</sub> <sup>2-</sup> [mM]	CH <sub>4</sub> [μM]	SO <sub>4</sub> <sup>2-</sup> [mM]	CH <sub>4</sub> [μM]	SO <sub>4</sub> <sup>2-</sup> [mM]	CH <sub>4</sub> [μM]	SO <sub>4</sub> <sup>2-</sup> [mM]	CH <sub>4</sub> [μM]
0	30	7	29	0	~30	0	~32	~30	~21	n.d.
10	21	18	19	0	n.d.	n.d.	n.d.	n.d.	~19	n.d.
20	11	8	20	6	~21	~30	n.d.	n.d.	~16	n.d.
50	n.d.	n.d.	12	25	~17	~10	~19	~110	n.d.	n.d.

#### 4.1.2 Microbial abundance, culturability, and viability in Tamar sediments

##### a) Microbial abundance and viability in Tamar sediments

Increasing depth was significantly, inversely correlated with the total number of cells ( $p < 0.05$ ; Fig 5.2a), which decreased from  $4 \times 10^9$  cells  $g^{-1}$  sediment at one cmbsf to  $2 \times 10^9$  cells  $g^{-1}$  sediment at 19 cmbsf. Slightly lower but also decreasing cell numbers were observed in tidal flat sediments of the German Wadden Sea (site NN: from  $8 \times 10^8$  cells  $g^{-1}$  at 0.5 cmbsf to  $2 \times 10^8$  cells  $g^{-1}$  sediment at 50 cmbsf, Köpke *et al.*, 2005; site JS: from  $6 \times 10^8$  cells  $g^{-1}$  at the surface to  $5 \times 10^8$  cells  $g^{-1}$  sediment at 50 cmbsf, Gittel *et al.*, 2008).

Although not significant by statistical means, with increasing depth higher percentages of dead cells and lower percentages of dividing cells occurred (calculated  $r$  values, 0.491 and 0.627, respectively; critical value, 0.632;  $n = 10$ ) indicating a loss of viability with burial. Queric *et al.* (2004) reported that in Arctic Ocean sediments with increasing water and sediment depths the percentages of dead

cells increased. In the shallowest samples (~1260 m water depths) the percentages of dead cells rose from ~26% at 1 cmbsf to ~60% at 5 cmbsf. Compared to these values viability in Tamar sediment is relatively high (only between 3% and 8% dead cells; Fig. 5.2b). From starvation-survival experiments (Chapter IV) it is known that especially Gram-positive bacteria (*Firmicutes* and *Actinobacteria*) showed increasing numbers of dead cells in the cultures (up to an average of 36% after one year; Fig. 4.55a) whereas representatives of the *Proteobacteria* exhibited much lower proportions (maximum 6%).

For FISH detectability, a significant, inverse correlation with depth was observed (calculated  $r$  value, 0.997; critical value, 0.997;  $n = 3$ ). Viability of bacteria based on FISH detectability decreased with depth (from 64% at 1 cmbsf to 27% at 19 cmbsf). Similar trends were observed for Wadden Sea sites JS (from ~80% at the sediment surface to ~35% at 20 cmbsf, Ishii *et al.*, 2004) and in the upper 5 cm for site Dangast (maximum 80%, Böttcher *et al.*, 2000). During starvation (Chapter IV), FISH detectability based on AODC total counts generally decreased with time. After 50 days, between 1.9% (*Gammaproteobacteria*) and 195% (*Firmicutes*) of total cells were FISH detectable (Fig. 4.55b). If FISH detectability in Tamar sediment is calculated based on AODC similar values to those of *Gammaproteobacteria* are observed (1 cmbsf, 1.9%; 9 cmbsf, 0.9%; 19 cmbsf 0.3%). These estimates are based on the eubacterial probe alone, which does not detect all bacteria (Daims *et al.*, 1999) and additionally, *Archaea* were not accounted for. Hence FISH detectability and thus viability of the prokaryotic community in Tamar sediment is most likely higher than reported here.

During starvation experiments no significant correlation was found between the averaged proportions of dead and FISH detectable cells. Although the trend suggested an inverse relationship for pure cultures (calculated  $r$  value -0.35; critical value, 0.71;  $n = 8$ ) and for Tamar sediments (calculated  $r$  value -0.98; critical value, 0.99;  $n = 3$ ).

#### b) Microbial culturability in Tamar sediments

Microbes rapidly utilise readily available substrates thus with increasing depth only recalcitrant organic matter remains (Hedges and Keil, 1995). Although the here sampled sediment column was not very deep, when calculating Tamar sediment ages

based on the highest sedimentation rate published by Müller and Suess (1979), the sediment might have been between 7 years (1 cmbsf) and 143 years (20 cmbsf) old. Due to potential bioturbation (down to 20 cmbsf; Kristensen, 2000), tidal effects, and storm events, however, age determinations for tidal sediments are difficult to obtain (Wilms *et al.*, 2006a). These disturbances by mechanical over-throwing of the upper sediment layers (resuspension of large sediment patches) might introduce new organic matter into near-surface sediments, which then leads to the formation of microenvironments with different physico-chemical and biological characteristics (Wilms *et al.*, 2006a) thus potentially influencing microbial viability on a relatively small scale.

The most noticeable geochemical characteristic of the investigated sediment was the presence of both sulphate and methane throughout the upper 20 cm (see above). Methane was produced in the presence of slow sulphate reduction (Fig. 5.1 a and c). This could indicate i) that non-competitive substrates are utilised by these two physiological groups (e.g. trimethylamine or betaine; King, 1984) or ii) that competitive substrates are present in sufficient concentrations to support activity and even growth in both groups (e.g. acetate or hydrogen; Fig. 5.1 a and c). Molecular studies on Wadden Sea sediments suggested that the competition for substrates shaped the depth profiles of these two physiological groups (Wilms *et al.*, 2007).

Culturable cell numbers of both methanogens ( $1 \times 10^4$  cell ml<sup>-1</sup>) and sulphate reducers ( $4 \times 10^4$  cell ml<sup>-1</sup>) in the shallowest sample were relatively high suggesting their coexistence *in situ* (Fig. 5.3). At 9 cmbsf, however, growth in SRP medium was present but low and FeS precipitation was absent indicating the lack of culturable sulphate producers. For methanogens the number of culturable cells from this layer was elevated ( $1 \times 10^5$  cell ml<sup>-1</sup>) compared to 1 cmbsf suggesting that these two groups might have been competing for acetate *in situ* (acetoclastic methanogens e.g. *Methanosarcina* sp., 1 cmbsf, versus acetate-consuming sulphate reducers, not detected). Hydrogen can also be utilised by these two groups of organisms (e.g. *Desulfurivibrio* sp., 1 cmbsf and *Methanogemium* sp., 5 cmbsf) and concentrations were low throughout the sediment (on average 3 µM between 1 and 17 cmbsf). However, the highest hydrogen concentration was detected at 19 cmbsf (28 µM; Fig. 5.1c) from which layer neither methanogens nor sulphate reducers grew in MPN dilution series (Fig. 5.3). Methanogens utilising hydrogen (e.g. *Methanospirillum*

*hungateii*, 99%), however, were isolated by direct enrichments from this sediment layer (Table 5.2).

Culturable heterotrophs were present throughout the sediment column with highest numbers near the surface ( $4.6 \times 10^5$  cell ml<sup>-1</sup>). Sequences for both heterotrophic *Archaea* and *Bacteria* were detected in Tamar sediments (e.g. *Thermocodium* sp., 85% at 5 cmbsf and *Natronocella* sp. 93%, 1 cmbsf; Table 5.3 and 5.4) suggesting their coexistence.

### c) Cultivation efficiencies compared to other sites

An extensive cultivation study on tidal flat Wadden Sea sediment (site NN and a site nearby) was conducted by Köpke *et al.* (2005). They isolated 112 pure cultures grouped into to 53 different OTUs and achieved cultivation efficiencies of up to 23% of the total count in the upper 2 m of the sediment. They reported that aerobic and anaerobically determined MPNs without metal oxides resulted in similar culturable cell numbers for the two uppermost layers (0.5 and 5 cmbsf) but were up to three orders of magnitude lower than anaerobic MPNs with metal oxides (iron hydroxide and manganese oxide) for these horizons.

**Table 5.9: Cultivation efficiencies of Tamar sediment based on AODC and FISH counts.** 1) this study, 2) Köpke *et al.* (2005). Note that different media were used in these two studies for the determination of culturable cells.

Medium for	Depth [cmbsf]	Tamar Sediment Cultivation Efficiency [%] <sup>1)</sup>	Depth [cmbsf]	Wadden Sea Sediment (site NN) Cultivation Efficiency [%] <sup>2)</sup>
SRPs	1	0.001	0.5	0.05
Heterotrophs		0.01		0.5
SRPs	9	$1.6 \times 10^{-7}$	5	0.005
Heterotrophs		$2.5 \times 10^{-4}$		0.006
SRPs	19	-	50	0.03
Heterotrophs		$5.5 \times 10^{-4}$		0.008

The cultivation efficiencies for tidal flat sediments obtained by Köpke *et al.* (2005) are between one and four orders of magnitude higher than those from Tamar sediments (Table 5.9). This discrepancy could be due to the different media used for the determination of culturable heterotrophs and sulphate reducers. Köpke *et al.* (2005) used the basal medium supplemented with a monomer mix (with and without sulphate, see Chapter II 3.3.2) while anoxic YPGL medium was applied during this

study (see Chapter II 3.2.1) for heterotrophs and anoxic basal medium (see Chapter II 3.3.1) supplemented with lactate and acetate. The monomer mix contains a broader range of substrates (e.g. more carboxylic acids and alcohols) and might have thus stimulated growth in more physiological groups resulting in higher culturabilities.

#### d) Microbial abundance and viability linked to geochemical depth profiles

The concentration of porewater acetate was significantly, inversely correlated to sediment depth ( $p < 0.05$ ) but neither to total cell numbers nor percentages of dividing, dead, or FISH detectable cells.

#### 4.1.3 Microbial diversity in Tamar sediments

The microbial diversity in Tamar sediments was investigated by molecular biological techniques (PCR-DGGE) for both *Bacteria* and *Archaea* while the cultivated diversity was restricted to *Archaea* (Andrew J. Watkins, PhD Thesis, Cardiff University, in preparation).

##### a) Archaeal diversity in Tamar sediments

The detected archaeal diversity generally increased with sediment depth (based on number of bands, Fig. 5.17a). The shallowest sediment layer (1 cmbsf) showed a remarkable limited diversity with only four bands being prominent and was dominated by *Crenarchaeota* (Fig. 5.17a). Bands A1-1 to A1-3 belonged to the recently discovered genus *Nitrosopumilus* (strain NM25, 94%, *Crenarchaeota*, Table 5.3), a marine autotrophic ammonia oxidiser so far only known from the water column. More intriguingly, in the recently published genome of *N. marinus* (Walker et al., 2010) only one 16S rRNA operon was reported, yet three different bands with 100% sequence identity (149 bp) were detected (Fig. 5.4a). The fourth band (A1-4, Table 5.3) in this sediment layer was affiliated with a strain (DJ3.25-13, 83%) isolated from the anoxic hypolimnion of Solar Lake (Sinai, Egypt; Cytryn *et al.*, 2000) belonging to Group II of the Euryarchaeota. Bands A1-3 and A1-4 were both

present in every sediment horizon investigated. While signal intensity for the former increased with depth, the opposite was true for the latter. This could indicate that the corresponding archeon of band A1-3 thrives in the sediment whilst the other was merely buried and disappeared with increasing age and depth, although it was reported that related organisms are widespread in marine sediments (Cytryn *et al.*, 2000).

Deeper layers were dominated by *Euryarchaeota* (Fig. 5.17a), especially sequences related to afore mentioned Solar Lake strain DJ3.25-13 and another Solar Lake strain J4.75-15 were detected several times (eight and nine times in total, respectively) and in each sediment horizon below 1 cmbsf but different sequences were obtained. Sequences related to *Methanococcoides methylutens*<sup>T</sup> (93%-100%) were detected five times at 3 and 5 cmbsf. This genus was readily isolated with methylamine from 3 and 19 cmbsf (Table 5.2). With one sequence each, the genera *Methanogenium* (87%, 5 cmbsf) and *Methanobacterium* (82%, 19 cmbsf) were also detected and a strain belonging to the latter genus was isolated from 3 cmbsf (Table 5.2).

Besides the genus *Nitrosopumilus* other cultivated representatives of the *Crenarchaeota* were detected in deeper layers. Two sequences related to *Thermocodium modestius*<sup>T</sup> (84%-85%) were present in 5 and 9 cmbsf, one sequence at 5 cmbsf related to *Thermofilum* sp. (88%), and another one from 9 cmbsf was affiliated to the genus *Staphylothermus* (84%). All these strains have been isolated from (acidic) hot springs in Japan or Russia (Zillig *et al.*, 1983; Fiala *et al.*, 1986; Itoh *et al.*, 1998; Table 5.3).

In addition, a number of methanogenic genera were isolated by Andrew J. Watkins that evaded molecular biological detection (Table 5.2). Amongst them are two species of known methylotrophs (*Methanosarcina* sp., 3 and 19 cmbsf; Kendall and Boone, 2006) while others use H<sub>2</sub>/CO<sub>2</sub> and/or acetate for methane production such as *Methanoplanus limicola* (98%, 3 cmbsf; Wildgruber *et al.*, 1982), *Methanobacterium subterraneum* (99%, 3 cmbsf; Kotelnikova *et al.*, 1998), *Methanoculleus* sp. (99%, 3 cmbsf; Maestrojuan *et al.*, 1990), and *Methanospirillum hungatei* (95%, 19 cmbsf; Ferry *et al.*, 1974).

While some *Archaea* were detected several times by molecular (e.g. Solar Lake strains) and cultivation techniques (e.g. *Methanosarcina* sp.) others were only

detected once or twice (e.g. *Methanoplanus* sp. or *Thermocladium* sp.). Frequent detection might suggest dominance of the respective affiliates or repeated burial of the same organism while less frequent detection, especially in deeper layers (e.g. *Methanobacterium* sp. 82%, 19 cmbsf) might be related to *in situ* activity.

Sequences belonging to the genera *Methanosarcina* (84% to 85% between 200 to 360 cmbsf) and *Methanococoides* (83% to 94% between 0 and 360 cmbsf) were also detected in intertidal mudflat sediments of the German Wadden Sea site NN (Wilms *et al.*, 2006b). Their presence in greater depth and in other tidal flat sediments than in the Tamar Estuary suggests that they play an ecological role within intertidal sediments.

#### b) Bacterial diversity in Tamar sediments

The bacterial diversity appears to be lower than the archaeal diversity in Tamar sediments (Fig. 5.4b). However, quite a number of bands were barely visible in the DGGE gel and could thus not be cut out for sequence analysis. The low signal intensities might have been caused by a high number of 16S rRNA gene sequences present in the original sample. If many gene templates are present the primer specificity towards certain sequences (less mismatches) plays an important role during PCR amplification (Wintzingerode *et al.*, 1997). Thus certain sequences, despite being abundant might not have been amplified to a detectable level.

The detected bacterial diversity increased with depth (based on number of bands, Fig 5.17b). A change in community composition occurred between the upper 5 cm and below 9 cm of the sediment (Fig. 5.4b). The number of *Chloroflexi*-related sequences increased in deeper layers while the sequences related to the *Cytophaga-Flavobacteria* group disappeared. Only one sequence of the phylum *Firmicutes* was detected and was related to the genus *Desulfotomaculum* (86%, at 9 cmbsf), a thermophilic, spore-forming sulphate reducer (Nilsen *et al.*, 1996). *Firmicutes*, known for their spore-forming ability, often dominate culture collections of deeper sediment layers from tidal flats (Köpke *et al.*, 2005) and in deep subsurface sediments (Batzke *et al.* 2007, ODP Leg 201, site 1229).

As observed for archaeal diversity, a number of bacterial sequences (three in total) were present throughout the entire sediment column. The first two sequences

belong to the *Gammaproteobacteria*. Band B1-1 is affiliated to *Alkalispirillum* sp. (94%), an obligate aerobe utilising acetate and other carboxylic acids (Rijkenberg *et al.*, 2001) while band B1-7 is related to *Natronocella* sp. (82%), a nitrile-degrading, obligate heterotroph (Sorokin *et al.*, 2007). The final band that was present throughout the sediment column is affiliated with *Dehalogenimons* sp. (92%, *Chloroflexi*) known to reductively dehalogenate polychlorinated aliphatic alkanes (Moe *et al.*, 2009). Other bands that also relate to this organism were detected in sediment layers between 9 and 19 cmbsf but showed varying sequence similarities to each other (79% to 90%, ~140bp). A further *Chloroflexi*-related sequence was detected in 9 cmbsf related to the genus *Dehalococcoides* sp., which can oxidise hydrogen and halogenated organic compounds (Maymo-Gatell *et al.*, 1997). Another hydrogen utilising bacterium belonging to the genus *Geobacter* (67%, band B19-1; Lovley *et al.*, 1993) was also detected.

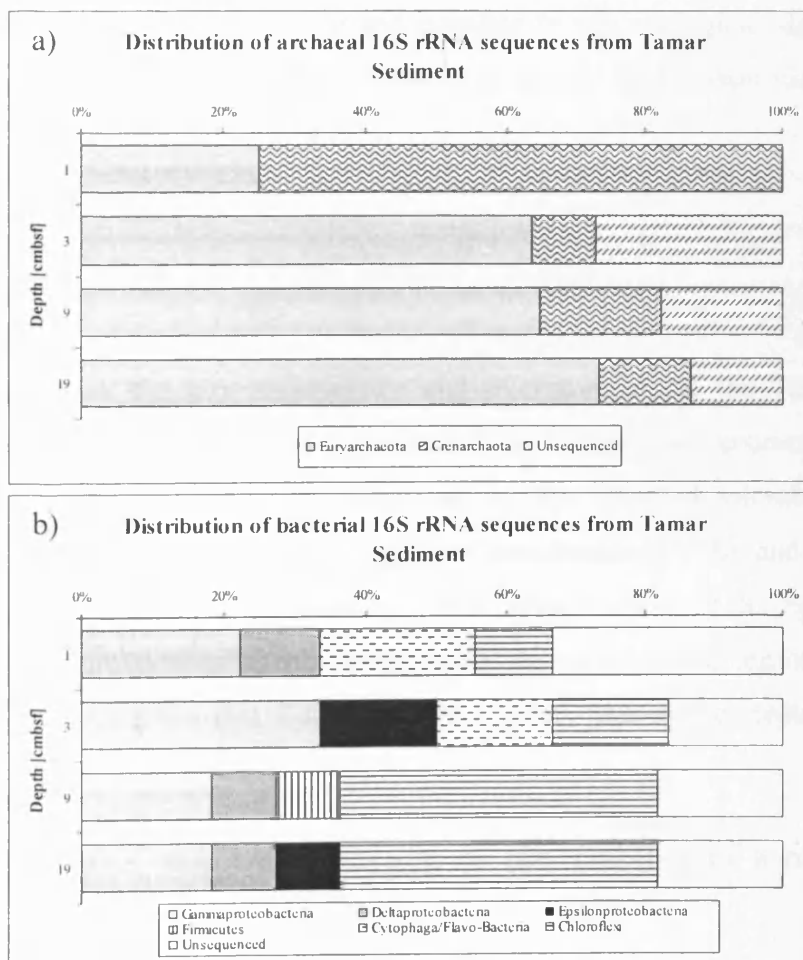


Fig. 5.17: Distribution of prokaryotic diversity based on 16S rDNA sequences in Tamar sediment. a) Archaeal diversity; b) Bacterial diversity. Number of sequenced bands is given in brackets.



The presence of a variety of hydrogen and acetate utilising bacteria might indicate that methanogens and sulphate reducers do not just compete with each other for these substrate but also that other members of the microbial community (e.g. *Chloroflexi*) consume these electron donors. This might explain the lack of culturable sulphate reducers in depths below 1 cmbsf (Fig. 5.3). This possible competition also seems to favour the presence of culturable methanogens able to use non-competitive substrates, which is supported by the isolation of *Methanococoides* strains grown on methylamine or *Methanoculleus* and *Methanospirillum* strains grown on formate (Table 5.2) while the latter two are also able to use hydrogen for methane production.

Wilms *et al.* (2006a, b) detected sequences related to *Dehalococcoides* (86% to 89%, between 0 and 380 cmbsf), *Desulfotomaculum* sp. (85%, between 20 and 380 cmbsf), and *Alkalispirillum* sp. (90% to 92%, between 0 and 180 cmbsf) at site NN and *Cytophaga* sp. (90% between 0 and 200 cmbsf) at site JS of Wadden Sea. This indicates the widespread occurrence and possible *in situ* ecological significance of these phylotypes in intertidal sediments even at greater depths than sampled in the Tamar Estuary.

#### 4.1.4 Summary of the Tamar Estuary study

After investigating the biogeochemistry and microbiology of the Tamar Estuary sampling site it can be concluded that this habitat shares many characteristics with other intertidal mudflats. This is supported by the detected microbial diversity (molecular biological and cultivated), similar abundances of cells and their *in situ* viability as well as *in vitro* culturability. Thus, effects observed during subsequent sequential heating experiments mimicking burial on the microbial community should be representative for processes that would occur in any near-surface sediment.

## 4.2 The effects of sequential heating on the viability of a near-surface microbial community

The Tamar Estuary near-surface microbial community is similar to other intertidal

mudflats (see above). To investigate what effects burial and concomitant increasing temperature have on such a population, sequential heating experiments were conducted. The main focus lay on changes in culturability and viability to help understand the processes observed in the deep biosphere.

#### 4.2.1 Biogeochemical alterations during mimicked burial and their effect on the microbial community

During the sequential heating experiment, geochemical changes in the sediment slurry occurred. At higher temperatures ( $>70^{\circ}\text{C}$ ) acetate and hydrogen concentrations started to increase (Fig. 5.5) as was observed by Parkes *et al.* (2007). Average acetate concentrations below  $70^{\circ}\text{C}$  were similar in both slurries ( $\sim 22\ \mu\text{M}$ ) and comparable to those detected in Tamar sediments ( $13\ \mu\text{M}$ ). At temperatures above  $70^{\circ}\text{C}$ , however, acetate increased by up to two orders of magnitude in both slurries to maximum concentrations of  $840\ \mu\text{M}$  to  $1100\ \mu\text{M}$ .

The increasing concentrations of acetate are significantly ( $p < 0.05$ ) inversely correlated with the AODC total counts and also significantly, but positively, correlated with increasing temperature. However, as AODC total counts are also significantly inversely correlated with temperature ( $p < 0.05$ ), the correlation between increasing acetate concentrations and decreasing cell numbers is most likely spurious. Both are affected by increasing temperature and could be unrelated to each other. It has been postulated that acetate is released from organic matter present in marine deep subsurface sediments (Wellsbury *et al.*, 1997). However, the lysis of microbial cells due to higher temperatures could also add to the observed acetate pool.

Hydrogen concentrations in Tamar sediments were on average  $6\ \mu\text{M}$  (maximum  $28\ \mu\text{M}$ ). Between  $15^{\circ}\text{C}$  and  $70^{\circ}\text{C}$  average concentrations in the slurries were generally higher than observed in the original sediment ( $\sim 20\ \mu\text{M}$ ). An increase to maximum values of  $682\ \mu\text{M}$  (slurry I) and  $137\ \mu\text{M}$  (slurry II) occurred at temperatures above  $70^{\circ}\text{C}$  (Fig. 5.5 a-b). Although hydrogen concentrations were not significantly correlated to increasing temperatures (calculated  $r$  value =  $0.459$  for slurry I and  $0.591$  for slurry II; critical value =  $0.602$ ;  $n = 11$ ), hydrogen production

has been linked to aromatisation of organic matter at higher temperatures (Parkes *et al.*, 2007).

Increasing concentrations of hydrogen and acetate at incubation temperatures above 70°C possibly stimulated activity in (hyper-) thermophilic microorganisms that were present in the original Tamar sediment. A likely candidate that might have been stimulated is a thermophilic, spore-forming sulphate reducer with an upper temperature limit of 75°C (*Desulfotomaculum thermocisternum*, Nilsen *et al.*, 1996). This species might have taken advantage of the produced hydrogen at higher temperatures. However an increase in sulphate reduction at elevated temperatures was not observed (Fig 5.6) although it has been shown that it can occur up to 102°C (Elsgaard *et al.*, 1994). Instead, sulphate was slowly but consistently removed during the sequential heating experiment. This is consistent with the observed, slow sulphate removal in the original sediment column (Fig. 5.1 a-b).

A hyperthermophilic archaeon was also detected with molecular biological methods. *Thermocodium modestius* is an anaerobic heterotroph with a growth range of 45°C to 82°C (optimum 75°C; Itoh *et al.*, 1998). This species reduces sulphur, thiosulphate and nitrate or FeCl<sub>3</sub> under a H<sub>2</sub>/CO<sub>2</sub> atmosphere and might have also been stimulated under the increasing temperature regime.

It is conceivable that other microorganisms, which were not detected with molecular biological methods in the original sediment, also used the thermogenically produced substrates. A variety of acetate and hydrogen utilising prokaryotes were detected. Many of them, however, are characterised as being psychro- to mesophilic, for example, the cultivated methanogens (Table 5.2), *Desulfurivibrio* sp., *Geobacter* sp. or strains belonging to the phylum *Chloroflexi*, which dominated deeper sediment layers. It cannot be ruled out that representatives of these groups exist that can be active at elevated temperatures. A molecular biological analysis of the microbial community over the course of the heating experiment was not conducted albeit samples were taken for this purpose.

#### 4.2.2 Effects of increasing temperatures on microbial abundance and viability

During sequential heating experiments the number of total cells increased by 135% (from 1.2 to 1.8×10<sup>8</sup> cells ml<sup>-1</sup>; not significant; temperature constant) between the

first to samplings while FISH detectability decreased (from 79% to 32% based on DAPI counterstain or from 13% to 8% based on AODC total count). These two trends are contradictory. Between these two samplings the FISH count slightly decreased by 15% (from  $1.5$  to  $1.3 \times 10^7$  cells  $\text{ml}^{-1}$ , not significant) while the DAPI count increased by 212% (from  $1.9$  to  $4.1 \times 10^7$  cells  $\text{ml}^{-1}$ ; not significant), thereby following the trend seen with AODC. This could indicate that either the proportion of sedimentary *Archaea* increased during this time as only the eubacterial probe EUBI was used for FISH and/or that bacterial groups not detected with this probe (e.g. *Planctomycetales* and *Verrucomicrobia*; Daims *et al.*, 1999) increased in abundance. Due to homogenisation of the sediment during the slurry setup, microenvironments were removed thereby potentially providing limited, starved cells with new substrates and thus causing the observed increase in abundance. The continued decrease of FISH detectability at higher temperatures is consistent with the decrease in AODC total counts albeit for the last sampling DAPI showed a higher count than AODC (Fig. 5.7).

Cell counts using the LIVE/DEAD<sup>®</sup> Kit were only obtained at incubation temperatures of 15°C. At higher temperatures (>40°C) background fluorescence was increased and neither cells nor sediment particles were visible (Fig. 5.8). Between the two samplings of 15°C the LIVE/DEAD<sup>®</sup> counts decreased by 24% (from  $9.4$  to  $7.2 \times 10^7$  cell  $\text{ml}^{-1}$ ) contradicting the observed increase in AODC total count. At the same time the percentages of detected dead cells in the samples decreased (from 10% to 5%) supporting the increasing AODC total cell count and culturability.

Why the LIVE/DEAD<sup>®</sup> Kit failed to stain cells at elevated temperatures is unclear. No reports have been found in the scientific literature that connect higher temperatures with insufficient staining. The kit has been successfully used to study deep biosphere sediments exhibiting elevated temperatures (60°C to 100°C; Roussel *et al.*, 2008). Biggerstaff *et al.* (2006), however, reported that when the LIVE/DEAD<sup>®</sup> Kit was applied to matrices consisting of inorganic and organic compounds (activated sludge) PI showed significantly greater non-specific binding to the matrix and caused increased background fluorescence. Surface sediments from the Tamar estuary most likely have a higher organic matter concentration than several million year old, deep subsurface sediments. Heating Tamar sediments could have increased unspecific binding of the two positively charged dyes to reactive

organic matter or sediment particles resulting in poor quality of the staining. Stained slurry samples from 42°C and above exhibited a greenish “veil” when observed under the microscope, indicating that Syto9<sup>®</sup> might have been more affected by this than PI.

a) Development of total counts in different heating experiments

In experiments using a thermal gradient system, Tamar sediment was amended with minerals (basalt, silica) and 1% sediment from the Guaymas Basin (known for its thermophilic microbial community, Jorgensen *et al.*, 1992) and incubated for ~100 days (Cathal Linnane, PhD Thesis, Cardiff University). Instead of slow heating, the thermal gradient system instantly heats parallel incubations of slurry aliquots to selected temperatures. Results from this experimental setup are similar to sequential heating experiments in regard to the geochemical stages that occur at respective temperatures (i.e. sulphate reduction; Parkes *et al.*, 2007). In both experiments sulphate was significantly removed (< 2 mM), acetate was produced at higher temperatures (~6-10 mM). The only differences observed were the production of hydrogen (only present in silica slurry, ~600 µM at 90°C) and methane (only present in hematite slurry with local maxima of ~120 µM at 70°C and ~ 270 µM at 100°C).

For these two temperature gradient experiments, total counts were determined (Fig. 5.18). Although initial cell concentrations in the mineral-containing slurries with hyperthermophilic inoculum were higher, the percentage-based decreases with increasing temperature were more drastic than in the unamended, sequentially heated slurry (this study; Table 5.10). In all slurries, however, there appear to be two stages of cell loss. The first stage is a slow decrease until approximately 75°C, which is close to the transition of thermo- to hyperthermophilic conditions. Above this, in the second stage the rate of cell loss is increased (Fig 5.18). Cell counts for slurries containing 1% Guaymas Basin sediment remained above the “pure” Tamar slurry at all temperatures. At higher temperatures, this might be due to the initial addition of (hyper-) thermophilic microorganisms. However, considering that mainly psychro- to mesophilic microbes were present in original Tamar sediment (Table 5.3 and 5.4) the differences between total counts of the “pure” slurry and slurries with hydrothermal inoculum are not too pronounced. Adding (hyper-) thermophiles does not seem to tremendously increase total counts at higher temperatures. This gives room for

questions in regard to the deep biospheres such as: Do parts of the microbial community adapt to increasing temperatures (stimulating evolution due to changing environmental parameters over geological timescales; see Chapter VI, 3.5.2)? Or, are only already heat-adapted microbes that become buried able to survive (here represented by the hydrothermal Guyamas basin inoculum)? And if so, how do they maintain their viability at deep seawater temperatures (4°C) until conditions become more favourable?

The nearly parallel development of cell counts with increasing temperature in slurries containing Tamar sediments with and without the addition of (hyper-) thermophiles and in different incubation systems underlines the reproducibility and thus reliability of such experiments.

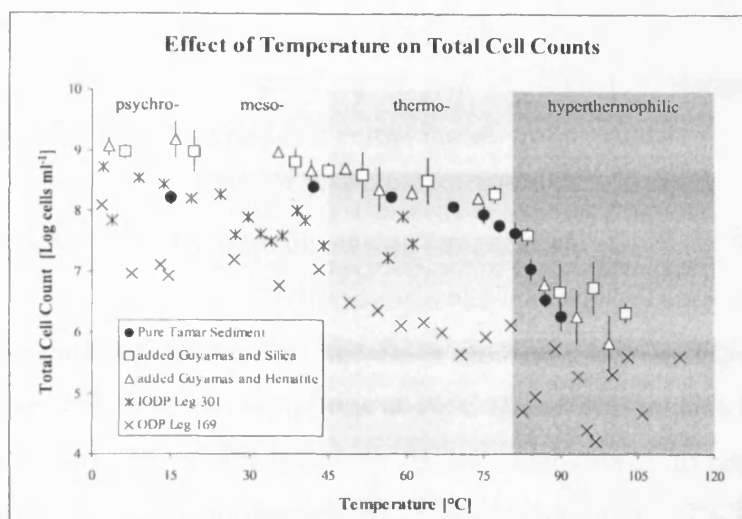
#### b) Cell counts of heating experiments compared to *in situ* studies of hot sediments

The development of total cell counts in different slurry heating experiments has been compared (see above). But how does the *in vitro* survivability of heated near-surface microbes compare to *in situ* cell counts?

Hydrothermal sediments from the north-east Pacific were sampled during ODP Leg 169 (Middle Valley, site 1035) and IODP Leg 301 (Juan de Fuca ridge, site U1301) and total cell numbers were determined in sediment layers with increasing temperatures (Cragg *et al.*, 2000 and Engelen *et al.*, 2008, respectively). Engelen *et al.* (2008) reported increasing sediment temperatures from ~2°C (1 mbsf) to 61°C (260 mbsf) at the eastern flank of the Juan de Fuca Ridge, spanning three temperature classes (psychro- to thermophilic).

A steeper temperature gradient was present at Middle Valley from 2°C near the surface to 113°C at 168 mbsf (Cragg *et al.*, 2000) also including the hyperthermophilic temperature class. Total counts from both sites are below total counts from slurry experiments (Fig. 5.18). Although total cell counts for Juan de Fuca sediments were higher than those for Middle Valley (Fig. 5.18) the percentages of cells loss with increasing temperature and depth were quite similar in the transition from psychrophilic to mesophilic temperatures (Table 5.10). However, for Middle Valley a greater cell loss was observed in the thermophilic range. The percentage of remaining cells (0.6%) is similar as observed in slurries amended with

hyperthermal inoculum (0.2% to 1%) but lower than in the pure Tamar sediment slurry (11%).



**Fig. 5.18: Effect of temperature of total cell counts.** Total cell counts from sequential heating experiment with “pure” Tamar sediment (this study) are compared to temperature gradient experiments with Tamar sediment amended with Basalt or Silica and 10% Guaymas Basin sediment (Cathal Linnane, PhD Thesis, Cardiff University) and to *in situ* cell counts of hydrothermal sediments from the Juan de Fuca Ridge (Engelen *et al.*, 2008) and Middle Valley (Cragg *et al.*, 2000). 95% confidence limits are given for slurry counts.

**Table 5.10: Changes in cell counts depending on temperature.**

Category	Temp. [°C]	Tamar slurry experiments			<i>In situ</i> studies	
		Pure	Guaymas and Silica	Guaymas and Hematite	ODP Leg 196	IODP Leg 301
Psychrophilic	< 20	100%	100%	100%	100%	100%
Mesophilic	20 – 45	180%	59%	54%	28%	25%
Thermophilic	46 – 80	78%	32%	21%	3%	15%
Hyperthermophilic	> 80	11%	1%	0.2%	0.6%	-

Decreasing cell counts with increasing temperatures were observed in sequential heating experiments and hydrothermal sediments. The latter showed lower total cell numbers than observed with simulated burial (up to 2.5 orders of magnitude). Reasons for this are probably the different times that cells are exposed to these high temperatures in combination with substrate availability. High temperature exposure times in the experiments were relatively short (100 to 343 days) and tidal mudflats contain organic matter (TOC ~0.9% dry sediment for Wadden Sea site NN, Freese *et al.*, 2008a), which is probably more accessible especially when heated, thus potentially feeding the prokaryotic population. In up to 111 million year old deep-sea sediments (Roussel *et al.*, 2008) the organic matter (1.45 wt%, IODP Leg 210,

Arnaboldi and Meyers, 2006) is most likely more recalcitrant and hence maintaining viability would be more challenging. Thus, over geological time spans more cells will die than during the short incubation times of heating experiments resulting in the lower numbers observed.

Nonetheless, there is a good agreement between the changes in total cell counts observed during mimicked and actual burial.

### c) Loss of FISH detectability with increasing temperature

It was already outlined above that the most dramatic loss in FISH detectability appeared between the first two samplings at 15°C (from 79% to 32% based on DAPI counterstain or from 13% to 8% based on AODC total count). In comparison FISH detectability only decreased moderately at higher temperatures (7% based on DAPI at 90°C).

Generally, it is virtually impossible to detect and phylogenetically identify 100% of a mixed population by using FISH. Biddle *et al.* (2006) approximately detected only 10% of the total prokaryotic population using general probes for *Bacteria* and *Archaea* yet concluded that the latter dominate the investigated sediment because a higher percentage of *Archaea* was detected. Hybridisation success depends on many variables (e.g. permeability, probe specificity) but mainly, as observed during starvation experiments (Chapter IV), on the physiological state of cells. Starved cells decrease their ribosomal content (Kjelleberg *et al.*, 1987; Fig. 4.48b) and although a number of cells might still be FISH detectable this does not predict their activity (e.g. *D. profundus*, Fig. 4.29c and 4.42c). However, if 90% of cells are not detected with FISH for whichever reason it cannot be concluded that these are inactive. A study on hydrothermal sediments (~80°C; Rusch and Amend, 2008) used a variety of probes (12 in total) to identify hyperthermophilic groups. Despite this effort only 49% of the total sediment microbial community were detected. The authors, however, did not calculate substrate formation rates, based on the presumably active cell count (FISH) but on the total cell count instead as they concluded that “the fraction of active cells can vary over time and FISH detectability does not depend on physiological activity alone”.

The decreasing FISH detectability observed during the sequential heating experiment is most likely a function of stressed or starved psychro- and mesophiles



reducing their ribosomal content or simply dying. It could also indicate that hyperthermophilic microorganisms, which might not be detected by the probe, assume an increasingly important role at higher temperatures. These prokaryotes would most likely be *Archaea* but not methanogens (due to the absence of methane production in slurry at higher temperatures) or possibly *Verrucomicrobia*, which have been found in hydrothermal environments (Schlesner *et al.*, 2006) but not *Plantomycetales* (Ward *et al.*, 2006), neither of which are detected with the probe EUBI (Daims *et al.*, 1999).

#### 4.2.3 Effects of increasing temperatures on microbial culturability

The estimation of culturable cells with increasing sediment slurry temperature showed the predominance of psychro- to mesophilic microorganisms even in samples from (hyper-) thermophilic regions (Fig. 5.15). The highest cultivation efficiencies based on AODC total counts were achieved for MPN incubations from 15°C samples (56 days) incubated at 15°C and generally in 40°C incubations (Fig. 5.19a, b, d). If the FISH count is used as a basis for calculating cultivation efficiencies the percentage values generally increase but the overall pattern does not change (Fig. 5.19c and e).

It appears that for heterotrophs two active populations of psychro- and mesophiles were present. This is indicated by a shift in cultivation efficiencies (Fig. 5.15d). High cultivation efficiencies in 15°C samples incubated at 15°C (0.1% to 0.7%) were followed by subsequent low culturability at higher temperatures (< 0.005%). The mesophilic population (MPNs incubated at 40°C) took over in samples above 42°C and showed highest cultivation efficiencies in samples from 57°C to 81°C (0.4% to 1.4%). Maybe a third group (thermophilic) then replaced the mesophilic population as 90°C samples incubated at 69°C also showed relatively high cultivation efficiencies (0.25%, Fig. 5.19d). This pattern is partly present in MPNs for methanogens and sulphate reducers (Fig. 5.19 a-b). Here, the highest cultivation efficiencies for 15°C MPN incubations were present in 15°C samples (~0.005% and 0.15%, respectively) and for 40°C MPN incubations in 42°C samples. Again, at higher slurry temperatures higher cultivation efficiencies were present in higher MPN incubation temperatures (e.g. for SRP medium, 90°C samples incubated

between 40°C and 90°C, 0.02% to 13%; methanogen medium, 90°C samples incubated between 40°C and 69°C, 0.01% to 0.1%). This suggests that a dormant (hyper-) thermophilic community must have been present in Tamar surface sediment, which was stimulated by increasing temperatures. Two likely candidates were detected with molecular biological methods: *Thermocladium modestius* (85%), an anaerobic, heterotrophic archeon and *Desulfotomaculum thermocisternum* (86%), a sulphate-reducing, spore-forming bacterium.

The presence of spore-forming prokaryotes in the slurry and their potential germination with increasing temperature is currently being investigated in our laboratory (by Dr. Louise O'Sullivan) and results are pending. It was, however, noted that even after heating the slurry to 90°C relatively high cultivation efficiencies were achieved at psychrophilic temperatures, which would indicate temperature-dependant germination of psychrophilic spores (equivalent to pasteurisation). Köpke *et al.* (2005) were able to revive and cultivate between 1080 (anaerobic) and 3170 (aerobic) spores g<sup>-1</sup> pasteurised sediment from Wadden Sea site NN (pasteurisation: 70°C for 15 minutes; incubation temperature 20°C). This equates to 0.0001% and 0.0004% revived spores of the total count, respectively, and to approximately 1% of the culturable cell count (Köpke *et al.*, 2005). Most cultivation efficiencies during the sequential heating experiment are several orders of magnitude above these estimates but mainly cultivation efficiencies of heterotrophs and sulphate-reducers incubated at 15°C fall in or below this range. Fichtel *et al.* (2008) quantified endospores in intertidal sediments of the Wadden Sea (sites NN and JS) via the detection of dipicolinic acid, a universal and specific component of bacterial endospores (Powell, 1953). They detected between 1×10<sup>5</sup> to 2×10<sup>7</sup> spores g<sup>-1</sup> sediment dry weight making up < 1% of total cell numbers in uppermost 50 cm of the sediment. If Tamar sediment contains similar numbers of spores, these could represent a significant source for culturable cells in MPNs from slurry samples at elevated temperatures, which were incubated at lower (spores of psychro- and mesophiles) as well as at higher temperatures (spores of thermophiles).

#### a) Cultivation efficiencies of heating experiments compared to *in situ* studies

There are a number of environments on the planet where steep temperature gradients exist (e.g. hot springs in the Yellowstone national park, Barns *et al.*, 1994;

hydrothermal vents and sediments in the deep sea, Pagé *et al.*, 2008, Teske *et al.*, 2002; and hot oil reservoirs, L'Haridon *et al.*, 1995). Although a variety of microorganisms have been cultivated from these habitats (e.g. de la Torre *et al.*, 2008; Dahle *et al.*, 2008), not many microbiological studies investigated the effects of the increasing temperature on culturability (e.g. Harmsen *et al.*, 1997).

Takai *et al.* (2004) investigated the microbial diversity of hydrothermal vent emissions at the Central Indian Ridge. They detected between  $10^3$  to  $10^6$  cells ml<sup>-1</sup> in 365°C hot fluids and were furthermore able to obtain isolates from some of the hottest samples. The culture collection was dominated by *Archaea* (especially hyperthermophilic methanogens) in hotter samples while their relative abundance decreased with decreasing sample temperature. Takai *et al.* (2004) concluded that a hydrogen-based hyperthermophilic subsurface lithoautotrophic ecosystem (HyperSLiME) exists beneath the hydrothermal field, with members of the *Thermococcales* being most frequently isolated but only representing 4% to 7.5% of the total cell counts. Cultivation efficiencies of 90°C slurry samples in methanogen medium were significantly lower than that (maximum 0.1% for incubations at 69°C, Fig. 5.19a).

Sievert *et al.* (1999) investigated the culturability of a variety of physiological groups (including sulphate-reducers) along a temperature gradient within a hydrothermal vent. They reported that both total and MPN count increased in the transition zone between hydrothermally influenced sediment and normal conditions (Table 5.11). Cultivation efficiencies for psychrophiles in sulphate-reducer medium are higher in slurry samples incubated at *in situ* temperature than observed by Sievert *et al.* (1999), 0.15% compared to 0.0004% to 0.03%, respectively. The same is true for mesophilic to thermophilic temperatures, 0.06% cultivation efficiency in slurry at *in situ* temperature (and 0.002% for 57°C samples incubated at 40°C, Fig. 5.19b) versus merely 0.00003% to 0.0003% in hydrothermal sediment. The higher cultivation efficiencies seen in slurry MPNs could be due to the additional presence of lactate in the medium used for MPN determination during this study, while Sievert *et al.* (1999) only used acetate and hydrogen (also used during this study).

**Table 5.11: Cultivation efficiencies for sulphate reducers from heating experiment compared with *in situ* studies.** Cultivation efficiencies are presented for slurry MPNs incubated at respective *in situ* slurry temperature and for two sites with differing distances from the centre of the hydrothermal vent (10 cm and 235 cm) incubated at near *in situ* temperature (Siefert *et al.*, 1999)

Sequential heating experiment		Hydrothermal vent sediment				
		10 cm from centre		235 cm from centre		
Temperature [°C]	Cult. Eff. [%]	Depth [cmbsf]	Temperature [°C]	Cult. Eff. [%]	Temperature [°C]	Cult. Eff. [%]
15	0.15	0 - 1	45	0.0003	20	0.03
40	0.06	1 - 2	50	0.00005	20	0.006
57	n.a.	2 - 3	55	0.00003	20	0.0004

A study by Engelen *et al.*, (2008) applied the MPN technique to estimate heterotrophic culturable prokaryotes in hydrothermal sediments from the Juan de Fuca ridge (IODP Leg 301, MPN incubation temperature 20°C). Their cultivation efficiencies showed no correlation with increasing depth or temperatures. Comparing their cultivation efficiencies to those from heating experiments (incubated at 15°C; Table 5.12) shows lower culturabilities at 14°C in the sediment compared to 15°C in the slurry (0.03% and 0.7%, respectively). At higher temperatures, however, cultivation efficiencies for sediment samples were higher than for the slurry experiment.

Cultivation efficiencies for heterotrophs in the original Tamar sediment were between 0.0003% and 0.01% of the AODC total count (Table 5.1). These values were systematically one order of magnitude lower than those reported by Köpke *et al.* (2005) for heterotrophs in Wadden Sea sediments site NN (Table 5.9). This discrepancy was most likely caused by different media used. The monomer medium used by Engelen *et al.* (2008) was similar to that of Köpke *et al.* (2005). Even if the observed discrepancy caused by different media is taken into account, the cultivation efficiencies at higher slurry temperatures still remain significantly below *in situ* values observed by Engelen *et al.* (2008).

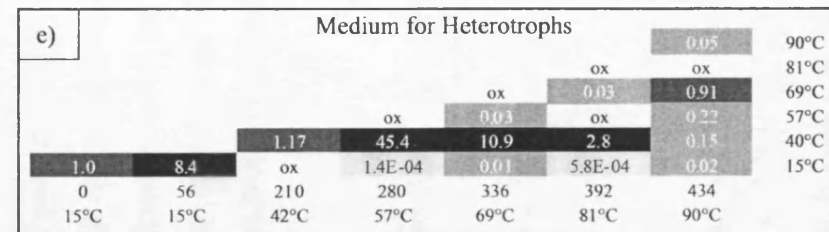
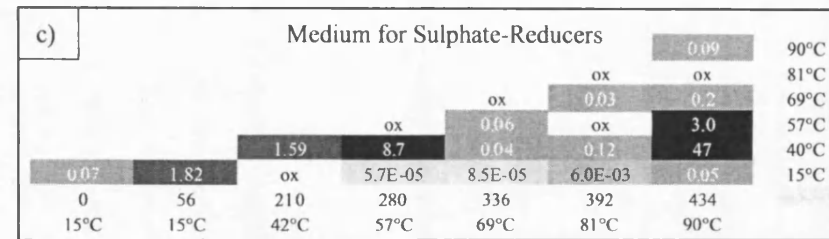
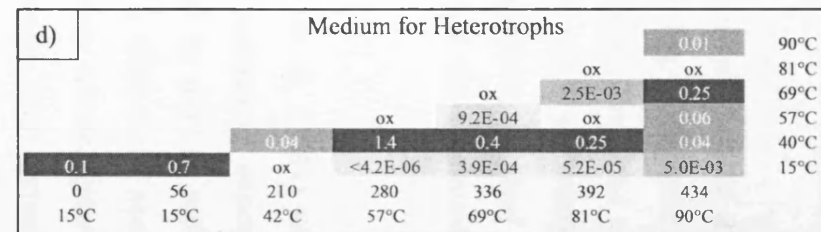
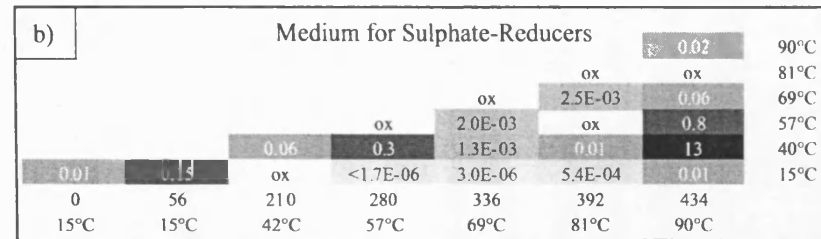
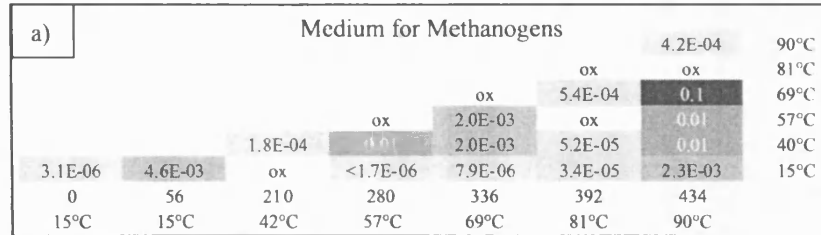
Cultivation efficiencies for heterotrophs during sequential heating experiments ranged between  $4.2 \times 10^{-6}\%$  and 1.4% of the total count. Thus it can be concluded that parts of the microbial community were stimulated to grow at increasing temperatures whilst other were negatively affected (e.g. psychrophiles, only little growth at temperatures above 15°C).

### Cultivation Efficiencies [%] based on AODC total count

### Cultivation Efficiencies [%] based on FISH count

Slurry incubation Time [days] and Temperature [°C]

Slurry incubation Time [days] and Temperature [°C]



Legend: oxidised <0.001 0.001-<0.01 0.01-<0.1 0.1-<2 >2

Fig. 5.19: Cultivation efficiencies during sequential heating experiment in different media based on AODC total counts and FISH counts.

**Table 5.12: Cultivation efficiencies for anaerobic heterotrophs from heating experiment compared with *in situ* studies.** Cultivation efficiencies are presented for slurry MPNs incubated at 15°C and at respective *in situ* temperature. Incubation temperature for IODP Leg 301 samples was 20°C (Engelen *et al.*, 2008).

Sequential heating experiment			IODP Leg 301		
Temperature [°C]	Cult. Eff. [%] at 15°C	Cult. Eff. [%] at respective <i>in situ</i> temperature	Depth [mbsf]	Temperature [°C]	Cult. Eff. [%] at 20°C
15	0.7	0.7	52	14	0.03
40	n.a.	0.04	169	40	< 1
57	$< 4.2 \times 10^{-6}$	n.a.	240	56	2.7
69	0.0004	n.a.	260	61	0.7

#### 4.2.4 Metabolic activity of “aged” sediment microbial community compared with viability and culturability

Potential metabolic activities of the microbial community were determined at 90°C after sequential heating of the Tamar sediment slurry. At this stage culturable cells were still detected in the three media used (240 cells ml<sup>-1</sup> for heterotrophs, 460 cells ml<sup>-1</sup> for sulphate-reducers [FeS precipitation], and ~8 cells ml<sup>-1</sup> for methanogens). FISH detectability was at a minimum with merely 7% of DAPI stained cells but 27% of AODC total counts detected (Fig. 5.7 and 5.10). Both the presence of culturable and FISH detectable cells is an indication for maintained viability despite increasing temperature stress.

Radioactively labelled lactate and glucose were metabolised at higher rates (3.3 pmol ml<sup>-1</sup> d<sup>-1</sup> and 2.0 pmol ml<sup>-1</sup> d<sup>-1</sup>, respectively; Fig. 5.16a and c) than acetate (0.7 pmol ml<sup>-1</sup> d<sup>-1</sup>; Fig. 5.16b). Glucose was most likely fermented to acetate and CO<sub>2</sub> before the produced acetate was also mineralised to CO<sub>2</sub>, potentially by sulphate reducers. The presence of sulphate reducers was confirmed by final MPNs using medium targeting SRP (FeS precipitates present) and the continued removal of sulphate throughout the experiment (Fig. 5.6).

Almost identical processes were observed by Tor *et al.* (2003) who subjected sediments from a shallow hydrothermal vent to radiotracer experiments (also incubated at 90°C). These sediments were dominated by sulphate reduction as the terminal oxidation process, which accounted for up to 63% of the acetate removal. Tor *et al.* (2003) detected glucose fermentation to mainly acetate, hydrogen and CO<sub>2</sub> before the acetate was also removed by sulphate reducers. Both acetate and glucose

were metabolised within 24 hours whereas during the Tamar slurry experiments this was only observed for glucose while acetate showed a lag phase (< 9 days). Unfortunately, the authors did not calculate metabolic rates of substrate removal.

A subsequent study on sediment incubations in specifically designed culture media by Rusch and Amend (2008) revealed that predominantly heterotrophic *Bacteria* (genus *Thermus*) but also *Archaea* metabolised glucose to acetate (production rate: 18 fmol cell<sup>-1</sup> day<sup>-1</sup>) and lactate (production rate 2.6 fmol cell<sup>-1</sup> day<sup>-1</sup>). The *Archaea* then also metabolised the lactate (5.6 fmol cell<sup>-1</sup> day<sup>-1</sup>). The production rates of acetate and CO<sub>2</sub> from the Tamar glucose slurry were five orders of magnitude lower (0.6 and 0.3 amol cell<sup>-1</sup> day<sup>-1</sup>, respectively) than those reported by Rusch and Amend (2008). The same applies to the lactate consumption rate (1.7 amol cell<sup>-1</sup> day<sup>-1</sup>). The rates of Rusch and Amend (2008), however, were not obtained from *in situ* radiotracer studies but from the added substrate in the media. Thus it is not surprising that these rates are higher than observed for the heating experiment in this study as substrate was present in excess but limited in the latter.

In the radiotracer slurry experiments, glucose and lactate were metabolised at similar rates compared to potential, minimum rates observed in starved cultures (for glucose 2.0 and 1.1 pmol ml<sup>-1</sup> d<sup>-1</sup>, respectively; for lactate 3.3 and 2.0 pmol ml<sup>-1</sup> d<sup>-1</sup> [without strain R002], respectively; Chapter IV Table 4.4). Furthermore, these rates are comparable to *in situ* rates of marine sediments determined at psychrophilic temperatures (e.g. Mediterranean Sea, Coolen *et al.*, 2002 and Atlantic Ocean, Wirsén and Jannsch, 1986; see Chapter IV Table 4.4). This underlines the transferability of results obtained in sequential heating experiments to processes in the deep biosphere.

## 5. Summary and Conclusions

This study is the first comprehensive investigation into viability, culturability, and potential metabolic activity of a near-surface microbial community exposed to increasing temperatures to mimic burial as it occurs over millions of years in marine sediments.

The experiments revealed the resilience of the psychro- and mesophilic community towards high temperatures. This was shown: i) by the presence of significant total cell numbers compared to other slurry experiments with hydrothermal inoculum and *in situ* studies (Fig. 5.18) and ii) by detectable culturability in samples from (hyper-) thermophilic regime incubated at lower temperatures (Fig. 5.15) and similar cultivation efficiencies compared to *in situ* studies (Table 5.12). However, FISH detectability using the eubacterial probe EUBI decreased rapidly (Fig 5.7), which might indicate the predominance of non-methanogenic *Archaea* or *Verrucomicrobia*. During this project, the contribution and of spore-forming microorganisms to total cell counts and culturability was not assessed. However, quantification of spores in the slurry is currently being conducted. As experimental design resembles pasteurisation, culturability at low temperatures after heating above 70°C is most likely linked to germination of spores.

Radiotracer experiments confirmed the viability of the stressed microbial community and rates of substrate utilisation were surprisingly similar to those of starved, pure cultures but significantly lower than of a culture-based study with marine sediment at comparable temperatures (Rush and Amend, 2008; see 4.2.4). This shows that an *in situ* microbial community is much better adapted to harsh conditions than it can be mimicked with sequential heating experiments.

Despite shorter time-spans compared with actual *in situ* processes in the deep subsurface, parallel developments were shown (e.g. loss of cell numbers and culturability). Acetate produced from recalcitrant organic matter and/or organic molecules produced by autotrophic organisms such as acetogens or methanogens potentially stimulate activity of the entire population at elevated temperatures (Jorgensen and Bak, 1991; Wellsbury *et al.*, 1997; Sievert *et al.*, 1999) and help maintain cellular integrity and viability. These processes might also be responsible for providing deep subsurface microbial communities with energy over geological timescales (Wellsbury *et al.*, 1997).

It can be concluded that sequential heating experiments are a good way of assessing the *in situ* challenges that marine sedimentary microorganisms face. Future experiments should include MAR-FISH or stable isotope probing to phylogenetically identify the active microorganisms at certain stages of incubation.



## **Chapter VI:**

### **General Discussion**

#### **1. Introduction**

The deep biosphere is possibly the largest prokaryotic habitat on the planet (Whitman *et al.*, 1998). Despite organic matter being present, cells most likely starve due to its recalcitrance, especially in deeper layers (Hedges and Keil, 1995). However, prior to this study, the ability to endure starvation has not been investigated on pure cultures of anaerobic sedimentary microorganisms. Furthermore a number of techniques are commonly applied to mixed microbial communities in the deep biosphere investigating abundances, viability, and activity without ever having been thoroughly tested on starving isolates from this environment. This hitherto existing lack of knowledge necessitated the experiments conducted during this project. The main objectives were:

- To isolate novel deep biosphere prokaryotes from one of the most organic matter-poor, marine habitats (Challenger Mound, IODP Leg 307).
- To then investigate the survivability of the obtained isolates alongside other representative deep biosphere strains as well as selected near-surface relatives in long-term, anaerobic, starvation-survival experiments.
- To assess any potential differences in the starvation responses of the pure cultures, applying commonly used methods of cell detection, viability, and activity used in deep biosphere research and to test their effectiveness.
- To furthermore investigate the changes in viability and culturability of a near-surface microbial community subjected to increasing temperature stress as it occurs during burial in deep marine sediments with the same methods applied to pure cultures.

In the beginning of this chapter the main findings are summarised. This is followed by a general discussion trying to link principal themes stretching through the three main chapters. Finally, future work is recommended.

## 2. Summary of main findings

During the course of this project knowledge of the deep biosphere was expanded via the isolation of novel deep biosphere strains from one of the most oligotrophic marine habitats ever investigated (a cold-water coral, buried, coral carbonate mound). Three genera of the *Gammaproteobacteria* were obtained directly from the mound. Besides *Shewanella* and *Vibrio* strains, a representative of the genus *Raoultella* was isolated from within the mound structure. *Raoultella* species were previously not known to inhabit marine sediments. The culture collection of IODP Leg 307 consisted mainly of facultative anaerobes (14 strains), one microaerophilic strain (*Arcobacter* sp.), one obligate anaerobe (*Desulfovibrio* sp.) and three obligate aerobes (*Pseudomonas* spp. and *Ornithinimicrobium* sp.). Amongst them is putatively a new species belonging to the genus *Ornithinimicrobium*. However, the marine origin of all strains was supported by the determined temperature (< 2°C to ~40°C) and salinity ranges ( $\geq 3.5\%$ ) which allowed growth at near to *in situ* conditions. Furthermore, all strains used a variety of substrates and electron acceptors and produced a range of extracellular enzymes, which potentially could aid growth and survival in deep marine sediments.

Subsequently, selected isolates were subjected to long-term starvation experiments alongside other typical cultured deep biosphere representatives and their near-surface relatives. To the best of my knowledge, this is the first study that has comprehensively examined the starvation-survival of facultative and obligately anaerobic marine sedimentary bacteria. All isolates were able to maintain a high level of viability as indicated by generally constant total cell counts, and generally low, but slowly increasing proportions of dead cells in the cultures throughout starvation (270 days to up to three years). For some strains FISH detectability decreased continuously, while it stabilised in others after an initial decrease. Similar patterns occurred for culturability throughout starvation but culturability rarely became zero. No significant correlations between FISH detectability and other measures of viability occurred. Instead starvation time was significantly positively correlated with percentages of dead cells and significantly inversely correlated with culturability ( $p < 0.05$ ). After starvation, potential metabolic rates were determined and surprisingly a number of strains were able to utilise the added low substrate

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concentrations (glucose 3.8 nM, lactate, 0.4 nM) very rapidly, (within a few hours, fastest rate was 33.3 pmol ml<sup>-1</sup> d<sup>-1</sup>) without an apparent lag phase, while other strains showed very low activities (lowest rate was 0.001 pmol ml<sup>-1</sup> d<sup>-1</sup>). No differences were observed for starvation survivability between near-surface and deep biosphere isolates. Instead it appears that different phylogenetic groups responded differently to starvation. Techniques, such as LIVE/DEAD<sup>®</sup> staining and FISH, commonly applied to investigate the microbial viability in marine sediments were assessed in regard to their effectiveness. Generally, a variety of different starvation responses of the pure cultures was detected using these measures of viability. The results will help to better understand viability assessments of mixed microbial communities in the future. Some of the dis-/advantages of the methods have already been discussed but will be more thoroughly evaluated in this chapter.

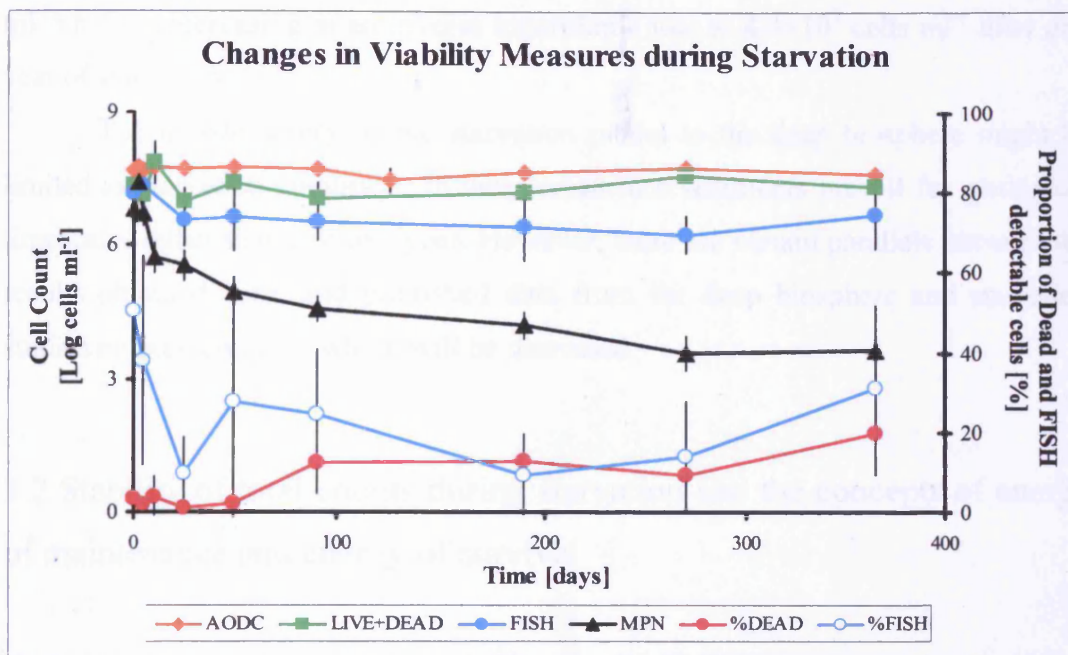
Furthermore, for the first time an exhaustive investigation into the viability and culturability of a near-surface sediment microbial community during increasing temperatures was conducted. The sequential heating experiments simulate temperature stress as it occurs during burial in marine deep sediments. Total cell counts were inversely correlated with increasing temperature and significantly decreased during mimicked burial. Although viability (detection with FISH) initially decreased rapidly it was maintained at a low level even at higher temperatures (90°C). The presence of active cells was also supported by the detection of culturable cells across the entire temperature range even at highest slurry temperatures (90°C). Cultivation results indicated that several subpopulations of psychro-, meso-, and thermophiles existed and were active at different times and temperatures throughout the experiment. This indicates a relatively rapid adaptation of the *in situ* microbial community to changing environmental factors, in this case temperature. Concluding experiments using radioactively labelled substrates (glucose [1.5 pM], lactate [4.3pM], acetate [8.2 pM]; incubated at 90°C) revealed measurable metabolic rates, similar to those in starving cultures. These experiments confirmed that (metabolic) activity was maintained in at least a subpopulation of the originally psychro- to mesophilic microbial community at increased temperatures (90°C). The contribution of germinated spores to the detected viability and culturability remains to be illuminated.

### **3. Suitability of the methods used to investigate starvation-survival and transferability of pure culture results to *in situ* deep biosphere communities**

In this section observations and patterns of starvation-survival revealed during this study will be critically assessed in regard to the robustness of the methods used, integrated with published data on pure culture starvation experiments and linked, where possible, to results of deep biosphere research. Concomitantly, differences in starvation responses of individual strains will be discussed in relation to a model that combines data of all strains and serves as a template for the “standard” starvation response of environmental, sedimentary bacteria. This model was already presented in parts during the discussion in Chapter IV but will be shown in its entirety here (Fig. 6.1).

#### **3.1 Model for starvation response in environmental strains**

A mixed microbial community in the environment consists of cells exhibiting different physiological states. All assessments of this community (e.g. metabolic rates or culturability) will, therefore, be determined as an average of active, dormant, or even dead cells. In the deep biosphere where starvation is commonplace due to highly recalcitrant organic matter (Jorgensen and D’Hondt, 2006), this might lead to the underestimation of the activity of the actual viable and active proportion of the community present. During starvation experiments different measures of viability were applied to pure cultures to investigate the different responses to substrate limitation of typical representatives of deep biosphere isolates. Some of these differences were discussed in Chapter IV and will be again below. If, however, all the data from individual strains and starvation treatments is combined, this would be similar to the results obtained when investigating a mixed microbial community and represent a model for the “standard” starvation response of environmental, sedimentary bacteria. This model, however, excludes possible *in situ* microbial interaction such as syntrophy or quorum sensing.



**Fig. 6.1: Changes in viability measures during starvation.** Averaged data from all strains and starvation treatments is presented to give an overview of their response towards starvation as it was assessed using different measures of viability. 95% confidence limits are given for AODC, LIVE, FISH counts, and for MPNs, if not visible they fall within the presented data point. Percentages of dead cells were calculated from the sum of “live” and “dead” scored cells while the proportion of FISH detectable cells was calculated from AODC total counts due to poor DAPI staining during starvation. For more detailed description refer to text.

In this model artificial microbial community, no significant change in AODC total counts occurred during starvation (on average  $5.1 \times 10^7$  cells ml<sup>-1</sup>;  $p < 0.05$ ; Fig. 6.1). The LIVE/DEAD<sup>®</sup> total counts were on average 49% of the AODC total counts ranging between 19% and 129% but also no significant change occurred with time ( $p < 0.05$ ). The proportions of dead cells in the cultures, however, increased significantly from  $< 4\%$  and almost linearly during starvation to a maximum of 20% after one year (based on LIVE/DEAD<sup>®</sup> counts;  $p < 0.05$ ; Fig. 6.1). The FISH counts decreased significantly between  $t_0$  and 25 days of starvation (from  $3.9 \times 10^7$  to  $7.8 \times 10^6$  cells ml<sup>-1</sup>). Subsequently, they remained relatively stable (average  $1.6 \times 10^7$  cells ml<sup>-1</sup>) until an increase occurred for the final sample (to  $3.3 \times 10^7$  cells ml<sup>-1</sup>). FISH detectability based on AODC total counts decreased significantly and sharply between  $t_0$  and 25 days of starvation (from 50% to 10%;  $p < 0.05$ ). However, subsequently the proportions of FISH detectable cells were very variable (between 9% and 28%) as indicated by high confidence limits. Like the FISH count, the percentages of FISH detectable cells increased in the final sample (31%). Culturability initially increased (MPNs from  $6.6 \times 10^6$  cells ml<sup>-1</sup> at  $t_0$  to  $2.6 \times 10^7$  cells

ml<sup>-1</sup>) before decreasing at an inverse logarithmic rate to  $4.4 \times 10^3$  cells ml<sup>-1</sup> after one year of starvation.

The transferability of the starvation model to the deep biosphere might be limited as starvation conditions in deep subsurface sediments prevail for geological timescales rather than just one year. However, there are certain parallels between the results obtained here, and published data from the deep biosphere and starvation studies on pure cultures, which will be discussed.

### 3.2 Stability of total counts during starvation and the concepts of energy of maintenance and energy of survival

The most obvious discrepancy between the model of starving pure cultures (Fig. 6.1) and the deep biosphere is the lack of significantly changing total cell counts during starvation observed with both AODC and the LIVE/DEAD<sup>®</sup> Kit. A reduction in total cell counts commonly occurs on a logarithmic scale in the marine sediments (Parkes *et al.*, 1994). This is a clear indication that during the experiments conducted here, microbes were not stressed beyond their capabilities of maintaining an intact cell that contained DNA, which is recognised by nucleic acids stains such as AO and Syto<sup>®9</sup>.

Stable total counts occurred in almost all strains and starvation treatments. Sometimes an initial increase in cell counts was observed in treatments “C” (unwashed cells). This was, however, most likely related to the consumption of substrate, which was still present in the medium as indicated by VFA production during the time of increase (e.g. *Photobacterium* sp. S11 Fig. 4.10; *Raoultella* sp. F17VIII, Fig. 4.15; *Desulfovibrio* sp. F16I, Fig. 4.26). Thus from the four different types of total cell count developments during starvation reported by Morita (1985) only one occurred during this project (Chapter I, Fig 1.3, pattern B). This pattern was previously only observed by Jones and Morita (1985) who starved a marine ammonium oxidiser (*Nitrosomonas* sp. 4W30) for 25 weeks. However, all other patterns summarised by Morita (1985) also occurred in marine isolates (e.g. pattern A, in five unidentified isolates, Amy and Morita, 1983b; pattern C, in *Vibrio* sp. Ant-300, Novitsky and Morita, 1977 and in a *Pseudomonas* sp. Kurath and Morita, 1983; pattern D, in several unidentified isolates, Amy and Morita, 1983b and in *Vibrio* sp.

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CNPT-3. Rice and Oliver, 1992). Hence, the development of total counts during starvation differed amongst different phylogenetic groups despite their common origin (all marine strains). During this project, however, stable total counts occurred in all isolates and thus this development seems to be independent of phylogeny.

### 3.2.1 Energy of maintenance and energy of survival

The occurrence of stable cell concentrations during long-term starvation (up to three years) raises questions about maintenance energy requirements of these environmental strains. As extraneous substrates were depleted in starving cultures (Chapter IV) cells must have used endogenous substrates to maintain cellular integrity and function. Morita (1997), however, distinguished between energy of maintenance and “energy of survival” in starving bacteria. The former is required for osmotic regulation, maintenance of intracellular pH, turnover of macromolecules and even motility (Price and Sowers, 2004) while the energy of survival is solely used for the repair of damage to macromolecules (e.g. amino acid racemisation and DNA depurination; Morita, 1997). Price and Sowers (2004) compared and grouped metabolic rates depending on their energy requirements. In addition to the group of metabolic rates typical for bacterial growth they confirmed the existence of Morita’s proposed groups of metabolic rates during starvation. Metabolic rates sufficient for maintenance were up to three orders of magnitude below rates for growth. The third group of rates, which allows mere survival, was a further two to three orders of magnitude below rates for maintenance. Experimental and calculated data from bacteria trapped in ice and marine sediments clustered above energy requirements needed to avoid DNA depurination and below values where amino acid racemisation begins, hence rates of repair of molecular damage are similar to the rate at which the damage occurs (Price and Sowers, 2004). The authors concluded that due to extremely low energy expenditures during the “phase of metabolic arrest” (Morita, 1997) microbial communities are able to survive “indefinitely” and this at temperatures as low as -40°C. Tijhuis *et al.* (1993) calculated maintenance energy requirements for anaerobic microorganisms to be  $127 \text{ J (g dry biomass)}^{-1} \text{ h}^{-1}$  at 25 °C. However, other studies have estimated this to be two to three order of magnitude lower (Peters *et al.*, 1998; Scholten and Conrad, 2000). Despite my best efforts, I did

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not find a study that revealed how much energy can actually be obtained from the anaerobic metabolism of endogenous substrates (i.e. cellular constituents). Thus, investigations are needed to quantify the obtainable energy from these compounds under anaerobic starvation conditions for upkeep of cellular maintenance, which delays the phase of metabolic arrest. However, Morita (1997) described “energy of survival” as a phase where endogenous metabolism is in all probability absent (analogous to the bacterial spore), cells are dormant, and even die. He also stated that cells of *Vibrio* sp. ANT-300 entered this phase after 70 days of starvation. Following Morita’s (1997) definition that starving cells are not dormant as they possess the ability to take up utilisable substrates immediately, only a few starving cultures might have reached the phase of metabolic arrest during this study (lag phase cultures during <sup>14</sup>C-radiotracer experiments, Chapter IV). According to the rates of Price and Sowers (2004) for energy of maintenance and energy of survival it would take a cell between ~1.1 and ~1100 years at 25°C and between ~6.3 and 11,000 years at 10°C to completely digest itself. Although this is a very abstract concept and the maximum values are unlikely to occur for a number of reasons, it shows that the rates for energy of maintenance must be even lower than estimated by Price and Sowers and thus closer to values for metabolic rates of energy of survival. This is supported by the fact that some of the cultures starved during this project were still in the phase of energy of maintenance, according to Mortita’s definition, when subjected to radiotracer experiments and had surpassed the lower theoretical threshold (1.1 years) at the time of analysis for potential metabolic activity (e.g. *Marinilactibacillus* sp. G8a3, and *Acetobacterium* sp. R002).

### 3.3 Applicability of the LIVE/DEAD<sup>®</sup> Kit and mortality during starvation

Differences between total count estimates using AO and the LIVE/DEAD<sup>®</sup> Kit were observed in almost every sample during starvation and sequential heating experiments. A main difference between these two cell counts was the sample fixation conducted for AODC while the LIVE/DEAD<sup>®</sup> count was performed shortly after sampling without fixation. Fixation using aldehydes leads to a permeabilisation



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(Lebaron and Joux, 1994) and thus allows AO to enter cells without hindrance potentially caused by the cell membrane. Although Syto<sup>®</sup>9 is a cell permeant dye, maybe certain cell membrane characteristics such as increased hydrophobicity (Kjelleberg and Hermansson, 1984) or decreased fluidity caused by starvation (Rice and Oliver, 1992), negatively affect penetration and thus result in lower numbers determined. However, it was noted for some strains (e.g. *Ornithinimicrobium* sp. F18IV, Fig. 4.3; *Pseudomonas* sp. NA101, Fig. 4.12; *Shewanella* SCSA3; Fig. 4.17; *Arcobacter* p. F17IX, Fig. 4.31) that the LIVE/DEAD<sup>®</sup> total count actually increased with time, contradicting these assumptions. No correlation between LIVE/DEAD<sup>®</sup> total count and the proportion of dead cells during this time was apparent. Some reports, however, suggested that with continued starvation the membrane fluidity increased due to the presence of short-chain fatty acids (C<sub>12:0</sub> and C<sub>14:0</sub>, *Vibrio* sp. S14, Malmcrona-Friberg *et al.*, 1986) or increased proportions of unsaturated fatty acids (C<sub>16:1</sub>, *Vibrio* sp. ANT-300, Oliver and Stringer, 1984). This could explain better staining of starved cells with Syto<sup>®</sup>9 leading to total cell counts more comparable to AODC. Species or even strain specific variations in rearranging the cell membrane in response to starvation could explain why detectability increased in some strains but not in others.

### 3.3.1 Absence of mortality during starvation experiments?

During starvation the proportions of dead cells would sporadically be increased at some timepoints but not at subsequent samplings (e.g. *Ornithinimicrobium* sp. F18IV “B”, Fig. 4.3; *Shewanella* sp. SCSA3, Fig. 4.17; *Arcobacter* sp. F17IX “C”, Fig. 4.31). As no significant changes in AODC total counts occurred, these “dead” cells might have been falsely labelled as such. It was already discussed that as a response to starvation membrane alterations are commonplace (Malmcrona-Friberg *et al.*, 1984; Oliver and Stringer, 1984; Rice and Oliver, 1992). Maybe just the restructuring of the membrane itself allowed PI to temporarily enter otherwise intact cells. However, metabolic activity, as indicated by the production or consumption of VFAs, did not seem to be connected to these sudden increases in dead cells. In addition, it was noticed that especially Gram-positive bacteria showed elevated numbers of dead cells (e.g. *S. marinus*, >80% after 180 days, Fig. 4.1;

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*Acetobacterium* sp. R002, >90% after 365 days, Fig. 4.33; comparison between groups, Fig. 4.53 a) despite stable total counts during starvation experiments. Potentially membranes of these microorganisms became more permeable to PI than those of Gram-negative bacteria. Furthermore, Davey and Hexley (2010) reported that membranes of stressed *Saccharomyces cerevisiae* cells were temporarily PI permeable. On average 7% of “dead”-scored cells had the ability to repair their membrane and were subsequently not permeable to PI. They conclude that it is possible for cells to be “red but not dead”.

### 3.4 Patterns of FISH detection during starvation

During starvation of isolates, different patterns of FISH counts and detectability occurred. Strains belonging to the first category (e.g. both *Acetobacterium* strains, Fig.4.32c and 4.33c; *Desulfovibrio* sp. F16I, Fig. 4.26c; *Shewanella* sp. F17V, Fig. 4.2 c; *Raoultella* sp. F17VIII, Fig. 4.15c) showed almost no decrease in FISH detection during starvation up to one year (no significant changes for *Acetobacterium* sp. R002 and *Desulfovibrio* sp. F16I). However, this was not correlated to metabolic activity during radiotracer experiments (e.g. no activity in *Desulfovibrio* sp. F16I, Fig. 4.42d; medium rate in *A. malicum* [ $0.014 \text{ pmol ml}^{-1} \text{ d}^{-1}$ ], Fig. 4.44a; high rates in others [ $> 3.2 \text{ pmol ml}^{-1} \text{ d}^{-1}$ ], Fig. 4.39b, 4.40b, and 4.44b) or growth (consistently low culturability in *Desulfovibrio* sp. F16I, always below  $10^4 \text{ cells ml}^{-1}$ , Fig. 4.26; consistently high culturability in *Raoultella* sp. F17VIII, always above  $10^4 \text{ cells ml}^{-1}$ , Fig. 4.15). Approximately one third of the strains followed the second pattern, which thus resembles the representative pattern observed for investigated strains (Fig. 6.1; e.g. *Arcobacter* sp. NA105, Fig. 4.30c.; *Pseudomonas* sp. F18V, Fig. 4.14c). In this pattern FISH detection initially decreased, for most strains within the first 50 days of starvation, and remained relatively stable thereafter. The third pattern is an inverse logarithmic decrease of FISH detection (e.g. *D. profundus*, Fig. 4.29c; *Shewanella* sp. SCSA3, Fig. 4.17c) whereby the asymptotically approached minimum was reached after ~100 days of starvation. The final pattern was a linear decrease but different rates of loss of FISH detectability occurred in different strains (e.g. *Photobacterium* sp SAMA2, Fig. 4.4c, 12% per year; *Marinilactibacillus* sp. G8a3,

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Fig.4.35 c. 60% per year; *Arcobacter* sp. F17IX, Fig. 4.31c, 91% per year). For none of the last three patterns a correlation to metabolic activity or growth was apparent.

### 3.4.1 FISH as a tool to assess viability in pure culture experiments and the role of ribosomes during starvation – “To degrade, or not to degrade: that is the question”

Ribosomes play a crucial role in protein synthesis but consist themselves of protein and RNA thus representing a valuable energy resource for endogenous metabolism. It has been shown that at the beginning of starvation the intracellular protein and RNA content is reduced as a whole in aerobically starved bacteria (Boylen and Ensign, 1970; Amy and Morita, 1983a; Hood *et al.*, 1986), and in addition, that polysome levels also decrease (Dresden and Hoagland, 1967). Although at the onset of starvation *de novo* protein synthesis decreases logarithmically (Almiron *et al.*, 1992; Rockabrand *et al.*, 1995) certain proteins are produced for the first time (starvation induced proteins; Jaan *et al.*, 1986; Givskov *et al.*, 1994a; Jewett *et al.*, 2009). This *de novo* protein synthesis is a significant process during the adaptation to substrate limitation and it has been shown that it amounts up to twice the mass of the bacterial cell (during 35 days of starvation, Rockabrand *et al.*, 1995). As ribosomes present an excellent substrate for long-term endogenous metabolism (Boylen and Ensign, 1970; Morita, 1997) starving bacteria need to strike a balance between reducing the ribosomal content to partly gain energy for restructuring processes during the starvation response and maintaining a certain level of ribosomes for *de novo* protein syntheses to firstly enable these changes, and to secondly, be able to quickly respond to sudden substrate availability.

If the FISH signal is a proxy for cellular ribosome content, several different patterns of ribosomal degradation were observed in different strains during starvation experiments (see section 3.4 and Chapter IV). Stable FISH counts indicate the lack of an appreciable decrease in ribosomal content and thus some strains maintain a large excess of ribosomes throughout starvation (Koch, 1971) that was sufficient for active growth, (e.g. four strains during this study, see above; *E. coli*, Yamagashi *et al.*, 1993; *P. putida*, Givskov *et al.*, 1994b). Other strains decreased their ribosomal content to possibly fuel the above mentioned cellular changes during starvation. Even when ribosome content was reduced, it most likely still remained above the

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level necessary for protein synthesis during starvation (Flårdh *et al.*, 1992; Givskov *et al.*, 1994b). During this project FISH detection did not cease completely for any strain investigated, but the minimum proportions of cells detected with FISH were 0.05% of the AODC total count in *Shewanella* sp. F17V ( $\sim 10^4$  FISH detectable cells  $\text{ml}^{-1}$  after 90 and 343 days of starvation). This strain, however, metabolised added radioactive glucose rapidly without an apparent lag phase during radiotracer experiments at a rate of  $3.2 \text{ pmol ml}^{-1} \text{ d}^{-1}$  (Fig. 4.40b). This highlights that FISH cannot be used to predict metabolic activity in starving pure cultures and therefore probably also not in the deep biosphere. It would be interesting to know why some cells in pure cultures (clones with identical genomes) decreased their ribosomal content below the FISH detection limit ( $\sim 370$  rRNA molecules per cell in a pure culture of *E. coli*, Hoshino *et al.*, 2008) while others did not.

Some studies have shown that the growth conditions prior to starvation have an impact on survivability during substrate limitation (e.g. substrate concentrations of the medium and thus growth rate; Rogers and Zilm, 1995; Eguchi *et al.*, 1996; Oda *et al.*, 2000). During this study relatively substrate-poor media (for heterotrophs:  $\sim 1.2$  ml glucose, 0.06 g peptone, 0.03 g yeast extract per litre medium; for SRBs and acetogens:  $\sim 20$  mM lactate) were used for general maintenance of isolates and for biomass production prior to starvation experiments. Hence, strains probably grew at a low rate during which they had time to adapt to limiting conditions potentially leading to increased survivability during starvation.

Although growth conditions prior to starvation might have a possible impact on subsequent starvation-survival, within a pure culture, however, these conditions should be almost identical. Thus there must be also a genetic element involved (maybe a stochastic one) that regulates whether and so, by how much a cell decreases its ribosome content upon substrate limitation. This underlying maybe random mechanism could potentially cause the weak correlations between FISH and other measures of viability (e.g. dead cells, culturability, and metabolic activity; Chapter IV, 4.1.2), which was also observed by Oda *et al.* (2000).

The different patterns of FISH count development and the underlying mechanisms controlling the intracellular ribosomal content obviously have a great impact on all *in situ* studies using FISH to determine microbial viability in the environment. For example, growth conditions are likely to differ tremendously for different members of the community at different times let alone the different

durations that these members might have been exposed to starvation (e.g. geological timescales in the deep biosphere). Hence, estimates of community compositions based on FISH detection (e.g. *Bacteria* versus *Archaea*) should be considered with great caution given that even in pure culture studies it is unclear why some clonal cells cannot be detected with this method (up to 99.95%) while others maintain a high enough ribosome content for detection during long-term starvation.

### 3.4.2 FISH as a tool to investigate viability in the deep biosphere

Both Schippers *et al.* (2005) and Biddle *et al.* (2006) showed relatively stable distributions of FISH and CARD-FISH detectable cells throughout the sediment column, although reporting different domains of prokaryotes to be more abundant using their respective methods. Schippers *et al.* (2005) approximately detected  $10^5$  to  $10^6$  bacterial cells  $\text{cm}^{-3}$  sediment up to 420 mbsf but no *Archaea* (CARD-FISH; ODP Leg 201, sites 1225, 1226, 1227, and 1230; probes Arch915-HRP and EUB-HRP). Biddle *et al.* (2006) reported average archaeal cell numbers of  $2.9 \times 10^6$   $\text{cm}^{-3}$  sediment in samples from ODP Leg 201 (Sites 1227, 1229, and 1230; minimum depth, 0.7 mbsf; maximum depth, 121.4 mbsf; probe Arch915-Cy3). These patterns are very similar to the stable average FISH counts in starving cultures between 25 and 365 days ( $1.8 \times 10^7$  cells  $\text{ml}^{-1}$  equal to 20% of AODC total count; Fig. 6.1) and in sequential heating experiments between 15°C and 69°C ( $7.2 \times 10^6$  cells  $\text{ml}^{-1}$  equal to 28% of AODC total count; Fig. 5.7). Despite the apparent stability of FISH detectability in both pure culture and mixed microbial community experiments, other measures of viability (e.g. culturability and metabolic activity) often varied and could thus not be correlated with FISH. This clearly shows the discrepancy between what appear to be stable viable counts indicating the absence of variation and the actual heterogeneity of viability present within a microbial community.

The FISH detection of high cell numbers in ancient sediments shows that a small proportion of cells is either active (Biddle *et al.*, 2006; ~10% of the total count) and potentially growing or that it has maintained viability over possibly millennia under slow energy supply and thus near to starvation conditions. In the deep biosphere, however, it will most likely be unknown proportions of active and surviving/starving cells that contribute to FISH counts. As the community

composition often changes with depths or at geological interfaces, shown by molecular investigations (Parkes *et al.*, 2005; Fry *et al.*, 2008), microbes that thrive in deeper sediment layers will contribute to the FISH detection as well as starving cells that merely persist.

Surprisingly, CARD-FISH counts from within the Challenger Mound (IODP Leg 307; Webster *et al.*, 2009), a representative of one of the organic-matter poorest marine habitats, are in a similar range ( $10^5$  to  $10^6$  cells  $\text{cm}^{-3}$  sediment) as those obtained for open ocean and ocean margin sites (Schippers *et al.*, 2005). For the Flank site (Fig. A3.1b), however, there is a significant increase between CARD-FISH detected cell numbers from sediments within the carbonate structure ( $2.3 \times 10^5$  cells  $\text{cm}^{-3}$  sediment) to the underlying sediments ( $3.1 \times 10^6$  cells  $\text{cm}^{-3}$  sediment;  $p < 0.05$ ). This is concomitant with an increase in detectability from 8% to 88% of AODC total counts and thus could indicate that more starved (not even detectable by CARD-FISH) than active cells are present in the organic-matter poor carbonate than in underlying sediments (92% compared to 12%). However, this was not observed at the Mound site. Webster *et al.* (2009) stated that many correlations between measures of the prokaryotic community composition and bacterial CARD-FISH counts were negative and concluded that CARD-FISH did not detect the complete prokaryotic community. Considering that CARD-FISH is a very sensitive technique with a lower detection limit of only 9 to 54 16S rRNA copies per cell (Hoshino *et al.*, 2008) shows the extent to which microbes in the deep biosphere are starved and how careful data need to be interpreted when trying to estimate community compositions via *in situ* hybridisation techniques.

### 3.5 Patterns of culturability, the VBNC state, and cryptic growth during starvation

Generally, up to four stages of culturability were observed during starvation: i.a) initial increase (not always present), i.b) initial stability (not always present), ii) decrease, and iii) late stability (often with a certain degree of variation; Fig. 6.1; Table 6.1). Unfortunately, cultivation efficiencies based on the total or a viability

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count are rarely given in the literature, which makes comparisons difficult and thus culturable cell numbers are presented in Table 6.1.

The initial increase in culturability occurred during this project in a number of strains and was in some cases linked to an initial increase in total counts (e.g. *Marinilactibacillus* sp. G8a3 “A” and “C”, Fig. 4.34; *Pseudomonas* sp. F18IV “C”, Fig. 4.14) but not in others (e.g. *Ornithinimicrobium* sp. F18IV “A”, “B”, and “C”, Fig. 4.3; *D. acrylicus* “A”, “B”, and “C”, Fig. 4.25). This phase was also reported in *Vibrio* sp. CNTP-3 (Rice and Oliver, 1992), *Vibrio fluvialis* (Amel *et al.*, 2008; Table 6.1), and *Deleya aquimarina* (Joux *et al.*, 1997; Table 6.1). Lebaron and Joux (1994) explained this phenomenon with the starvation induced cell division also known as reductive cell division. However, during this project the initial increase in MPNs is more likely related to the fact that growth had not completely ceased at the onset of starvation as indicated by either slightly increasing total counts or the continued consumption or production of VFAs at the beginning of starvation. The latter occurred in all of the strains and treatments listed above.

In some strains the initial increase in culturability was absent and instead a phase of constant culturability was present for a certain amount of time (e.g. *Pseudomonas* sp. F18IV “A” and “B”, stable for five days, Fig. 4.14; *Photobacterium* sp. SAMA2 “A” and “B”, relatively stable for 180 days, Fig. 4.4). [Note that *Serinicoccus marinus* (Fig. 4.1) is not included in this list as initially culturable cell numbers were higher than the upper detection limit of the MPN method used ( $10^8$  cell ml<sup>-1</sup>).] However, this pattern occurred, for example, in *P. abyssi* starved at 4°C for 30 days (Marteinsson *et al.*, 1997; Table 6.1) and *Vibrio* sp. Ant-300 starved at 5°C for 42 days (Amy *et al.*, 1983; Table 6.1).

In other strains an immediate decrease of culturability occurred after the onset of starvation (e.g. *A. malicum*, Fig. 4.32; *D. profundus*, Fig. 4.29; *Raoultella* sp. F17VIII, Fig. 4.15; *Shewanella* sp. F18III, Fig. 4.19). Regardless of the initial increase being present or absent, two categories of subsequent decrease occurred. The first category is a continuous decrease gently leading into the late stability phase (e.g. *Acetobacterium* sp. R002, Fig. 4.33; *Pseudomonas* sp. NA101, Fig. 4.12; *S. marinus*, Fig. 4.1). This was also observed for *V. fluvialis* by Amel *et al.* (2008; Table 6.1). Strains belonging to the second category lost culturability rapidly and

**Table 6.1: Changes in culturability of starving marine isolates under various incubation conditions.** Legend: Starv., starvation n.d., no data

<b>This study</b>		<b>Amy <i>et al.</i> (1983)</b>		<b>Amel <i>et al.</i> (2008)</b>		<b>Vattakaven <i>et al.</i> (2006)</b>				
Sedimentary <i>Bacteria</i> (averaged data)		<i>Vibrio</i> sp. Ant-300		<i>Vibrio fluvialis</i>		<i>Vibrio tasmaniensis</i>			<i>Vibrio shiloi</i>	
Starv. Time [days]	MPN at 10°C-25°C [cells ml <sup>-1</sup> ]	Starv. Time [days]	CFU at 5°C [cells ml <sup>-1</sup> ]	Starv. Time [days]	CFU [cells ml <sup>-1</sup> ]	Starv. Time [days]	CFU at 4°C [cells ml <sup>-1</sup> ]	CFU at 20°C [cells ml <sup>-1</sup> ]	CFU at 4°C [cells ml <sup>-1</sup> ]	CFU at 20°C [cells ml <sup>-1</sup> ]
0	6.6×10 <sup>6</sup>	7	3×10 <sup>7</sup>	0	10 <sup>8</sup>	0	10 <sup>10</sup>	10 <sup>9</sup>	10 <sup>9</sup>	10 <sup>9</sup>
2	2.6×10 <sup>7</sup>	14	3×10 <sup>7</sup>	25	10 <sup>4</sup>	2	10 <sup>8</sup>	10 <sup>7</sup>	10 <sup>8</sup>	10 <sup>7</sup>
5	5.2×10 <sup>6</sup>	28	2×10 <sup>7</sup>	50	10 <sup>4</sup>	12	10 <sup>9</sup>	10 <sup>8</sup>	10 <sup>10</sup>	10 <sup>9</sup>
10	5.2×10 <sup>5</sup>	42	1×10 <sup>7</sup>	150	10 <sup>3</sup>	85	10 <sup>9</sup>	10 <sup>6</sup>	10 <sup>9</sup>	10 <sup>10</sup>
25	3.5×10 <sup>5</sup>	56	8×10 <sup>6</sup>	210	< 10 <sup>2</sup>	100	10 <sup>9</sup>	10 <sup>6</sup>	10 <sup>8</sup>	10 <sup>10</sup>
50	9.2×10 <sup>4</sup>					125	10 <sup>9</sup>	10 <sup>5</sup>	0	0
90	3.8×10 <sup>4</sup>					157	10 <sup>8</sup>	10 <sup>3</sup>	n.d.	n.d.
190	1.6×10 <sup>4</sup>									
270	3.8×10 <sup>3</sup>									
365	4.4×10 <sup>3</sup>									

<b>Jones and Morita (1983b)</b>		<b>Rice and Oliver (1992)</b>		<b>Marteinsson <i>et al.</i> (1997)</b>			<b>Joux <i>et al.</i> (1997)</b>		
<i>Nitrosomonas</i> sp. 4W30		<i>Vibrio</i> sp. CNPT-3 (piezophilic)		<i>Pyrococcus abyssi</i>			<i>Deleya aquamarina</i>		
Starv. Time [days]	MPN at 5°C [cells ml <sup>-1</sup> ]	Starv. Time [days]	CFU 5°C, 40 MPa [cells ml <sup>-1</sup> ]	Starv. Time [days]	MPN at 4°C [cells ml <sup>-1</sup> ]	MPN at 95°C [cells ml <sup>-1</sup> ]	Starv. Time [days]	CFU at 5°C [cells ml <sup>-1</sup> ]	CFU at 20°C [cells ml <sup>-1</sup> ]
0	1×10 <sup>6</sup>	0	1×10 <sup>6</sup>	0	10 <sup>8</sup>	2×10 <sup>8</sup>	0	10 <sup>7</sup>	10 <sup>7</sup>
7	2×10 <sup>6</sup>	2	2×10 <sup>6</sup>	2	10 <sup>8</sup>	1×10 <sup>8</sup>	15	10 <sup>7</sup>	10 <sup>7</sup>
175	2×10 <sup>5</sup>	5	6×10 <sup>5</sup>	7	10 <sup>8</sup>	3×10 <sup>7</sup>	35	3×10 <sup>6</sup>	2×10 <sup>6</sup>
		10	3×10 <sup>5</sup>	30	10 <sup>8</sup>	r. d.	42	2×10 <sup>6</sup>	1×10 <sup>6</sup>
		25	3×10 <sup>4</sup>				95	2×10 <sup>6</sup>	1×10 <sup>6</sup>



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subsequently recovered before entering the late stability phase (e.g. *Arcobacter* sp. NA105, Fig. 4.30; *D. desulfuricans*, Fig. 4.23; *Photobacterium* sp. F18I, Fig. 4.8). This pattern also occurred in *V. tasmaniensis* and *V. shiloi* (Vattakaven *et al.*, 2006; Table 6.1). Generally, the loss of culturability was concomitant with decreasing FISH counts and thus FISH detectability, albeit not statistically significant (Fig. 6.1a).

The phase of late stability occurred in a number of strains e.g. *S. marinus* sp. ( $\sim 10^4$  cells ml<sup>-1</sup>, aerobic, up to 270 days), *Raoultella* sp. F17VIII ( $\sim 10^5$  cells ml<sup>-1</sup>, aerobic and anaerobic, up to 270 days), and *Photobacterium* sp. SAMA2 “B” and “C” ( $\sim 10^5$  cells ml<sup>-1</sup>, aerobic, up to three years). A number of studies observed this phenomenon of continuously stable culturable cells during advanced starvation albeit during shorter incubation times (e.g. Amy and Morita, 1983b, < 200 days; Moyer and Morita, 1989b, < 100 days) and Joux *et al.* (1997) described it as “constant culturable cell fraction” (starvation time < 100 days).

Despite having had higher percentages of dead cells in their cultures, culturability in Gram-positive strains did not differ from Gram-negative isolates and ranged between the lowest (*Deltaproteobacteria*) and highest culturable cell numbers (*Gammaproteobacteria*) occurring during the late-stationary phase (Chapter IV, Fig. 4.53b).

### 3.5.1 Cultivation efficiencies, the VBNC state, and dormancy

During starvation a loss of culturability occurred (Fig. 6.1). During advanced stationary phase (more than 20 days of starvation) the constant culturable cell fraction was higher in starvation treatment “B” than in “A” and “C” (Fig. 4.56b). However, average cultivation efficiencies of these treatments (Table 6.2) were calculated based on the also averaged AODC total counts, the LIVE counts, and the FISH counts for the “standard” starvation response model (Fig. 6.1). For most of the timepoints during starvation (seven out of nine; Table 6.2) cultivation efficiencies showed the following pattern: lowest when calculated from AODC counts, then when based on LIVE counts, and highest when based on FISH counts. This appears to be logical as AO stains all cells including dead ones, the LIVE count should exclude dead cells based on membrane integrity, while the FISH counts only

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represent live cells containing a detectable number of ribosomes for protein synthesis. In the discussion of Chapter IV it was thus argued that cultivation efficiencies should be calculated from a cell estimate that is based on viable cells rather than on the AODC total count.

**Table 6.2: Averaged cultivation efficiencies and percentages of viable but nonculturable cells based on different cell counts methods.** Legend: Cult. Eff., cultivation efficiencies; n.d., not determined

Starvation Time [days]	Cultivation Efficiencies and VBNC based on					
	AODC		LIVE		FISH	
	Cult. Eff. [%]	VBNC [%]	Cult. Eff. [%]	VBNC [%]	Cult. Eff. [%]	VBNC [%]
0	13	87	31	69	43	57
2	46	54	n.d.	n.d.	n.d.	n.d.
5	10	90	88	12	23	77
10	0.9	99.1	0.8	99.2	n.d.	n.d.
25	0.7	99.3	3	97	10	90
50	0.2	99.5	0.4	99.6	2	98
90	0.07	99.93	0.4	99.6	1	99
190	0.03	99.97	0.1	99.9	0.6	99.4
270	0.006	99.994	0.01	99.99	0.2	99.8
365	0.01	99.99	0.04	99.96	0.08	99.92

The same would apply, as the name suggests, when calculating the number of “viable but nonculturable” proportions of a sample. This term was coined by Roszak and Colwell (1987) and applies to cells that have a detectable metabolism but are not culturable by available methods. This state is regarded as a survival strategy and can be induced in cells by (sudden) changes in environmental conditions (e.g. temperature or nutrient concentration; Colwell, 2000). But what constitutes a viable cell? Is a cell that has an intact membrane viable? Not necessarily. Although an intact and functioning cell membrane is essential for many cellular processes (e.g. cell energetics) it does not mean that every cell with an intact membrane is indeed viable. The cellular processes might have come to a standstill due to, for example, starvation or increasing temperature and the cell might already be beyond its capability of recovery, yet the membrane could have remained intact. Hence additional indications are needed to safely assume cellular viability. These could comprise assessment of ribosomal content or activity measurements. Therefore, cultivation efficiencies and proportions of VBNC numbers should be based on the (CARD-) FISH count or MAR-positive cells thereby discounting live cells with no potential of recovery and, of course, dead cells.

During starvation a significant number of cells were still culturable even after one year of incubation (Fig. 6.1). Taking an arbitrary cell concentration of  $10^7$  cells  $\text{ml}^{-1}$  as an example often,  $10^4$  cell  $\text{ml}^{-1}$  would still be culturable. This, however, corresponds to a VBNC fraction of 99.9%. In Table 6.2, the percentages of VBNC cells are given. These were already relatively high shortly after the onset of starvation, for example between 12% and 77% after only five days, depending on which viability method is used for determination (LIVE or FISH count). These VBNC proportions continued to increase during substrate limitation. Nishino *et al.* (2003) stated that a pure culture consists of clonal cells in different physiological states. This phenomenon has already been touched upon in regard to differences in FISH detectability (see 3.4.1). However, Nishino *et al.* (2003) separated stationary phase *V. parahaemolyticus* cells according to their buoyant density. Although it is not fully understood how buoyant density is related to cell culturability and stress resistances, Nishino *et al.* (2003) found that the majority of cells present in the low-density fraction from the stationary phase had the highest stress resistances and the lowest culturability. In contrast, high-density cells represented a much smaller fraction yet exhibited much higher culturability. This method of buoyant density-dependant separation of viable and culturable cells from viable but nonculturable cells could facilitate investigations into the underlying principles of the VBNC state and why some cells enter this state and some of their clonal kin do not.

Although more and more cells entered the VBNC state as a response to continued starvation (Table 6.2) or increasing temperature the complete absence of culturability was rarely observed, not even at highest temperatures of the heating experiment ( $90^\circ\text{C}$  incubated at  $15^\circ\text{C}$  and  $90^\circ\text{C}$ ; Fig. 5.19). During this project, culturability was only absent for *D. profundus* and *D. desulfuricans*. However, as the latter was originally revived from a 12-year old culture during a repeated attempt of transferring a 5 ml inoculum into fresh medium while no growth occurred during a first attempt and in a conducted MPN dilution series (0.1 ml inoculum) suggests that there is a stochastic element involved in the resuscitation of VBNC cells. Two possible explanations spring to mind.

i) Firstly, non culturable cells fall into two categories. The first category would be cells in the VBNC state while the second one would represent cells starved beyond their ability to recover and are thus equal to dead cells (although might not immediately be scored as such with the LIVE/DEAD Kit<sup>®</sup>). Contributions of these

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two categories within the non culturable fraction would with time shift from predominantly VBNC cells to predominantly dead cells. Thus, with increasing starvation time the chance to transfer a VBNC cell into fresh medium, which at least theoretically possesses the ability to re-grow decreases. Hence a larger inoculation volume increases the chances to detect growth (for *D. desulfuricans* a 50 times higher volume led to cultivation success). This, however, also means that inoculation using a larger volume of the starving culture might have also led to higher MPN counts during this project.

ii) The second explanation could be that dormant cells awake randomly. The two *Desulfovibrio* species mentioned above were, according to Morita's definition, dormant as only starved cells can immediately respond to available substrate, which these two strains were not capable of, according to the radiotracer experiments (Chapter IV, Fig. 4.41). Epstein (2009) called the stochastic awakening of a single cell within a dormant culture a "scout". As dormancy has been described as a strategy to withstand unfavourable conditions (e.g. starvation; Wright, 1978) a scout that encounters conditions supporting survival might either start a new population or "wake up" the remaining hitherto dormant populations via growth-inducing signalling compounds (Epstein, 2009). Depending on the frequency of scout cell formation this theory could explain why *D. desulfuricans* was revived after 12 years of starvation when using a 50 times larger volume for inoculation than was used for MPN analysis.

One of these two concepts of the influence of chance on resuscitation of VBNC or dormant cells could also explain why in the radiotracer experiments for *D. profundus* (Fig. 4.42c) and *A. malicum* (Fig. 4.44a) metabolic activity was only detected in some of the vials.

During this project it was shown that continued starvation (Chapter IV) and increasing temperatures (Chapter V) will cause cells to enter the VBNC state. As these conditions prevail in the deep biosphere high percentages of cells are likely present in this state. This impedes cultivation efforts from ancient, deeply buried sediments even if appropriate cultivation media are used and viability measurements such as FISH and potential metabolic activities are detectable.

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### 3.5.2 The GASP phenotype, cryptic growth, and the role of endogenous metabolism

These topics are discussed in the Appendix.

### 3.6 Radiotracer experiments and general activity of starved prokaryotes in pure cultures and the deep biosphere

Morita (1997) distinguished starved from dormant cells by the ability of the former to immediately respond to utilisable substrate. This immediate response was seen in a number of strains during radiotracer experiments, which surprisingly lacked a detectable lag phase (e.g. *Photobacterium* sp. F18I, Fig. 4.36c; *Raoultella* sp. F17VIII, Fig. 4.38; *Shewanella* sp. F17V, Fig. 4.36b; *Vibrio* spp., Fig. 4.40; *D. acrylicus*, Fig. 4.41a; *Acetobacterium* sp. R002, Fig. 4.43b; *Marinilactibacillus* sp. G8a3, Fig. 4.43; Chapter IV). Almost immediate potential microbial activity for glucose and lactate was also present after sequential heating of a psychro- to mesophilic microbial community to hyperthermophilic temperatures in sediment slurry experiments (90°C; Fig. 5.16a and c).

To the best of my knowledge this study represents the first investigation into metabolic activity after long-term anaerobic starvation. The absence of an apparent lag phase as well as the relatively high rates in some strains was unexpected and highlight how well sedimentary bacteria are adapted to starvation and in spite of substrate limitation maintain the potential for metabolic activity over long periods of time.

In most cultures changes in VFA concentrations occurred throughout the starvation period indicating a constant metabolic activity of starving cells, not dormancy. In *Photobacterium* sp. F18I "C", for example, lactate and formate concentrations continuously decreased throughout starvation (Fig. 4.8f), although this strain produced these VFAs from glucose under anaerobic conditions (Fig. 4.37c) indicating a "cryptic" metabolic activity, hidden during rapid growth. This metabolic activity, however, did not appear to be linked to the presence or absence of a lag phase during radiotracer experiments nor to the rate at which the added substrate was metabolised. Constantly changing VFA concentrations (e.g. increasing lactate in

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*Shewanella* sp. SCSA3, from 0.6 mM to 2.1 mM between 90 and 343 days of starvation, Fig. 4.17f) nonetheless indicate ongoing metabolic processes during starvation like continued restructuring of cellular constituents (e.g. cell membrane) and endogenous metabolism (see above), both of which might have lead to the release or uptake of VFAs into or from the medium.

Radiotracer studies have been routinely applied to samples from various sub-seafloor sediments (e.g. Cragg *et al.*, 1990, 1995; Wellsbury *et al.*, 2002; Coolen *et al.*, 2002). Rates determined using starved isolates and a temperature stressed microbial community fit in well with some of the published rates (Chapter IV, Table 4.4; Chapter V, section 4.2.4) thus confirming *in vitro* what has been observed *in situ*, that (starved) cells in the deep biosphere are able to quickly respond to available substrate and occasionally even at relatively high rates (e.g. in organic-matter rich sapropel layers of the Mediterranean Sea; Coolen *et al.*, 2002). In order to rapidly utilise available substrate, cells that did not have a lag-phase after substrate addition must have maintained significant cellular enzyme and ribosome content. But what distinguishes these strains from those that only started consuming added substrate after a certain lag phase and then only at relatively low rates? Furthermore, which intracellular processes needed to happen in the lag-phase strains in order for them to eventually be able to consume the substrate? Answering these questions is an exciting prospect for the future but certain aspects have already been discussed above (e.g. scout formation, genetic disposition).

Radiotracer studies, however, do not reveal the identity nor which proportion of the microbial community is responsible for the detected activity. Karner and Fuhman (1997), however, showed similar proportions of cells detected by FISH and autoradiography (separate experiment using incorporation of <sup>3</sup>H-labeled amino acid mixture) in marine waters, which suggests that these two methods counted the same active subpopulation. The application of MAR-FISH (Nielsen *et al.*, 1999) would have been beneficial to investigate whether the FISH detectable subpopulation of a starving culture is responsible for metabolic activity. After all, a very small number of highly active cells could have caused the rapid removal of the substrate as well as all cells being active at a lower rate. Thus MAR-FISH should be applied in future studies investigating metabolic activities in starving cultures (samples from this study have been stored for later analysis) or indeed in mixed microbial communities such as the slurry experiments conducted in Chapter V or the deep biosphere. This

would not only evaluate the use of FISH as a proxy for activity but also reveal the identity of active subpopulations using probes targeting specific phylogenetic groups.

### 3.7 Summary

Data gathered during this project shed light on the variety of starvation responses of anaerobic sedimentary bacteria as well as the adaptability of a psychro- to mesophilic microbial community to increasing temperature stress as it occurs during burial of marine sediments. The applicability of commonly used techniques to study the deep biosphere was generally confirmed. However, great caution must be taken when interpreting results from *in situ* studies because even under controlled pure culture conditions a variety of contrasting starvation responses occurred, some even in phylogenetically closely related strains.

### **4. Effect of environmental parameters on starvation-survival**

During this project investigating the effects of temperature, oxygen, and pressure on starvation-survival was not a specific objective. However, it is conceivable that these environmental parameters influence the survivability of deep biosphere strains as some incubation conditions were different between starvation incubations. For example, for some of the starving *Photobacteria* cultures, oxygen was unwittingly present in the headspace, and additionally, they were incubated at 10°C (close to optimum temperature for these strains) while other *Gammaproteobacteria* were starved at 25°C. Furthermore, it was possible to catch a glimpse on the effects of pressure on a starving, mixed microbial community from the Gulf of Mexico (Gulf of Mexico Hydrates Joint Industry Project, Parkes *et al.*, 2009) in a fortuitous additional study. These topics are discussed in the Appendix.

## 5. Outlook and future work

This project has shed first light on the survivability of marine sedimentary microbes exposed to starvation and temperature stress under predominantly anaerobic conditions. Although a variety of strains representing major phylogenetic and physiological groups were tested, many others could have been investigated in their place. Thus future work should not only include more bacterial representatives but also *Archaea* (e.g. methanogens, MCG), more representatives of which also need isolation. Techniques like MAR-FISH (Nielsen et al., 1999) or NanoSIMS combined with FISH (Behrens *et al.*, 2008) should be applied to starved cultures. This would not only reveal whether cells detected by MAR are also detected by FISH but also whether metabolic activity in starved cultures is due to all cells being active or only caused by a subset of the population. Another intriguing aspect is the maintenance of metabolic activity in some strains but not others. Metagenomic studies could reveal what causes cells to maintain viability under prolonged substrate limitation. This leads to the question of how long could a laboratory prokaryotic culture starve under anaerobic conditions before all cells have truly died. This, I would speculate, could be an investigation that might span several academic careers.

Cryptic growth and the “recycling” of dead cells need further investigating. So far the importance of these processes is unresolved. Questions to be answered are: To which extent are these processes present in starving cultures? How much energy can be obtained from anaerobic growth on necromass? How many dead cells need to be recycled for the development of one new cell? The answers to these questions would impact significantly on deep biosphere research if neighbouring cells constitute a possible source of energy in deeply buried marine sediments.

Sediment slurry experiments could present a good way of enriching certain physiological groups. Previously mentioned methods like MAR-FISH could help to guide isolation of so far “uncultivated” phylogenetic groups such as JS1 coupled with single-cell isolation. Different substrates could be tested and their suitability to stimulate growth could be rapidly assessed. The close to *in situ* conditions in sediment slurries potentially are the perfect basis for such directed isolation attempts (e.g. Webster *et al.*, 2011).



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The influence of pressure on the survivability of microorganisms was barely touched upon during this project but preliminary results suggest a positive impact on survival. Future work should not disregard this defining feature of deeply buried marine sediments.

Personally, I would also recommend studying the underlying genetic principles of starvation-survival in environmental isolates. These might differ greatly from the so far obtained knowledge for starving *E. coli* cultures.

Future work specifically regarding this project would include i) finalising the species description for *Ornithinimicrobium* sp. F18IV (GC-content, peptidoglycan analysis, deposition at two culture collections), ii) PFLA analysis of starved cultures to compare potential changes to results of non-starved cultures, iii) PCR-DGGE for sequential heating experiments to follow the community changes on the molecular biological level and subsequent sequencing to identify likely active members at increasing temperatures, and iv) investigate significance of spore-forming prokaryotes during sequential heating experiment (currently being conducted).

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## Appendix

### A1. 16S rDNA sequences of isolates obtained from IODP Leg 307

#### A1.1 *Ornithimicrobium* sp. F18IV

TGCTTAACACATGCAAGTCGAACGATGAAGCGGTGCTTGCACCGTGGATTAGTGGCGAA  
CGGGTGAGTAACACGTGAGTAACCTGCCCTTCACTCTGGGATAACTCCGGGAAACCGGT  
GCTAATACTGGATACGACCTCAACCTGCATGGGTATGGGGTGGAAAGCTTTTGTGGTGG  
GGATGGACTCGCGCCTATCAGCTTGTGGTGGGGTAATGGCCCACCAAGGCGACGACG  
GGTAGCCGGCCTGAGAGGGTGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCT  
ACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCC  
GCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCTCTGACGAAGCTTTATTG  
TGACGGTAGGAGCAGAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGTAATACGT  
AGGGTGCAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTTGTAGGCGGCTTGTCCGCT  
CTGCTGTGAAATCCCCGGGGCTCAACCTCGGGCTTGCAGTGGGTACGGGCAGGCTAGAGT  
ATGGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGGAATGCGCAGATATCAGGAGGAA  
CACCGATGGCGAAGGCAGGTCTCTGGGCCATCACTGACGCTGAGAAGCGAAAGCGTGGG  
GAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGTTGGGCGTAGGTTGTG  
GGTCCCATTCACGGGGTCCGTGCCGCAGCTAACGCATTAAGCGCCCCGCTGGGGAGTA  
CGGCCGCAAGGCTAAACTCAAAGGAATTGACGGGGGCCCGCACAAAGCGGCGGAGCAT  
GCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATGTACCGGAAACT  
CTCAGAGATGGGGGCCCGCAAGGTCCGTACACAGGTGGTGCATGGTTGTCTGTCAGCTC  
GTGTCTGTGAGATGTTGGGTAAAGTCCCAGCAACGAGCGCAACCCTCGTTCTATGTTGCCAG  
CACGTGATGGTGGGGACTCATAGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGAT  
GACGTCAAATCATCATGCCCCTTACGTCTTGGGCTTACGCATGCTACAATGGCCGGTAC  
AGAGGGCTGCGATACCGCGAGGTGGAGCGAATCCCTTAAAGCCGGTCTCAGTTCGGATT  
GGGGTCTGCAACTCGACCCCATGAAGTCCGAGTCGCTAGTAATCGCAGATCAGCAACGC  
TGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCAAGTCACGAAAGTCGGTAA  
CACCCGAAGCCGGTGGCCCAACCCCTTGTGGGAGGGGAGCCGTCGAAGGTGGGA

#### A1.2 *Shewanella* sp. F16III

ACACATGCAAGTCGAGCGGTAACACAAGGGAGCTTGCTCYtGaGGTGACGAGCGGGCGGA  
YGGGTGAGTAATGCCTAGGGATCTGCCAGTCGAGGGGGATAACAGTTGGAAACGACTG  
CTAATAACGCATACGCCCTACGGGGgAAAGGAGGGGACCTTCGGGCcTTCCGCGATTGGA  
TGAACCTAGGTGGGATTAGCTAGTTGGTGGGTAATGGCTCACCAAGGCGACGATCCCT  
AGCTGTTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACG  
GGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCCATGCCGCGT  
GTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGTAGGGAGGAAAGGTGATGTGT  
AATAGCACATTGCTGTGACGTTACCTACAGAAGAAGGACCGGCTAACTCCGTGCCAGCA  
GCCGCGGTAATACGGAGGGTCCGAGCGTAAATCGGAATTACTGGGCGTAAAGCGTGCGC  
AGGCGGTTTTGTTAAGCCAGATGTGAAATCCCCGGGCTCAACCTGGGAATTGCATTTGGAA  
CTGGCGAACTAGAGTCTTGTAGAGGGGGgTAGAATTCCAGGTGTAGCGGTGAAATGCGT  
AGATATCTGGAGGAATACCGGTGGCGAAGgGGCCCCCTGGACAAAGACTGACGCTCATG  
CACGAAAGCGTGGGGAGCAAACAGGAiTAGATACCCTGGTAGTCCACGCCGTAAACGAT  
GTC'TACTCGGAGTTTGGT'GACTTAGTCACTGGGCTCCCAAGCTAACGCATTAAGTAGACC  
GCCTGGGAG



## Appendix

### A1.3 *Shewanella* sp. F16IV

TACACATGCAGTCGAGCGGTAACACAAGGGAGCTTGCTCCTGAGGTGACGAGCGGGGA  
CGGGTGAGTAATGCCTAGGGATCTGCCAGTCGAGGGGGATAACAGTTGGAAACGACTG  
CTAATACCGCATAACGCCCTACGGGGGAAAGGAGGGGACCTTCGGGCCTTCCGCGATTGG  
ATGAACCTAGGTGGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCC  
TAGCTGTTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTAC  
GGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCCATGCCCGG  
TGTTGTAAGAAGGCCTTCGGGTGTAAAGCACTTTCAGTAGGGAGGAAAGGTGATGTGT  
TAATAGCACATTGCTGTGACGTTACCTACAGAAGAAGGACCGGCTAACTCCGTGCCAGC  
AGCCGCGTAATACGGAGGGTCCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCG  
CAGGCGGTTTGTAAAGCCAGATGTGAAATCCCCGGGCTCAACCTGGGAATTGCATTTGGA  
ACTGGCGAAGTACTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCG  
TAGATACTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCA  
TGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAACG  
ATGTCTACTCGGANTTTGGTGACTTAGTCACTGGGCTCCCAAGCTAACGCATTAAGTAGA  
CCGCCTGGGGAGTACGGCCGCAAGGTTAAACTCAAATGAATTGACGGGGGCCCGCACA  
AGCGGTGgAGCATGTGGNTT

### A1.4 *Shewanella* sp. F16V

CTACACATGCAAGTCGAGCGGTAACACAAGGGAGCTTGCTCCTGAGGTGACGAGCGGGC  
GACGGGTGAGTAATGCCTAGGGATCTGCCAGTCGAGGGGGATAACAGTTGGAAACGAC  
TGCTAATACCGCATAACGCCCTACGGGGGAAAGGAGGGGACCTTCGGGCCTTCCGCGATT  
GGATGAACCTAGGTGGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATC  
CCTAGCTGTTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCT  
ACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCCATGCCG  
CGTGTGTGAAGAAGGCCTTCGGGTGTAAAGCACTTTCAGTAGGGAGGAAAGGTGATGT  
GTTAATAGCACATTGCTGTGACGTTACCTACAGAAGAAGGACCGGCTAACTCCGTGCCAG  
CAGCCGCGTAATACGGAGGGTCCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGGTG  
CGAGGCGGTTTGTAAAGCCAGATGTGAAATCCCCGGGCTCAACCTGGGAATTGCATTTGG  
AACTGGCGAAGTACTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGC  
GTAGATACTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTC  
ATGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAC  
GATGTCTACTCGGAGTTTGGTGACTTAGTCACTGGGCTCCCAAGCTAACGCATTAAGTAG  
ACCGCTGGGGAGTACGGcCGCAAGGTTAAACTCAAATGAATTGACGGGGGCCCGCAC  
AAGCGGTGGAgCATGtgGTTAATTCGATGCAACGCGA

### A1.5 *Shewanella* sp. F17III

CTACACATGCAAGTCGAGCGGCAGCGGGAAGATAGCTTGCTATCTTTGCCGGCGAGCGG  
CGGACGGGTGAGTAATGCCTAGGGATCTGCCAGTCGAGGGGGATAACAGTTGGAAACG  
ACTGCTAATACCGCATAACGCCCTACGGGGGAAAGGAGGGGACCTTCGGGCCTTCCGCGA  
TTGGATGAACCTAGGTGGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGA  
TCCCTAGCTGTTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCC  
TACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCCATGCC  
GCGTGTGTGAAGAAGGCCTTCGGGTGTAAAGCACTTTCAGTAGGGAGGAAAGGTAGCA  
GCTTAATACGCTGTGTGCTGTGACGTTACCTACAGAAGAAGGACCGGCTAACTCCGTGCCA  
GCAGCCGCGTAATACGGAGGGTCCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGTG  
CGCAGGCGGTTTGTAAAGCCAGATGTGAAAGCCCCGGGCTCAACCTGGGAATTGCATTT  
GAACTGGCGAAGTACTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATG  
CGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCT  
CAGGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAC  
CGATGTCTACTCGGAGTTTGGTGCTTGAACACTGGGCTCTCAAGCTAACGCATTAAGTA

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NACCGCCTGGGGAGTACGGCCGCAAGGTAAAACCTCAAATGAATTGACGGGGGGCCCGCA  
CAAGCGG

### A1.6 *Shewanella* sp. F17V

CATGCAGTCGAGCGGCAGCGNNAANANAGCTTGCTMTCTTTGCCGGCGAGCGGGCGGACG  
GGTGANTAATGCCTANGGATCTGCCANTCGAGGGGGATAACAGTTGNAAACNACTGCT  
ANTACCGCATAACGCCCTACGGGGGAAAGGAGGGGACCTTCGGGCCTTCCGCGATTGGAT  
GAACCTANGTGGGATTANCTANTTGGTGAGGTAATGGCTCACCGNNGCNACNATCCCTA  
NNTGTTCTGANAGGATGATCAGCCNCTGGGACTGAGACACGGNCCANACTCCTACAG  
NAGGCAGCANTGGGGAATATTGCACAATGGGGGAAACCCTGATGCANCCATGCCGCGTG  
NGTGAANAANGCCTTCNGGTTGTAAAGCACTTTCAGTANGNAGGAAAGGTAGNANNTTA  
ATACGCTGTTGCTGTGACNTTNCCTACNNAANAAGGACCGGNTAACTCCGTGCCNNCNN  
CCNCGGTAATACGGANGGTCCGAGNGTTNNTCNGAATTACTG

### A1.7 *Shewanella* sp. F17VI

ACACATGCaAGTCGAGCGGTAACACAAGGGAGCTTGCTcTCTGAGGTGACGAGCGGGCGa  
cGGGTGAGTAATGCCTAGGGATCTGCCAGTCGAGGGGGATAACAGTTGGAAACGACTG  
CTAATACCGCATAACGCCCTACGGGGGAAAGGAGGGGACCTTCGGGCcTTCGCGATTGGA  
TGAACCTAGGTGGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCCT  
AGCTGTTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACG  
GGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCCATGCCGCGT  
GTGTGAAGAAGGCCTTTCGGGTTGTAAAGCACTTTCAGTAGGGAGGAAAGGTGATGTGT  
AATAGCACATTGCTGTGACGTTACCTACAGAAGAAGGACCGGCTAACTCCGTGCCAGCA  
GCCGCGTAATACGGAGGGTCCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGC  
AGGCGGTTTGTAAAGCCAGATGTGAAATCCCCGGGCTCAACCTGGGAATTGCATTTGGA  
CTGGCGAACTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGT  
AGATATCTGGAGGAATACCGGTGGCGAAGGcGGCCCCCTGGACAAAGACTGACGCTCAT  
GCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGA  
TGTCTACTCGGAGTTTGGTGACTTAGTCACTGGGCTCCCAAGCTAACGCATTAAGTAGAC  
CGCCTGGGGAGTACGgCcGCAAGgtTAAAACCTCAAATGaATTGACGGGGGGCCCGCACaAGC  
GtGGAGCATGTgGtTtAtTCGATGccAC

### A1.8 *Shewanella* sp. F17VII

ACACATGCAAGTCGAGCGGTAACACAAGGGAGCTTGCTCCTGAGGTGACGAGCGGGCGGA  
CGGGTGAGTAATGCCTAGGGATCTGCCAGTCGAGGGGGATAACAGTTGGAAACGACTG  
CTAATACCGCATAACGCCCTACGGGGGAAAGGAGGGGACCTTCGGGCCTTCCGCGATTGG  
ATGAACCTAGGTGGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCC  
TAGCTGTTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTAC  
GGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCCATGCCGCG  
TGTGTGAAGAAGGCCTTTCGGGTTGTAAAGCACTTTCAGTAGGGAGGAAAGGTGATGTGT  
TAATAGCACATTGCTGTGACGTTACCTACAGAAGAAGGACCGGCTAACTCCGTGCCAGC  
AGCCGCGTAATACGGAGGGTCCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCG  
CAGGCGGTTTGTAAAGCCAGATGTGAAATCCCCGGGCTCAACCTGGGAATTGCATTTGGA  
ACTGGCGAACTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCG  
TAGATATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCA  
TGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACG  
ATGTCTACTCGGAGTTTGGTGACTTAGTCACTGGGCTCCCAAGCTAACGCATTAAGTAGA  
CCGCTGGGGAGTACGGCCGCAAGGTAAAACCTCAAATGAATTGACGGGGGGCCCGCACA  
AGCGGtGGAGcATGtGgTTTAAATTCGATGCAACGCGAARAACCTTACCTACTCTTGACATC  
CACAGA

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### A1.9 *Shewanella* sp. F18III

tacACATGCAGTCGAGCGGTAACAGAGAGTAAGCTTGCTTACTTTGCTGACGAGCGGCGGA  
CGGGTGAGTAATGCCTAGGGATCTGCCAGTCGAGGGGGATAACAGTTGGAAACGACTG  
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ATGAACCTAGGTGGGATTAGCTAGTTGGTGAGGTAATGGCTACCAAGGCGACGATCCC  
TAGCTGTTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTAC  
GGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCCATGCCGCG  
TGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGTAGGGAGGAAAGGTGGTGTGT  
TAATAGCACAATTGCTGTGACGTTACCTACAGAAGAAGGACCGGCTAACTCCGTGCCAGC  
AGCCGCGGTAATACGGAGGGTCCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCG  
CAGGCGGTTTGTAAAGCCAGATGTGAAATCCCCGGGCTCAACCTGGGAATTGCATTTGGA  
ACTGGCGAACTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCG  
TAGAGATCTGGAGGAAATACCGGTGGCGAAGGCGGcCCCCTGGACAAAaGACTGACGCTC  
ATGCACGAAAAGcgtGGGGAGcAAACAGGatTagATACCCTGGTAGtCCacGCCGTAAAcgAtGTc  
tAcctccGcAcTTTtggtgectTTgAgcacTgG

### A1.10 *Vibrio* sp. F16II

CTTCGGNNGNNTTNAATGGGCGTCGAGCGGCGGACGGGTGAGTAATGCCTAGGAAATTGC  
CTTGATGTGGGGGATAACCAATTGGAACCGATGGCTAATACCGCATAATGCCTACGGGCC  
AAAGAGGGGGATCTTCGGACCTCTCGCGTCAAGATATGCCTAGGTGGGATTAGCTAGTTG  
GTGAGGTAATGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCA  
CACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCAC  
AATGGGCGAAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAA  
AGTACTTTCAGTTGTGAGGAAGGGTGTGTAGTTAATAGCTGCNTATCTTGACGTTAGCAA  
CAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAGCG  
TTAATCGGAATTACTGGGCGTAAAGCGCATGCAGGTGGTTCATTAAGTCAGATGTGAAA  
GCCCGGGGCTCAACCTCGGAACTGCATTTGAAACTGGTGAAGTACTAGAGTGTGTAGAGGG  
GGGTAGAAATTCAGGTGTAGCGGTGAAATGCGTAGAGATCTGAAGGAATACCAGTGGCG  
AAGGCGCCCCCTGGACAGACACTGACACTCAGATGCGAAAGCGTGGGGAGCAAACAG  
GATTAGATAACCCTGGTAGTCCACGCCGTAACGATGTCTACTTGGAGGTTGTGGCCTTGA  
GCCGTGGCTTTCGGAGCTAACCGTAAAGTAGACCGCCTGGGGAGTACGGTCCGAAGAT  
TAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCC  
ATGCAACCGGAAGAACCCTTACCTACTCTTGACATCCA

### A1.11 *Vibrio* sp. F17I

tacaCATGCAGTCGAGCGGAAACGACamyattgawyCTTCGgayGmkTTgwTGGGCGTCGAGCGG  
CGGACGGGTGAGTAATGCCTAGGAAATTGCCTTGATGTGGGGGATAACCAATTGGAACG  
ATGGCTAATACCGCATAATGCCTACGGGCCAAAGAGGGGGATCTTCGGACCTCTCGCGTC  
AAGATATGCCTAGGTGGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGAT  
CCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAAGTACTGAGACACGGTCCAGACTCC  
TACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCCATGCC  
GCGTGTATGAAGAAGGCCCTTCGGGTTGTAAAGTACTTTCAGTTGTGAGGAAGGGtGTGTA  
GTTAATAGCTGCrymTCTTGACGTTAGCAACAGAAGAAGCACCGGCTAACTCCGTGCCAG  
CAGCCGCGGTAATACGGAGGGTGCAGCGTTAATCGGAATTACTGGGCGTAAAGCGCAT  
GCAGGTGGTTCATTAAGTCAGATGTGAAAGCCCGGGGCTCAACCTCGGAACTGCATTTGA  
AACTGGTGAAGTACTAGAGTGTGTAGAGGGGGGTAGAATTTTCAGGTGTAGCGgTGAAATGC  
GTAGAGATCTGAAGGAATACCAGTGGCGAAGGCGGCCCCCTGGacAGACACTGACACTCA  
gATGCgAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGtAGTCCACGCCGTAAACGA  
TGTCTACTTgGaggTtGtGccttGAgCCGtgGcTTTcggAGCTaAcgctTagTacAccgcCTGGGGAG

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### A1.12 *Vibrio* sp. F17IV

tacACATGCAGTCGAGCGGAaCGACACTAACAATCCTTCGGGTGCGTTAwTGGGCGTCGAG  
CGGCGGACGGGTGAGTAATGCCTAGGAAATTGCCTTGATGTGGGGGATAACCAATTGGAA  
ACGATGGCTAATACCGCATAATGCCTACGGGCCAAAGAGGGGGATCTTCGGACCTCTCG  
CGTCAAGATATGCCTAGGTGGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGA  
CGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAAGTGGAGACACGGTCCAGA  
CTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCCA  
TGCCCGGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGTTGTGAGGAAGGGG  
GTAACGTTAATAGCGTTATCTCTTGACGTTAGCAACAGAAGAAGCACCGGCTAACTCCGT  
GCCAGCAGCCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAATTAAGGGCGTAAAG  
CGCATGCAGGTGGTTTCAATTAAGTCAGATGTGAAAGCCCGGGGCTCAACCTCGGAAGTGC  
ATTTGAAACTGGTGAAGTACAGTGTAGAGGGGGGTAGAATTTAGGGTGTAGCGGTG  
AAATGcGTAGAGATCTGAAGGAATACCAGTGGCGAAGGCGGCCCCCTGGacAGAcCaTGAC  
ACTCAgATGcGAAAAGCGTGGGGAgcAAACAGGATTAGATACCctGGTAGTCCACGCCgtAAA  
CGatgtCtAcTtgGAggtgacctTGAgCCgtgGcTTTcGgAGCTaAcgegTagTAgAccgcCTGGGGag

### A1.13 *Raoultella* sp. F17II

TACACATGCAGTCGAGCGGTARCAKARAGCTTGCTCTCGGGTGACGAGCGGCGGACG  
GGTGAGTAATGTCTGGgAAACTGCCTGATGGAGGGGGATAACTACTGGAAACNGNNCT  
AATACCGCATAACGTCNCANGACCAAAAGTGGGGACCTTCNGGCCTCNTGCCATCENNAT  
GTGCCNNTATGGNATTAGCTANTANGNGGGTAATGGCTCACCTANGCNACNATCCCTA  
NCTGGNCTGAAAGGATGACCANCCNCTGGANCTGANACACGGTCCANACTCCTACGG  
GAGGCANCNNGGGGAATATTGCNCANTGGGNNCAAGCCTGATGCANCCNTGCCGCGN  
GNATGAANAANGCCTTCGGGTTGTAAAGTACTTTCANCGAGGAGGAAGGCNTTAAGGTT  
AATAACCTTGGNGATTGACGTTACTCGCANAANAANCACCGGCTAACTCCGTGCCAGCA  
GCCGCGTAATACNGAGGGTGAAGCGNTANTCGGAATTAAGTGNNGTAAAGCGCACGC  
ANGCGTTTTGTTAANTCENATGTGAANTCCCCGGNNTCENCTGGGAAGTGCATNNNN  
ANTGNNAAGCTTGAGTCTTGTAGAGGGGGGTAGAATTCNNGGTGTAGCGNTGAAATGCN  
NANAGATCTGGAGGAATACCGGTGGCNAANGCAGCCCCCTGGAC

### A1.14 *Raoultella* sp. F17VIII

TACACATGCAGTCGAGCGGTAGCACAGAGAGCTTGCTCTCGGGTGACGAGCGGCGGACG  
GGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCT  
AATACCGCATAACGTCGCAAGACCAAAAGTGGGGACCTTCGGGCCTCATGCCATCAGAT  
GTGCCAGATGGGATTAGCTAGTAGGTGGGGTAATGGCTCACCTAGGCGACGATCCCTA  
GCTGGTCTGAGAGGATGACCAGCCACACTGGAAGTGGAGACACGGTCCAGACTCCTACGG  
GAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTG  
TATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGAGGAGGAAGGCCTTAAGGTTA  
ATAACCTTGGNGATTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAG  
CCGCGGTAATACGGAGGGTGAAGCGTTAATCGGAATTAAGTGGCGTAAAGCGCACGCA  
GGCGGTTTTGTTAAGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAAGTGCATTTGAAAC  
TGGCAAGCTTGAGTCTTGTAGAGGGGGGTAGAATTCAGGTGTAGCGGTGAAATGCGTA  
GAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGG  
TGCGAAAGCGTGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGAT  
GTGACTTGAgGTTGTTCCCTTGAGGAGTGGcTTCCgGgAGCTAACGCGTTAAGTCGACCG  
cCTGGGgAGTACgGgCCGCAAGGtAAAACCTCAAaTGaatTGAcGGGGgCCcGCccaAgCGGtgG

## Appendix

### A1.15 *Pseudomonas* sp. F18II

tacaCATGCAGTCGAGCGGtAGAGAGAAGCTTGCTTCTCTTGAGAGCGGCGGACGGGTGAG  
TAATGCCTAGGAATCTGCCTAGTGGTGGGGGATAACGTTTCGGAAACGGACGCTAATACC  
GCATACGTCTCTACGGGAGAAAGCGGGGGACCTTCGGGCCTCGCGCCATTAGATGAGCCT  
AGGTTCGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCTACGATCCGTAACCTGGTC  
TGAGAGGATGATCAGTCACACTGGAAGTGGAGACACGGTCCAGACTCCTACGGGAGGCAG  
CAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAG  
AAGGTC'TTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGTAGTAACCTTAATACGTTG  
CTACTTTGACGTTACCGACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAA  
TACGAAGGGTGC AAGCGTTAATCGGAATTA CTGGGCGTAAAGCGCGGTAGGTGGTTCA  
GTAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCAAAACCTGCTGAGCT  
AGAGTACGGTAGAGGGTAGTGGAAATTTCTGTGTAGCGGTGAAATGCGTAGATATAGGA  
AGGAACACCAGTGGCGAAGGCGACTACCTGGACTGGTACTGACACTGAGGTGCGAAAGC  
GTGGGGAGCAAACAGGATTAGATACCCTGgtAGTCCACGCCGTAAACGATGTCAACTAGC  
CGTTGGGagTCgTGaacTctTAgaGgagCAGCTAACGCATTaAgtTgACcGcCtGGGGAGTACgGcC

### A1.16 *Pseudomonas* sp. F18V

TACACATGCAGTCGAGCGGTAGAGAGAAGCTTGCTTCTCTTGAGAGCGGCGGACGGgTG  
AGTAATGCCTAGGAATCTGCCTAGTGGTGGGGGATAACGTTTCGGAAACGGACGCTAATA  
CCGCA TACGTCTCTACGGGAGAAAGCGGGGGACCTTCGGGCCTCGCGCATTAGATGAGC  
CTAGGTCGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCTACGATCCGTAACCTGG  
TCTGAGAGGATGATCAGTCACACTGGAAGTGGAGACACGGTCCAGACTCCTACGGGAGGC  
AGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGA  
AGAAGGTC'TTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGTAGTAACCTTAATACG  
TTGCTACTTTGACGTTACCGACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGG  
TAATACGAAGGGTGC AAGCGTTAATCGGAATTA CTGGGCGTAAAGCGCGGTAGGTGGT  
TCAGTAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCAAAACCTGCTGA  
GCTAGAGTACGGTAGAGGGTAGTGGAAATTTCTGTGTAGCGGTGAAATGCGTAGATATA  
GGAAGGAACACCAGTGGCGAAGGCGACTACCTGGACTGGTACTGACACTGAGGTGCGAA  
AGCGTGGGGAGCAAACAGGATTAGATACCCTGGtAGTCCACGCCGTAAACGATGTCAACT  
AGCcGTTGGGAGTCTTGaACTctTAgtTGGCgCAgCTAAcGCATTAAGTTGACGCCTGGGAGT  
ACGgccGCAaGGTTAAACTCAAATGaaTgacGGGGCCgc

### A1.17 *Photobacterium* sp. F18I

TACACATGCAGTCGAGCGGTAACAGGAATTAGCTTGCTAATTTGCTGACGAGCGGCGGA  
YGGGTGAGTAATGCCTGGGAATATGCCTTAGTGTGGGGGATAACTATTGGAAACGATAG  
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ATTAGCCCAGGTGGGATTAGCTAGTTGGTGAGGTAAGGCTCACCAAGGCAACGATCCC  
TAGCTGGTCTGAGAGGATGATCAGCCACTGGAAGTGGAGACACGGTCCAGACTCCTAC  
GGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCCATGCCGCG  
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acC

## Appendix

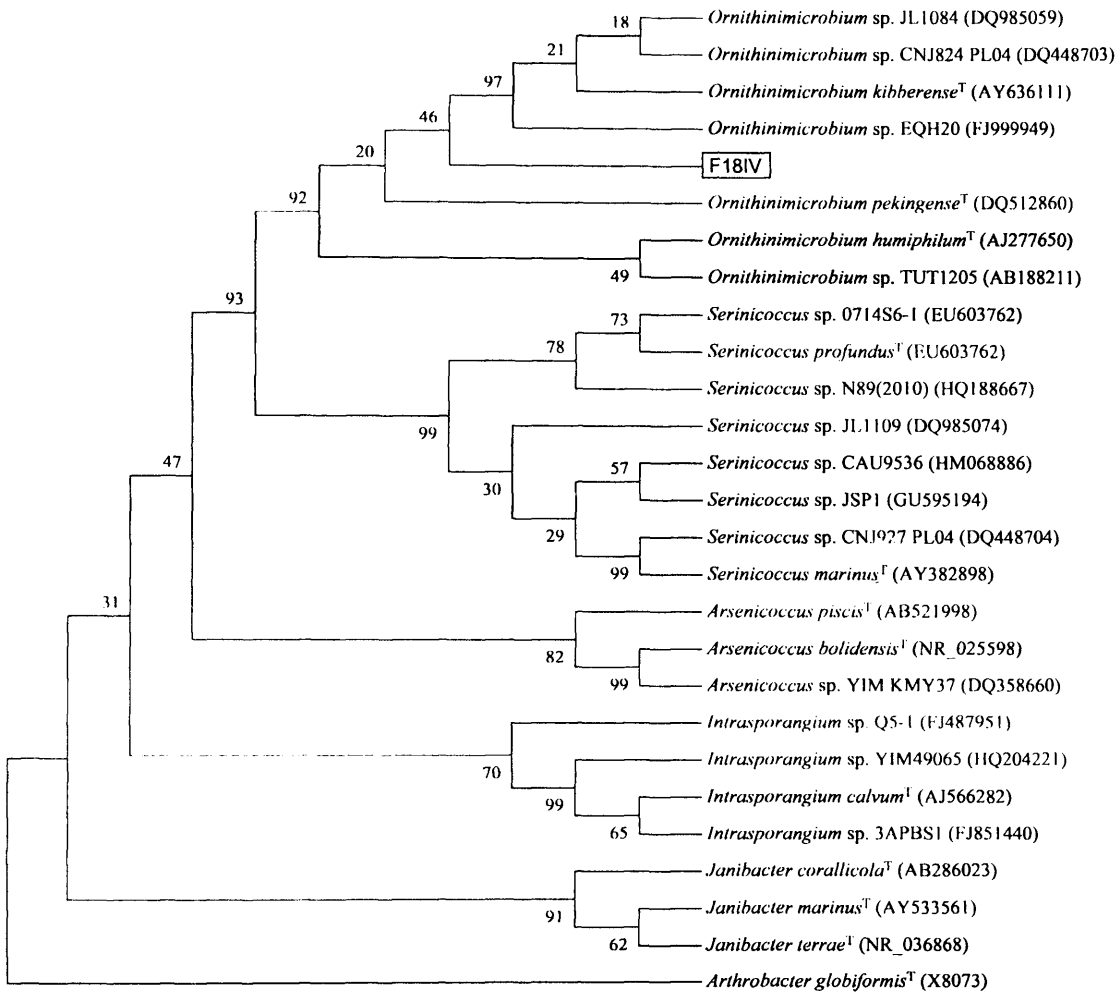
### A1.18 *Desulfovibrio* sp. F16I

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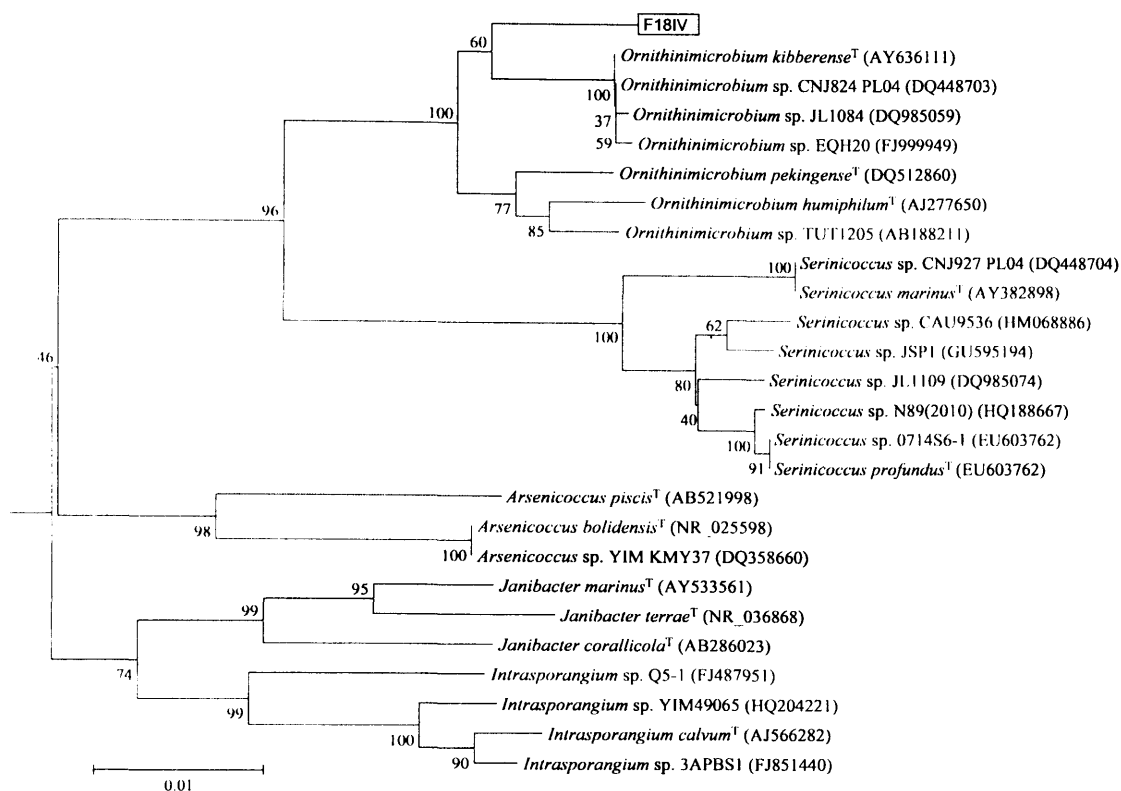
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**A2. Phylogenetic trees based on 16S rDNA sequences of  
*Ornithinimicrobium* sp. F18IV**



**Fig. A2.1** Phylogenetic tree of 16s rDNA sequences based on Maximum Parsimony algorithm of selected species of the family *Intrasporangiaceae* including the novel isolate **F18IV**. Outgroup: *Athrobacter globiformis*

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**Fig. A2.2** Phylogenetic tree of 16S rDNA sequences based on Minimum Evolution algorithm of selected species of the family *Intrasporangiaceae* including the novel isolate F18IV. Outgroup: *Athrobacter globiformis*

### A3. Results from IODP Leg 307

The data presented here was published by Webster *et al.* (2009) and is shown to provide background information to the subsequent results of isolation and physiological characterisation of strains originating from subsurface sediments drilled during IODP Leg 307. As a reminder, three sites were investigated, the Mound Site (U1317), the down-slope Flank Site (U1316), and an up-slope non-mound Reference Site (U1318). Due to drilling constraints multiple holes were drilled at the mound sites. To make the different recovered cores comparable to one another the data is expressed as “metres above mound base” (mamb; see also Chapter II.1.1)



## Appendix

### A3.1 Sediment geochemistry

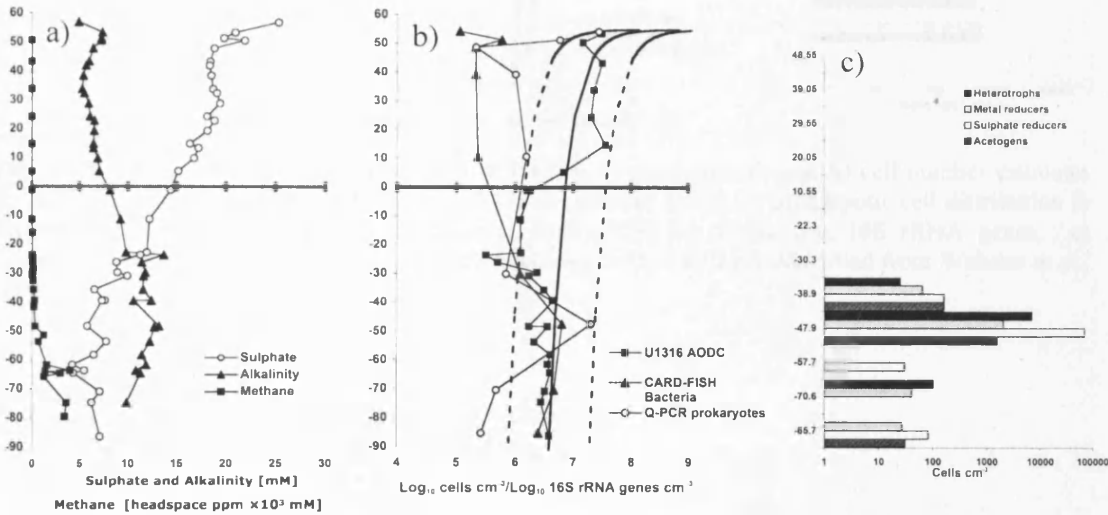
Sulphate concentrations at the Reference site (U1318, Fig A3.3a) decreased steeply in the top 14 metres from seawater values (26.6 mM) to 10.4 mM indicating active sulphate reduction (Fig. A3.3a). Below 14 mbsf, however, sulphate concentrations increased and fluctuated between 10 mM and 17 mM until the bottom of the sampled sediment column, with two local maxima at 30 mbsf and between 130-160 mbsf suggesting oxidation of previously formed metal sulphides (Webster *et al.*, 2009). Similar sulphate profiles were found at other Ocean Drilling sites (D'Hondt *et al.*, 2002) where microbial activity was consistently low and thus sulphate was present throughout the sediment column.

At the two Mound sites (Fig. A3.1a and A3.2a) the rate of sulphate removal was lower than in the top 14 metres of the Reference site but continued with depth. Sulphate removal was, however, much more pronounced at the Mound site than at the Flank with sulphate concentrations of 6.9 mM and 15.1 mM, respectively near the mound base (5 mamb). Below the mound sulphate decreased further reaching minimum concentrations of 0.4 mM at the Mound Site (-61.6 mamb) and 4 mM at the Flank site (-61.6 mamb) (Fig. A3.2a and A3.1a). At both sites sulphate concentrations increased slightly towards the bottom of the sampled sediment column. Also at both mound sites sulphate decreased almost linearly with depth suggesting a slow yet constant flux of sulphate throughout the Mound structure. This continued beyond the geological interface between Mound and sediment until near depletion of sulphate.

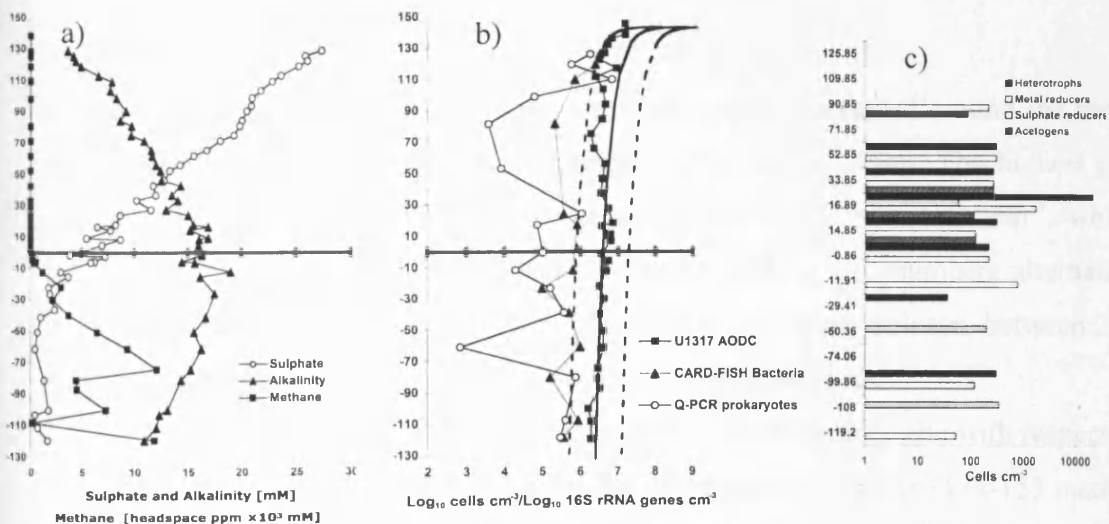
No methane was detected in the sediment column at the Reference site (Fig. A3.3a). This is most likely caused by the presence of sulphate and thus sulphate-reducing prokaryotes, which are known to out-compete methanogens for substrates (Lovley and Klug, 1983). Where sulphate was low, methane concentrations were elevated, which was the case for both Mound sites at depth below -36.3 mamb for the Mound (Fig. A3.2a) and -61.7 mamb at the Flank (Fig. A3.1a) creating sulphate-methane transition zones (SMTZ). Methane concentrations were generally higher at the Mound site than at the Flank with  $12 \times 10^3$  ppm and  $3.6 \times 10^3$  ppm headspace gas concentrations, respectively at around -74 mamb.

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Sediments with an SMTZ potentially harbour groups of microorganisms capable of anaerobic methane oxidation (AMO; Treude *et al.*, 2003, 2005a,b; Parkes *et al.*, 2005; Niemann *et al.*, 2006), a process associated with carbonate precipitation (Ritger *et al.*, 1987; Aloisi, 2002; Greinert *et al.*, 2002; Heijs *et al.*, 2006). Alkalinity mirrored the sulphate profile at all sites, indicating that it was produced by sulphate reduction.

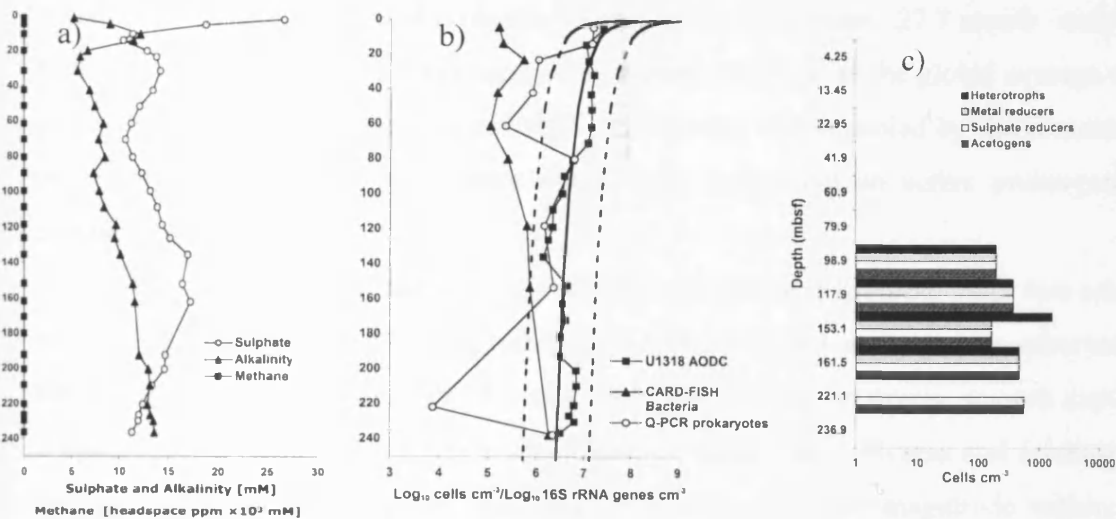


**Fig. A3.1: Depth profiles of Flank Site U1316.** a) geochemical data; b) cell number estimates for AODC (solid line represents Parkes *et al.* [2000] general model for prokaryotic cell distribution in marine sediments), CARD-FISH for Bacteria, and qPCR for prokaryotic 16S rRNA genes ; c) Culturable cell numbers from MPN enrichments using different media. Modified from Webster *et al.*, 2009.



**Fig. A3.2: Depth profiles of Mound Site U1317.** a) geochemical data; b) cell number estimates for AODC (solid line represents Parkes *et al.* [2000] general model for prokaryotic cell distribution in marine sediments), CARD-FISH for Bacteria, and qPCR for prokaryotic 16S rRNA genes ; c) Culturable cell numbers from MPN enrichments using different media. Modified from Webster *et al.*, 2009.

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**Fig. A3.3: Depth profiles of Reference Site U1318.** a) geochemical data; b) cell number estimates for AODC (solid line represents Parkes *et al.* [2000] general model for prokaryotic cell distribution in marine sediments), CARD-FISH for Bacteria, and qPCR for prokaryotic 16S rRNA genes ; c) Culturable cell numbers from MPN enrichments using different media. Modified from Webster *et al.*, 2009.

### A3.2 Abundance of prokaryotic cells

The abundance of prokaryotic cells was determined at all sites by acridine orange direct counts (AODC), CARD-FISH, and qPCR targeting the prokaryotic gene for the 16S ribosomal RNA.

#### a) Total cell counts (AODC)

At the Reference site (Fig. A3.3b) the total cell counts fluctuated around the mean global trend observed for marine sediments (Parkes *et al.*, 2000). The highest cell concentrations were detected near the sediment surface  $2.7 \times 10^7$  cells  $\text{cm}^{-3}$ , which subsequently decreased within the first 20 metres. Below, cell numbers alternately increased and decreased over the entire length of the sediment column, between 200 and 236 mbsf even exceeding the predicted numbers in the model.

The Mound site showed a similar profile to the Reference site with respect to fluctuation (Fig. A3.2b). Cell numbers in the upper sediment layers (138-153 mamb) were  $\sim 10^7$  cells  $\text{cm}^{-3}$ . With depth cell numbers declined to  $1.8 \times 10^6$  cells  $\text{cm}^{-3}$  at 75 mamb, thereby following the lower regression limits for global cell numbers. Subsequently cell numbers increased slowly and remained relatively stable for the

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remainder of the sediment column. Total counts between 27.7 mamb and -87.4 mamb, bridging the mound base, were higher or similar to the global average of cell numbers in the marine deep subsurface. This was accompanied by the presence of dividing cells (up to 14%, data not shown) suggesting an active prokaryotic population.

The profile of cell numbers at the Flank site differed from the other two sites in regard to the amplitude of the gradient (Fig. A3.1b). Cell numbers of the other two sites ranged between  $10^6$  and  $10^7$  cells  $\text{cm}^{-3}$  and form a relatively smooth curve whereas prokaryotic abundances at the Flank are much more diverse and increased and decreased more frequently spanning up to two orders of magnitude within a relative small distance (e.g. between 14.9 mamb and -23 mamb). At this site the smallest population was counted for all three sites ( $3.2 \times 10^5$  cells  $\text{cm}^{-3}$  at -23.6 mamb)

Generally the trends observed for total cell numbers did not seem to change significantly during the transition from mound structure to underlying sediment. There is, however, more variability of cell numbers at the Flank (U1316) than at the other two sites. Cell numbers for Mound and Reference site are relatively comparable considering the geological differences (e.g. sediment type, TOC) of the habitats and agree in most parts with predictions for marine sediments made by Parkes *et al.* (2000).

### b) CARD-FISH and prokaryotic quantitative PCR analysis

The determination of cell abundances with catalysed reporter deposition fluorescence *in situ* hybridisation (CARD-FISH) using probes targeting archaeal ribosomal RNA was unsuccessful for all three sites as counts remained below the detection limit of  $10^5$  cells  $\text{cm}^{-3}$  (Schippers *et al.*, 2005). Thus only counts using a mixture of bacterial probes (EUBI, II, III) are shown (Fig. A3.1b-A3.3b). For qPCR a universal primer set was used (Takai and Horikoshi, 2000).

At all sites cell estimates using CARD-FISH and qPCR generally remained below the total cell counts by one to two orders of magnitude difference. At the Reference site CARD-FISH cell counts increased from the sediment surface and reached a local maximum at 22.7 mbsf (Fig. A3.3b). At the Flank an increase in CARD-FISH counts of one order of magnitude between samples from above and

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below the mound base occurred with a local maximum at -47.6 mamb and remained almost constant towards the bottom of the sediment column (Fig. A3.1b). CARD-FISH profiles at the Flank and Reference site increased with depth thus reversing the trend observed for total cell numbers. This, however, indicates that more active microorganisms were present in deeper sediment layers as CARD-FISH aims at the cellular ribosome content, which is linked to viability and activity of cells (Schippers *et al.*, 2005). At the Mound site CARD-FISH cell estimates decreased continuously with depth showing only a few local maxima between 17.1 and -11.7 mamb and at -60.8 mamb and -107.7 mamb (Fig. A3.2b). However, no correlation was found between cell concentrations and sediment type (mound or underlying sediment) at this site.

Results of qPCR cell estimates were quite variable between sites and different depths at each site. At the Flank a steep decrease occurred in the top 10 metres of the sediment column (Fig. A3.1b). In deeper layers the number of detected 16S rDNA genes remained relatively stable with the exception of a peak at -47.6 mamb where the 16S rDNA gene copy numbers exceeded the total count. The Mound and Reference site showed much greater variation throughout the sediment column compared to the Flank. At the mound, cell estimates using qPCR increased and decreased drastically and repeatedly throughout the sediment column forming several local maxima (e.g. at -0.59 mamb and below -80.4 mamb; Fig. A3.2b). A similar but less drastic profile occurred at the Reference site with the highest cell estimates near the surface (4.25 mbsf) and local maxima at 79.7 mbsf and 237.4 mbsf (Fig. A3.3b). No apparent change in qPCR results occurred between Mound and underlying sediment at both Mound sites.

The detection of cells using CARD-FISH and qPCR proves that an active microbial community was present within and below the carbonate mound and at the reference site. Both these methods detected on average more cells per cm<sup>3</sup> sediment at the Flank and Reference site than at the Mound site.

### c) Culturable microbial diversity

To determine the anaerobic culturable microbial diversity within sediments from Leg 307 five different substrate combinations were used for most-probable number dilution series (MPN) targeting heterotrophic (fermenting), metal-reducing, sulphate-

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reducing, and acetogenic bacteria as well as methanogenic *Archaea* (Webster *et al.*, 2009).

At the Reference site no culturable prokaryotes were detected in the top 80 m of the sediment column using MPN (Fig. A3.3 c). Only incubations with samples from beneath the erosional surface (84 to 221 mbsf) resulted in positive most-probable numbers. For most depth horizons growth occurred in four of the five media targeting certain physiological groups. However, no growth occurred in medium targeting methanogens, which is true for all sites. In the deepest sample growth was detected in sulphate reducer and acetogen medium. Culturable cell numbers ranged from only  $10^2$  to  $10^3$  cells  $\text{cm}^{-3}$  sediment. Similarly at the Flank site culturable cells were only detected in deeper sediment layers, more specifically below the mound base (Fig. A3.1c). Cell numbers ranged from 10 to  $10^4$  cells  $\text{cm}^{-3}$  sediment and were highest at -47.9 mamb. Mound samples yielded more positive MPNs than other sites and were not restricted to layers beneath the mound base (Fig. A3.2c). MPN counts ranged between 10 to  $10^4$  cells  $\text{cm}^{-3}$  sediment but not every medium stimulated growth. For most samples at this site only one or two of the target groups were stimulated to grow (9 out of 12) and all four target groups together were only found in one sample (16.9 mamb), which incidentally showed the maximum MPN for heterotrophs.

These results clearly show that culturable and therefore most likely active bacteria are present within the carbonate mound and in the underlying sediments. Despite the low TOC (Table 3.1) bacterial MPN results from IODP Leg 307 ranged from 10 to  $10^4$  cells  $\text{cm}^{-3}$  sediment and are comparable to MPN counts obtained from other seafloor habitats. For shallow subsurface intertidal sediments in the German Wadden Sea anaerobic MPNs have been determined for the top six metres of the sediment ranging from  $10^2$  to  $10^6$  cells per gram of sediment (Köpke *et al.*, 2005). Engelen *et al.* (2008) reported similar results from the Juan de Fuca Ridge (IODP Leg 301) with cell numbers of  $10^1$  to  $10^6$  cells per  $\text{cm}^3$  sediment. Slightly elevated numbers have been found in some Mediterranean sediments with  $10^4$  to  $10^6$  cells per  $\text{cm}^3$ , probably due to the organic-matter rich sapropel layers (Table 3.1, Süß *et al.*, 2004).

Methanogenic *Archaea* were not enriched from any site despite the presence of methane in the sediment at the mound sites and positive radiotracer experiments for methanogenesis at all sites (from acetate and  $\text{H}_2/\text{CO}_2$ ).

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### A3.3 Rates of microbial activity

This section focuses on the prokaryotic activities within sediments of the Challenger Mound investigated by thymidine incorporation and acetate oxidation studies (Fig. A3.4). Although rates for methanogenesis were also determined they have been omitted as no methanogens were isolated from these sediments.

At the Flank site acetate oxidation rates were low within the mound but showed elevated activities in layers beneath the mound base. Thymidine incorporation decreased towards the mound base but subsequently increased towards the bottom of the sediment column. Two areas of local maxima for both thymidine incorporation and acetate oxidation were found at -47.9 mamb and -70.6 mamb (Fig. A3.4).

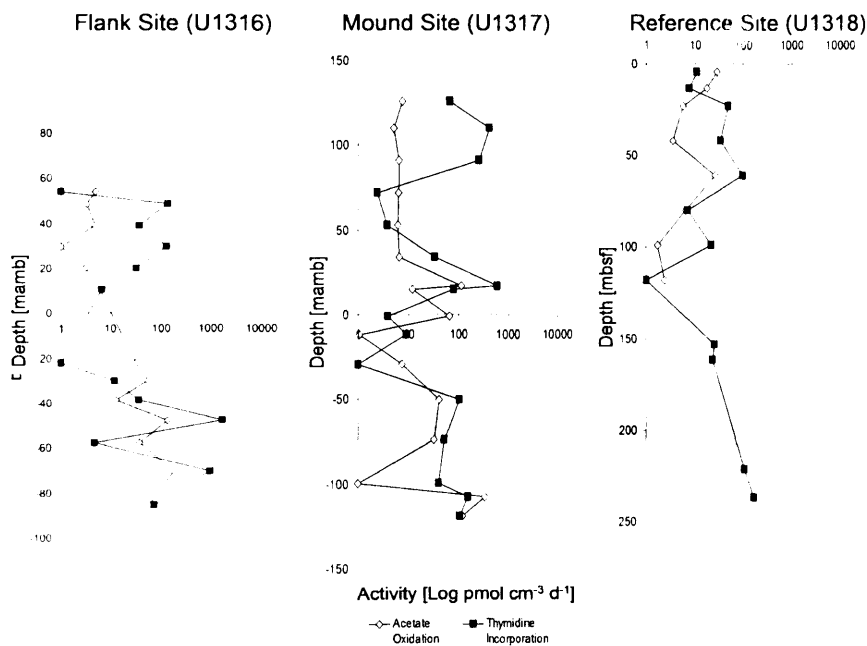


Fig. A3.4: Activity profiles of sediment samples from Leg 307. Data provided by Dr. G. Webster.

At the Mound site thymidine incorporation was high in shallow layers (between 109.9 mamb and 90.9 mamb) followed by a rapid decrease and a local maximum at 16.9 mamb followed once more by a decline bridging the mound base and almost reaching no detectable activity (Fig. A3.4). Subsequently thymidine incorporation increased resulting in local maxima at -50.4 mamb and -107.9 mamb. Some parallels to this pattern could be observed for acetate oxidation rates, which also had local

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maxima at 16.9 mamb, -50.4 mamb and -107.9 mamb but showed an additional peak at -0.86 mbsf.

At the Reference site acetate oxidation activities were highest near the surface (4.25 mbsf) and at 60.9 mbsf but showed a general decrease with depth (Fig. A3.4). Thymidine incorporation was lower in near-surface layers than between 22.9 mbsf and 60.9 mbsf. Below, activities decreased until 117.9 mbsf but subsequently increased again towards the bottom of the sediment column.

There was no apparent difference between activities within or below the Mound apart from increasing acetate oxidation rates across the Mound base at the Flank site. Activities of both measures were present yet variable throughout the entire sediment column (except acetate oxidation below 117.9 mbsf) at the Reference site.

### A3.4 Molecular microbial diversity

Investigating the microbial diversity within the Challenger Mound using molecular biological techniques was a crucial part of assessing the importance of these geological features as a deep biosphere habitat. Webster *et al.* (2009) applied qPCR to quantify the abundance of *Bacteria* and *Archaea* throughout the sediment column (data not shown). They also used nested PCR-DGGE of the 16S rRNA to investigate the prokaryotic diversity with general primers targeting the bacterial and archaeal 16S rRNA gene and specific primers targeting the 16S rRNA gene of candidate division JS1. Using this nested PCR-DGGE approach Webster *et al.* (2009) investigated sediment layers from each drilling site including samples from within and beneath the Challenger Mound (Fig. A3.5).

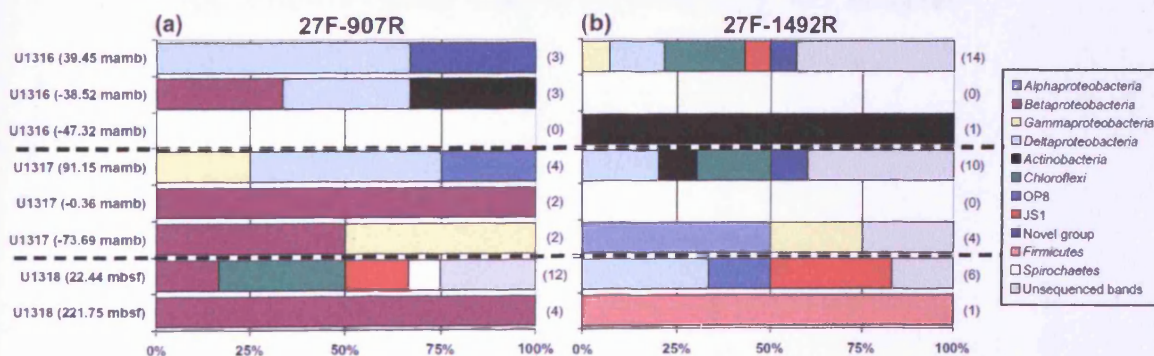
Sequences of *Archaea* were found in the sediment at all sites but in scarce numbers. As no representatives of the *Archaea* were isolated during the course of this project the results are only briefly described here. The analysed archaeal DGGE sequences were dominated by members of the South African Gold Mine Group 1 (SAGMEG-1) and detected at the two Mound sites (within the Mound) and below the Mound at Mound site (-73 mamb). No SAGMEG-1 sequences were found at the Reference site. Members of the Marine Benthic Group D (MBG-D) were detected within the Mound at the Flank site (39 mamb) and in sediment from the Reference



site (22 mbsf). Affiliates of the Miscellaneous *Crenarchaeota* Group (MCG) were also detected but only at the Reference site at 22 and 221 mbsf. No *Archaea* were found that are associated with AOM (e.g. ANME 1) despite methane-sulphate transition zones being present at both Mound sites and this process being linked to carbonate precipitation (Heijs *et al.*, 2006).

The diversity for *Bacteria* detected using nested PCR-DGGE was much higher than the diversity for *Archaea*. The Reference site showed a higher bacterial diversity at 22 mbsf than at 221 mbsf. This is true for both primer sets used (Fig. A3.5a and b). In the deeper layer the bacterial community was dominated by *Betaproteobacteria* and *Firmicutes* whereas in sediment from 22 mbsf sequences belonging to *Beta-*, *Deltaproteobacteria*, *Chloroflexi*, *Spirochaetes*, and candidate division JS1 and OP8 were present.

For the two Mound sites sediment layers within and beneath the Mound were chosen for investigation (Fig. A3.5). The two mound sites combined harboured members of the *Alpha-*, *Beta-*, *Gamma-*, and *Deltaproteobacteria*, *Actinobacteria*, *Chloroflexi*, candidate division JS1, and a novel bacterial group. The microbial community in sediment layers beneath the mound was strongly dominated by *Actinobacteria* and *Betaproteobacteria*, also present were *Alpha-*, *Gamma-* and *Deltaproteobacteria*.



**Fig. A3.5: Distribution of bacterial 16S rRNA gene sequences from Challenger Mound sites (U1316 and U1317) and Reference Site (U1318) at different sediment depths using nested PCR-DGGE analysis.** a) Nested PCR-DGGE analysis with primer sets 27F and 907R and 357F and 518R; b) Nested PCR-DGGE analysis with primer sets 27F and 1492R and 357F and 518R; Numbers of DGGE bands at each depth are shown in parentheses. Source: Webster *et al.*, 2009.

Some of the phyla were only detected at the Mound sites (e.g. *Actinobacteria*, candidate division OP8), others only at the reference site (*Spirochaetes*, *Firmicutes*). It is noteworthy that some phyla, for example *Actinobacteria*, although discovered at

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both Mound sites, were in one instance present within the Mound (Flank site) and in the other instant below the Mound (Mound site).

The molecular investigation revealed a diverse bacterial population within the Challenger Mound, its underlying sediments as well as at the Reference site. Typical subsurface groups, such as *Gammaproteobacteria*, which often dominate clone libraries (Fry *et al.*, 2008), were identified alongside less frequently detected sequences (e.g. *Actinobacteria*).

### A3.5 Summary

The results of Webster *et al.* (2009) show that the Challenger cold-water carbonate mound harbours a significant (cell counts and molecular results) and active (cultivation and radiotracer experiments) microbial community. With around 1600 similar mounds just in the Porcupine Basin, these mounds may represent a significant subseafloor habitat despite being an environment with low organic matter concentrations.

## **A4. Phenotypical data for IODP Leg 307 isolates**

**Table A4.1: Phenotypic properties of *Ornithinimicrobium* sp. F181V and related species of the family *Intrasporangiaceae*.**

Data for strain F181V, this study; *O. kibberense* DSM 17687<sup>T</sup>, data from Mayilraj *et al.*, 2006; *O. humiphilum* DSM 12362<sup>T</sup>, data from Groth *et al.*, 2001 and Mayilraj *et al.*, 2006; *O. pekingense* DSM 21552<sup>T</sup>, data from Liu *et al.*, 2008; *Serinicoccus marinus* DSM 15273<sup>T</sup>, data from Yi *et al.*, 2004 and Xiao *et al.*, 2011; *S. profundus* DSM 21363<sup>T</sup>, data from Xiao *et al.*, 2011.

Characteristic	Strain F181V	<i>O. kibberense</i>	<i>O. humiphilum</i>	<i>O. pekingense</i>	<i>S. marinus</i>	<i>S. profundus</i>
<b>Isolated from</b>	Deep Sea Sediment (Atlantic Ocean)	Indian Himalaya Soil	Garden Soil	Activated Sludge	East Sea (Korea)	Deep Sea Sediment (Indian Ocean)
<b>Growth Properties</b>						
Temperature Range	4° - 39°C	20°C - 37°C	<50°C	26°C - 38°C	5°C - 35°C	10°C - 35°C
Optimum	36°C	28°C	37°C - 42°C	33°C - 37°C	35°C	30°C
pH Range	5.9 - 9.4	≤ 9	< 9	6 - 9	6 - 11	6 - 11
Optimum	8.1			7.8 - 8.2	8	7
Salinity Range	3.5% - 7.5%	≤ 7%	≤ 6%		0% - 14%	0% - 14%
Optimum	3.5%	2% - 4%	2% - 4%	≤ 7%	2% - 3%	3% - 5%
<b>Acid Production from</b>						
L-arabinose	-	-	+	-	n.d.	n.d.
D-cellobiose	-	-	+	-	n.d.	n.d.
D-fructose	+	+	-	-	n.d.	n.d.
D-galactose	-	+	+	-	n.d.	n.d.
D-glucose	-	-	+	-	-	-
Lactose	-	-	-	-	n.d.	n.d.
D-maltose	-	+/-	+	-	n.d.	n.d.
D-mannose	-	-	-	-	n.d.	n.d.
L-rhamnose	-	-	-	-	n.d.	n.d.
D-ribose	-	n.d.	+	-	n.d.	n.d.
Sucrose	-	+/-	(+)	-	n.d.	n.d.
Trehalose	-	+/-	(+)	n.d.	n.d.	n.d.
D-xylose	-	-	(+)	-	n.d.	n.d.

**Legend:** +, Growth was scored positive; (+), weak positive; -, negative; +/-, variable results (scored positive and negative in separate experiments); +/- different results obtained from different experiments; n.d., data not determined or not available in publications.

Table A4.1. (cont.): Phenotypic properties of *Ornithinimicrobium* sp. F181V and related species of the family *Intrasporangiaceae*.

Characteristic	Strain F181V	<i>O. kibberense</i>	<i>O. humiphilum</i>	<i>O. pekingense</i>	<i>S. marinus</i>	<i>S. profundus</i>
<b>Growth on Substrates</b>						
Acetate	(+)/v	+/-	-	+	+	n.d.
L-arginine	-	n.d.	n.d.	n.d.	-	n.d.
Cellobiose	(+)/v	+	-	+	+	-
Citrate	-	+	-	+	-	-
Formate	-	n.d.	+	n.d.	n.d.	n.d.
D-fructose	-	+/-	-	+	+	(+)
D-galactose	-	+	-	+	+	n.d.
D-glucose	+	+	+	n.d.	+	+
L-glutamic acid	+v	-	n.d.	n.d.	n.d.	n.d.
Glycerol	-	n.d.	n.d.	n.d.	+	+
$\alpha$ -Ketoglutarate	-	-	+	n.d.	n.d.	n.d.
DL-malate	-	-	(+)	n.d.	n.d.	+
Maltose	-	+	n.d.	n.d.	n.d.	+
D-mannose	-	+	n.d.	n.d.	+	+
D-mannitol	-	-	n.d.	n.d.	+	-
L-phenylacetate	-	n.d.	n.d.	n.d.	n.d.	-
Propionate	-	+/-	+	n.d.	n.d.	n.d.
D-sorbitol	-	-	-	n.d.	+	(+)
Succinate	-	+	(+)	-	-	(+)
Sucrose	+v	+	n.d.	n.d.	+	+
D-xylose	-	+	n.d.	n.d.	n.d.	n.d.
<b>Electron acceptor utilisation</b>						
Nitrate	-	+	+	+	+	+

**Legend:** +, Growth was scored positive; (+), weak positive; -, negative; +/-, variable results (scored positive and negative in separate experiments); +/- different results obtained from different experiments; n.d., data not determined or not available in publications.

Table A4.1. (cont.): Phenotypic properties of *Ornithinimicrobium* sp. F181V and related species of the family *Intrasporangiaceae*.

Characteristic	Strain F181V	<i>O. kibberense</i>	<i>O. humiphilum</i>	<i>O. pekingense</i>	<i>S. marinus</i>	<i>S. profundus</i>
<b>Antibiotic MIC [<math>\mu\text{g/ml}</math>]</b>						
Chloramphenicol	1	n.d.	30	n.d.	n.d.	n.d.
Erythromycin	0.016	n.d.	15	n.d.	n.d.	< 15
Gentamycin	32	n.d.	10	n.d.	n.d.	n.d.
Kanamycin	> 256	n.d.	30	n.d.	n.d.	n.d.
Penicillin G [IU]	0.78	n.d.	2	n.d.	n.d.	10
Streptomycin	16	n.d.	10	n.d.	n.d.	n.d.
Tetracycline	16	n.d.	n.d.	n.d.	< 30	n.d.
Trimethoprim	> 256	n.d.	n.d.	n.d.	n.d.	n.d.
<b>Hydrolytic Properties/ Exo-Enzymes</b>						
Aesculin hydrolysis	-	n.d.	-	-	+	+
Agarose (liquefaction)	-	n.d.	-	n.d.	n.d.	n.d.
Alginate	-	n.d.	n.d.	n.d.	-	n.d.
<i>L</i> -arginine dihydrolase	-	n.d.	n.d.	n.d.	-	n.d.
<i>L</i> -arginine deaminase	-	n.d.	n.d.	n.d.	n.d.	-
Beta-galactosidase	+	n.d.	-	n.d.	+/v	+
Beta-glucuronidase	-	n.d.	-	n.d.	-	-
Cellulose	-	n.d.	n.d.	n.d.	-	n.d.
Chitin	+	n.d.	n.d.	n.d.	-	n.d.
DNase	-	n.d.	n.d.	n.d.	+	+
Esterase (C4)	-	n.d.	+	n.d.	+	+/v
Gelatinase	-	n.d.	+	+	+	+
Lipase (C14)	-	n.d.	-	n.d.	n.d.	-
Lipase tween 80	-	-	-	+	+	+
Phosphatase (acid)	-	n.d.	+	n.d.	-	n.d.
Phosphatase (alkaline)	+	n.d.	+	n.d.	-	+
Starch	+	+	+	+	+	n.d.
<i>L</i> -tryptophane deaminase	-	n.d.	n.d.	n.d.	n.d.	n.d.
Urease	-	n.d.	-	n.d.	-	-

**Legend:** +, Growth was scored positive; (+), weak positive; -, negative; +/-, variable results (scored positive and negative in separate experiments); +/- different results obtained from different experiments; n.d., data not determined or not available in publications.

Table A4.1. (cont.): Phenotypic properties of *Ornithinimicrobium* sp. F181V and related species of the family *Intrasporangiaceae*.

Characteristic	Strain F181V	<i>O. kibberense</i>	<i>O. humiphilum</i>	<i>O. pekingense</i>	<i>S. marinus</i>	<i>S. profundus</i>
<b>Other Phenotypic Properties</b>						
Catalase presence	n.d.	+	+	+	+	+
Flagella	-	n.d.	n.d.	n.d.	n.d.	n.d.
Gram Stain	+	+	+	+	+	+
Indol production	-	-	-	n.d.	n.d.	-
Methyl-Red Test	-	-	-	n.d.	n.d.	n.d.
Motility	-	-	-	-	-	-
Oxidase presence	n.d.	-	-	-	-	-
Sporulation	-	-	-	n.d.	-	-
Voges-Proskauer Test	-	-	-	-	n.d.	n.d.

**Legend:** +, Growth was scored positive; (+), weak positive; -, negative; +/-, variable results (scored positive and negative in separate experiments); +/- different results obtained from different experiments; n.d., data not determined or not available in publications.

**Table A4.2: Phenotypic properties of *Shewanella* sp. isolates and closely related affiliates.**

Strains: F16III, F16IV, F16V, F17III, F17V, F17VI, F17VII, F18III data from this study; SCSA3, data from this study and provided by Dr. Henrik Sass; *S. vesiculosa* CECT 7339<sup>T</sup>, data from Bozal *et al.*, 2009; *S. frigidimarina* DSM 12253<sup>T</sup>, data from Bowman *et al.*, 1997; *S. profunda* DSM 15900<sup>T</sup>, data from Toffin *et al.*, 2004a.

Characteristic	F16 III	F16 IV	F16 V	F17 III	F17 V	F17 VI	F17 VII	F18 III	SCSA3	<i>S. vesiculosa</i>	<i>S. frigidimarina</i>	<i>S. profunda</i>
<b>Isolated from</b>	Deep Sea Sediment (Atlantic Ocean)								Tidal Flat Sed. Wadden Sea Germany	Marine Sediment Antarctica	Antarctic Sea Ice	Deep Sea Sediment Nankai Trough
	-44 mamb	-44 mamb	-44 mamb	47 mamb	14 mamb	14 mamb	-1 mamb	4 mbsf				
<b>Growth Properties</b>												
Temperature Range	< 2°C - < 33°C	< 2°C - 39°C	< 2°C - 39°C	< 2°C - 39°C	< 2°C - 36°C	< 2°C - 36°C	< 2°C - 39°C	< 2°C - 33°C	< 2°C - 30°C	- 4°C - 30°C	- 14°C - 28°C	4°C - 37°C
pH Range	6.0 - 9.1	6.0 - 9.1	6.0 - 9.4	6.0 - 9.4	6.0 - 9.4	6.7 - 9.4	6.0 - 9.4	6.0 - 9.4	n.d.	6.0 - 8.5	n.d.	6.5 - 8.0
Optimum	7.3 - 8.4	8.4	7.3	8.4	8.4	8.4	8.4	7.3		7.5		7.0
Salinity Range	3.5% - 10%	3.5% - 10%	3.5% - 10%	3.5% - 10%	2.5% - 10%	3.5% - 10%	3.5% - 7.5%	3.5% - 7.5%	n.d.	0% - < 7%	≤ 9%	0% - 6%
Optimum	3.5%	3.5%	5% - 7.5%	7.5%	7.5%	7.5%	3.5%	3.5%		2%		0.5%
<b>Electron acceptor utilisation</b>												
Fe-citrate	-	-	-	-	-	-	-	-	n.d.	n.d.	n.d.	n.d.
Ferrihydrite	+	+	-	+	-	-	+	+	+	n.d.	+	+
Manganese(IV) oxide	+	+	-	+	(+)	+	+	+	+	n.d.	n.d.	n.d.
Nitrate	+	+	-	+	+	+	+	+	-	+	+	+
Thiosulphate	(+)/v	-	-	(+)	-	-	-	(+)	+	n.d.	n.d.	n.d.

**Legend:** +, Growth was scored positive; (+), weak positive; -, negative; +/-, variable results (scored positive and negative in separate experiments); +/- different results obtained from different experiments; n.d., data not determined or not available in publications.

Table A4.2 (cont.): Phenotypic properties of *Shewanella* sp. isolates and closely related affiliates.

Characteristic	F16 III	F16 IV	F16 V	F17 III	F17 V	F17 VI	F17 VII	F18 III	SCSA3	<i>S. vesiculosa</i>	<i>S. frigidimarina</i>	<i>S. profunda</i>
<b>Fermentations</b>												
Casamino Acids	+v	-	-	-	+	-	-	+v	n.d.	n.d.	n.d.	n.d.
D-glucose	+	-	+	-	+	+	(+)	+	+	n.d.	+	n.d.
Hydrogen production	+	+	+	+	-	+	+	+	+	n.d.	n.d.	n.d.
<b>Hydrolytic Properties/ Exo-Enzymes</b>												
L-arginine dihydrolase	-	-	n.d.	-	+	-	-	-	n.d.	n.d.	+	n.d.
Caseinase	+	+	+	+	+	+	+	+	n.d.	+	n.d.	n.d.
DNase	+	+	+	+	-	+	+	+	n.d.	n.d.	+	n.d.
Esterase (C4)	-	-	-	-	-	-	-	-	n.d.	+	n.d.	n.d.
Gelatinase	n.d.	+	+	+	+	+	+	+	-	+	n.d.	+
Lipase tween 20	+	+	+	+	-	+	-	+	+	n.d.	n.d.	n.d.
Lipase tween 80	-	+	+	+	-	+	+	+	+	+	n.d.	n.d.
Lysine decarboxylase	-	-	n.d.	-	-	-	-	-	n.d.	n.d.	-	n.d.
Ornithine decarboxylase	-	-	n.d.	+	(+)	-	-	-	n.d.	n.d.	+v	+
Phosphatase (acid)	-	-	-	(+)	+	-	-	-	n.d.	+	n.d.	n.d.
Phosphatase (alkaline)	+	+	+	+	+	+	+	+	n.d.	+	n.d.	n.d.
Starch	-	-	+	-	+	-	-	-	n.d.	-	-	n.d.
Urease	-	-	-	-	-	-	-	-	n.d.	-	-	n.d.
<b>Other Phenotypic Properties</b>												
Indol Production	-	-	-	-	+	-	-	-	n.d.	n.d.	-	n.d.
Motility	+	+	+	+	-	+	+	+	n.d.	+	+	+

**Legend:** +, Growth was scored positive; (+), weak positive; -, negative; +v, variable results (scored positive and negative in separate experiments); +/- different results obtained from different experiments; n.d., data not determined or not available in publications.



Table A4.2 (cont.): Phenotypic properties of *Shewanella* sp. isolates and closely related affiliates.

Characteristic	F16 III	F16 IV	F16 V	F17 III	F17 V	F17 VI	F17 VII	F18 III	SCSA3	<i>S. vesiculosa</i>	<i>S. frigidimarina</i>	<i>S. profunda</i>
<b>Antibiotic MIC [<math>\mu\text{g/ml}</math>]</b>												
Chloramphenicol	0.3	0.3	1.0	2.0	2.0	0.1	0.3	8	n.d.	n.d.	n.d.	n.d.
Erythromycin	0.3	2.0	2.0	8.0	4.0	2.0	2.0	4	n.d.	n.d.	n.d.	n.d.
Gentamycin	0.5	2.0	2.0	16.0	4.0	0.5	4.0	16	n.d.	n.d.	n.d.	n.d.
Kanamycin	2.0	16.0	8.0	64.0	4.0	8.0	8.0	64	n.d.	n.d.	n.d.	n.d.
Penicillin G [IU]	3.1	6.3	200.0	>3200	3.1	20.0	3200	100	n.d.	n.d.	n.d.	n.d.
Streptomycin	2.0	32.0	16.0	>256	4.0	16.0	16.0	128	n.d.	n.d.	n.d.	n.d.
Tetracycline	8.0	32.0	32.0	>256	16.0	32.0	32.0	32	n.d.	n.d.	n.d.	n.d.
Trimethoprim	>256	0.5	1.0	>256	256.0	0.3	0.3	>256	n.d.	n.d.	n.d.	n.d.
<b>Growth on Substrates</b>												
Acetate	+	+	+	+	+	+	+	+	+	n.d.	+	n.d.
DL-alanine	+	+	-	+	+	-	+	+	+	n.d.	-	n.d.
L-arabinose	-	-	-	-	-	-	-	-	-	n.d.	-	+
L-arginine	-	-	-	(+)	+	-	-	-	-	n.d.	n.d.	n.d.
Benzoate	-	-	-	+	-	-	-	-	-	n.d.	n.d.	n.d.
Betaine	-	-	-	-	-	-	-	+v	+	n.d.	n.d.	n.d.
<i>n</i> -Butanol	-	+v	-	+	-	-	-	-	-	n.d.	n.d.	n.d.
Butyrate	+	+	(+)	+	-	+	+	+	+	n.d.	+	n.d.
Caproate	+	+	(+)	+	-	+	+	+	+	n.d.	+v	n.d.
Caprylate	-	-	-	+	-	-	-	-	n.d.	n.d.	-	n.d.
Casamino acids	+	+	+	+	+	+	+	+	+	n.d.	n.d.	n.d.
Cellobiose	+	+	(+)	+	+	-	+	+	+	n.d.	+	n.d.
Cellulose	-	-	-	-	-	-	-	-	-	n.d.	n.d.	n.d.
Chitin	(+)	-	-	-	(+)	-	-	-	+	n.d.	n.d.	n.d.

**Legend:** +, Growth was scored positive; (+), weak positive; -, negative; +/v, variable results (scored positive and negative in separate experiments); +/- different results obtained from different experiments; n.d., data not determined or not available in publications.

Table A4.2 (cont.): Phenotypic properties of *Shewanella* sp. isolates and closely related affiliates.

Characteristic	III	IV	V	III	V	VI	VII	III	A3	<i>siculosa</i>	<i>timarina</i>	<i>ofunda</i>
<b>Growth on Substrates (continued)</b>												
Citrate	(+)	+v	-	+	+	-	+	-	+	n.d.	-	+
Crotonate	+	+	-	+	-	+	+	+	-	n.d.	n.d.	n.d.
Ethanol	-	+	-	+	-	-	-	-	-	n.d.	n.d.	n.d.
D-fructose	+	+	+	+	+	+	+	+	+	n.d.	+v	n.d.
Fumarate	+	+	(+)	+	+	+	+	+	-	n.d.	+	+
D-galactose	-	-	-	+	+	-	-	+	n.d.	+	+v	n.d.
D-gluconate	-	-	-	+	+	-	-	-	-	n.d.	n.d.	n.d.
D-glucosamine	+	+	+	+	+	+	+	-	+	n.d.	n.d.	n.d.
D-glucose	+	+	+	+	+	+	+	+	+	+	+	n.d.
L-glutamic acid	+	+	(+)	+	+	+	+	-	n.d.	n.d.	+v	+
L-glutamine	+	+	+	+	+	+	+	+	+	n.d.	n.d.	n.d.
Glycerol	+	+v	-	+	+	-	+v	-	-	n.d.	-	n.d.
Glycol	-	+	-	+	-	-	-	-	-	n.d.	n.d.	n.d.
Glycolate	-	-	-	+	-	-	-	-	-	n.d.	n.d.	n.d.
L-isoleucine	+	+	-	+	+v	-	+	+	+	n.d.	n.d.	n.d.
α-Ketoglutarate	+	+	-	(+)	+	+	+	+	+	n.d.	n.d.	n.d.
DL-lactate	+	+	-	+	+	+	+	+	+	n.d.	+	+
Lactose	+	+	+	-	+	-	+	+	n.d.	n.d.	-	n.d.
Laminarin	+	(+)	+	(+)	(+)	-	(+)	+	-	n.d.	n.d.	n.d.
DL-malate	+	+	(+)	+	+	+	+	+	-	+	+	n.d.
Malonate	-	-	-	(+)	-	-	-	-	-	n.d.	-	n.d.
Maltose	+	+	(+)	+	+	-	+	+	+	+	-	+
Mannitol	+	+	+	+	+	-	+	-	+	n.d.	+	n.d.

**Legend:** +, Growth was scored positive; (+), weak positive; -, negative; +v, variable results (scored positive and negative in separate experiments); +/- different results obtained from different experiments; n.d., data not determined or not available in publications.

Table A4.2 (cont.): Phenotypic properties of *Shewanella* sp. isolates and closely related affiliates.

Characteristic	F16 III	F16 IV	F16 V	F17 III	F17 V	F17 VI	F17 VII	F18 III	SCSA3	<i>S. vesiculosa</i>	<i>S. frigidimarina</i>	<i>S. profunda</i>
<b>Growth on Substrates (continued)</b>												
D-mannose	-	-	-	+	+	-	-	+	+	n.d.	n.d.	+
Methanol	-	+	-	+	-	-	-	-	-	n.d.	n.d.	n.d.
Methylamine	-	-	-	-	-	-	-	-	-	n.d.	n.d.	n.d.
Peptone	+	+	+	+	+	+	+	+	+	n.d.	n.d.	+
L-phenylalanine	-	-	-	+	-	-	-	-	+	n.d.	+/v	n.d.
<i>n</i> -propanol	-	+	-	+	-	-	-	-	-	n.d.	n.d.	n.d.
Propionate	+	+	(+)	+	+	+	+	+	-	n.d.	+	n.d.
L-Rhamnose	-	-	-	-	-	-	-	-	-	n.d.	-	n.d.
Salicylate	-	-	-	-	-	-	-	-	-	n.d.	n.d.	n.d.
L-serine	+	+	-	+	+	-	+	-	n.d.	n.d.	-	n.d.
D-sorbitol	-	-	-	+	-	-	-	-	n.d.	n.d.	-	+
Starch	+	+	-	-	+	-	+	+	-	n.d.	n.d.	n.d.
Succinate	+	+	(+)	+	+	+	+	+	+	n.d.	+	+
Sucrose	+	+	(+)	+	+/v	(+)	+	+	+	+	+	n.d.
Trehalose	-	-	-	-	-	-	-	-	+	n.d.	+	n.d.
L-tryptophane	-	-	-	-	-	-	-	-	+	n.d.	n.d.	n.d.
Tween 80	+	+	(+)	+	+	-	+	+	-	n.d.	n.d.	n.d.
Valerate	+	+	+	+	-	+	+	+	+	n.d.	+	n.d.
Xylan	-	-	-	-	-	-	-	-	+	n.d.	n.d.	n.d.
D-Xylose	-	-	-	((+))	-	-	-	-	-	n.d.	+/v	n.d.
Yeast Extract	+	+	+	+	+	+	+	+	+	n.d.	n.d.	n.d.

**Legend:** +, Growth was scored positive; (+), weak positive; -, negative; +/v, variable results (scored positive and negative in separate experiments); +/- different results obtained from different experiments; n.d., data not determined or not available in publications.

## Appendix

**Table A4.3: Phenotypic properties of *Vibrio* sp. isolates and closely related affiliates.**

Strains: F16II, F17I, and F17IV data from this study; SCSA1, data from this study and provided by Dr. Henrik Sass; *V. lentus* DSM 13757<sup>T</sup>, data from Macian *et al.*, 2001; *V. kanaloae* DSM 17181<sup>T</sup>, data from Thompson *et al.*, 2003.

Characteristic	F16 II	F17 I	F17 IV	SCSA1	<i>V. lentus</i>	<i>V. kanaloae</i>
<b>Isolated from</b>	Deep Sea Sediment (Atlantic Ocean)			Tidal Flat Sediment	Oysters	Diseased
	-27 mamb	115 mamb	14 mamb	Wadden Sea Germany	Spain	Oyster Larvae France
<b>Growth Properties</b>						
Temperature						
Range	< 2°C - 27°C	< 2°C - 27°C	< 2°C - 27°C	< 2°C - < 30°C	4°C - 30°C	n.d.
pH Range	5.9 - 9.4	5.9 - 9.4	5.9 - 9.4	n.d.	n.d.	n.d.
Optimum	7.3	7.3	9.1			
Salinity Range	3.5% - 10%	2.5% - 7.5%	3.5% - 10%	n.d.	n.d.	n.d.
Optimum	5%	3.5%	3.5%			
<b>Growth on Substrates</b>						
Acetate	+	+	+	+	-	+
L-alanine	+	+	+	-	+	+
L-arabinose	-	-	-	-	-	-
L-arginine	+	+	+	n.d.	-	n.d.
Benzoate	-	-	-	-	n.d.	n.d.
Betaine	-	-	-	+	n.d.	n.d.
<i>n</i> -Butanol	-	-	-	+	n.d.	n.d.
Butyrate	-	-	-	+	n.d.	n.d.
Caproate	+	+	+	+	n.d.	n.d.
Caprylate	-	-	-	n.d.	n.d.	n.d.
Casamino acids	+	+	+	+	n.d.	n.d.
Cellobiose	+	+	+	+	+	-
Cellulose	-	-	-	-	n.d.	n.d.
Chitin	-	-	-	+	n.d.	n.d.
Citrate	-	-	+	+	n.d.	n.d.
Crotonate	-	-	-	+	n.d.	n.d.
L-cysteine	-	-	-	-	n.d.	n.d.
Ethanol	-	-	-	+	n.d.	n.d.
Formate	-	-	-	-	n.d.	+
D-fructose	+	+	+	+	+	n.d.
Fumarate	+	+	+	+	+	
D-galactose	-	-	+	n.d.	+	+
D-gluconate	+	+	+	(+)	-	+
D-glucosamine	+	+	-	+	-	n.d.
D-glucose	+	+	+	+	-	n.d.
L-glutamic acid	+	+	+	n.d.	+	n.d.
L-glutamine	+	+	+	+	+	n.d.
Glycerol	+	+	+	+	+	n.d.
Glycol	-	-	-	+	n.d.	n.d.
Glycolate	-	-	-	-	n.d.	n.d.
Isoleucine	-	-	(+)	+	n.d.	n.d.

**Legend:** +, Growth was scored positive; (+), weak positive; -, negative; +/v, variable results (scored positive and negative in separate experiments); +/- different results obtained from different experiments; n.d., data not determined or not available in publications.

Appendix

**Table A4.3 (cont.): Phenotypic properties of *Vibrio* sp. isolates and closely related affiliates.**

Characteristic	F16 II	F17 I	F17I V	SCSA1	<i>V. lentus</i>	<i>V. kanaloae</i>
<b>Growth on Substrates (continued)</b>						
α-Ketoglutarate	+	+	+	+	+	+
DL-lactate	-	-	+	-	+	n.d.
Lactose	-	-	+	n.d.	-	n.d.
Laminarin	-	-	-	+	n.d.	n.d.
DL-malate	-	-	+	+	+	n.d.
Malonate	-	-	-	(+)	n.d.	n.d.
Maltose	+	+	+	+	+	n.d.
Mannitol	+	+	+	+	+	+
D-mannose	+	+	+	+	+	+
Methanol	-	-	-	+	-	n.d.
Methylamine	-	-	-	n.d.	n.d.	n.d.
Peptone	+	+	+	+	n.d.	n.d.
L-phenylalanine	-	-	-	+	n.d.	-
<i>n</i> -Propanol	-	-	-	+	n.d.	n.d.
Propionate	-	+	+	-	-	+
L-rhamnose	-	-	-	-	-	n.d.
Salicylate	-	-	-	-	n.d.	n.d.
L-serine	+	+	+	n.d.	+	+
D-sorbitol	-	-	-	n.d.	-	-
Starch	+	+	+	+	n.d.	n.d.
Succinate	-	-	+	+	+	n.d.
Sucrose	-	-	-	+	-	+
Trehalose	n.d.	n.d.	n.d.	+	-	n.d.
L-tryptophane	-	-	-	+	n.d.	n.d.
Tween 80	+	+	+	-	n.d.	n.d.
Valerate	-	+	+	-	n.d.	n.d.
Xylan	+	+	(+)	+	n.d.	n.d.
D-Xylose	-	-	-	-	-	n.d.
Yeast extract	+	+	+	+	n.d.	n.d.
<b>Electron acceptor utilisation</b>						
Fe-citrate	-	-	-	n.d.	n.d.	n.d.
Ferrihydrite	-	-	-	-	n.d.	n.d.
Manganese(IV)	-	-	-	-	n.d.	n.d.
Nitrate	-	-	+/-	+	+	n.d.
Thiosulphate	+/-	+/-	-	-	n.d.	n.d.
<b>Fermentations</b>						
Casamino acids	+	+	+	n.d.	n.d.	n.d.
D-glucose	+	+	+	+	+	n.d.
Hydrogen production	-	-	-	+	n.d.	n.d.
<b>Other Phenotypic Properties</b>						
Indol production	+	+	+		+	+
Flagella	not observed	single polar	not observed		single polar	single polar
Motility	not observed	not observed	not observed			

**Legend:** +, Growth was scored positive; (+), weak positive; -, negative; +/-, variable results (scored positive and negative in separate experiments); +/- different results obtained from different experiments; n.d., data not determined or not available in publications.

Appendix

**Table A4.3.: Phenotypic properties of *Vibrio* sp. isolates and closely related affiliates, continued.**

Characteristic	F16 II	F17 I	F17I V	SCSAI	<i>V. lentus</i>	<i>V. kanaloae</i>
<b>Hydrolytic Properties/ Exo-Enzymes</b>						
L-arginine dihydrolase	+	+	+	n.d.	+/v	+
Caseinase	-	-	-	n.d.	+	n.d.
DNase	-	-	-	n.d.	+	n.d.
Esterase (C4)	-	-	-	n.d.	n.d.	n.d.
Gelatinase	-	+	+	+	+	+
Lipase tween 20	-	-	-	-	n.d.	n.d.
Lipase tween 80	-	-	-	-	+by	n.d.
Lysine decarboxylase	-	-	-	n.d.	-	-
Ornithine decarboxylase	-	-	-	n.d.	-	-
Phosphatase (acid)	(+)	-	+	n.d.	n.d.	n.d.
Phosphatase (alkaline)	+	+	+	n.d.	n.d.	n.d.
Starch	+	+	+	n.d.	+	n.d.
Urease	-	-	-	n.d.	n.d.	n.d.
<b>Antibiotic MIC [µg/ml]</b>						
Chloramphenicol	n.d.	0.5	0.3	0.5	n.d.	sensitive
Erythromycin	n.d.	8.0	1.0	4	n.d.	n.d.
Gentamycin	n.d.	8.0	64.0	4	n.d.	n.d.
Kanamycin	n.d.	32.0	4.0	8	n.d.	n.d.
PenicillinG [IU]	n.d.	1.6	25.0	25	n.d.	n.d.
Streptomycin	n.d.	16.0	2.0	16	n.d.	n.d.
Tetracycline	n.d.	16.0	2.0	8	n.d.	sensitive
Trimethoprim	n.d.	4.0	0.5	32	n.d.	n.d.

**Legend:** +, Growth was scored positive; (+), weak positive; -, negative; +/-, variable results (scored positive and negative in separate experiments); +/- different results obtained from different experiments; n.d., data not determined or not available in publications.

## Appendix

**Table A4.4: Phenotypic properties of *Raoultella* sp. isolates and closely related affiliates.**

Strains: F17II and F17VIII data from this study; *R. planticola* DSM 3069<sup>T</sup>, data from Bagley *et al.*, 1982, Drancourt *et al.*, 2001, and Brisse *et al.*, 2006; *R. ornithinolytica* DSM 7464<sup>T</sup>, data from Sakazaki *et al.*, 1998, Drancourt *et al.*, 2001, and Brisse *et al.*, 2006; *R. terrigena* DSM 2687<sup>T</sup>, data from Izard *et al.*, 1981, Drancourt *et al.*, 2001, and Brisse *et al.*, 2006.

Characteristic	F17II	F17 VIII	<i>R. planticola</i>	<i>R. ornithinolytica</i>	<i>R. terrigena</i>
<b>Isolated from</b>	Deep Sea Sediment (Atlantic Ocean)		Radish root	urine	Drinking water
	66 mamb	-74 mamb			
<b>Growth Properties</b>					
Temperature Range	< 2°C - 36°C	< 2°C - 48°C	at 5°C	at 5°C, at -41°C	at 5°C
pH Range	5.9 - 9.1	5.9 - 9.4			
Optimum	8.1	8.4	n.d.	n.d.	n.d.
Salinity Range	3.5% - 7.5%	3.5% - 10%			
Optimum	3.5%	3.5%	n.d.	n.d.	n.d.
<b>Growth on Substrates</b>					
Acetate	+	+	n.d.	n.d.	+
L-alanine	+	+	+	+	+
L-arabinose	-	+	n.d.	n.d.	+
L-arginine	-	+	+	n.d.	n.d.
Benzoate	-	+	+	-	+
Betaine	-	-	n.d.	n.d.	-
<i>n</i> -Butanol	-	-	n.d.	n.d.	n.d.
Butyrate	+	-	n.d.	n.d.	-
Caproate	+	-	n.d.	+	-
Caprylate	-	-	n.d.	+/-	n.d.
Casamino acids	+	+	n.d.	n.d.	n.d.
Cellobiose	+	+	n.d.	n.d.	+
Cellulose	-	-	n.d.	n.d.	n.d.
Chitin	(+)	-	n.d.	n.d.	n.d.
Citrate	-	+	n.d.	+	+
<i>m</i> -Coumarate	n.d.	n.d.	+	+	-
Crotonate	+	-	n.d.	n.d.	n.d.
L-cysteine	-	-	n.d.	n.d.	n.d.
Ethanol	-	-	n.d.	n.d.	-
Formate	-	-	n.d.	n.d.	n.d.
D-fructose	+	+	n.d.	n.d.	n.d.
Fumarate	+	-	n.d.	+	+
D-galactose	-	+	n.d.	n.d.	+
D-gluconate	(+)	+	n.d.	n.d.	n.d.
D-glucosamine	+	+	n.d.	-	+
D-glucose	+	+	n.d.	n.d.	+
L-glutamic acid	+	+	n.d.	+	+
L-glutamine	+	+	n.d.	n.d.	n.d.
Glycerol	-	+	n.d.	n.d.	+
Glycol	-	-	n.d.	n.d.	n.d.

**Legend:** +, Growth was scored positive; (+), weak positive; -, negative; +/-, variable results (scored positive and negative in separate experiments); +/- different results obtained from different experiments; n.d., data not determined or not available in publications.

Appendix

Table A4.4 (cont.): Phenotypic properties of *Raoultella* sp. isolates and closely related affiliates.

Characteristic	F17II	F17 VIII	<i>R. planticola</i>	<i>R. ornithinolytica</i>	<i>R. terrigena</i>
<b>Growth on Substrates (continued)</b>					
Glycolate	-	+	n.d.	n.d.	n.d.
Histamine	n.d.	n.d.	+	-	+
Isoleucine	-	-	n.d.	n.d.	-
α-Ketoglutarate	+	(+)/v	n.d.	n.d.	
DL-lactate	+	+	n.d.	+	+
Lactose	+	+	n.d.	n.d.	+
Laminarin	+	-	n.d.	n.d.	n.d.
DL-malate	+	+	n.d.	+	+
Malonate	-	+	n.d.	+	+/-
Maltose	+	+	n.d.	n.d.	+
Mannitol	+	+	n.d.	n.d.	+
D-mannose	(+)/v	+	n.d.	n.d.	+
Methanol	-	-	n.d.	n.d.	n.d.
Methylamine	-	-	n.d.	n.d.	n.d.
Peptone	+	+	n.d.	n.d.	n.d.
L-Phenylalanine	-	+	n.d.	n.d.	-
n-Propanol	-	-	n.d.	n.d.	-
Propionate	+	-	n.d.	-	-
L-rhamnose	-	+	+	+	+
Salicylate	-	-	n.d.	n.d.	-
Serine	+	+	n.d.	+	+
D-sorbitol	+	+	+	+	+
Starch	-	-	n.d.	n.d.	n.d.
Succinate	+	+/v	n.d.	n.d.	+
Sucrose	+	+	+	+	+
Trehalose	+	-	n.d.	n.d.	+
L-tryptophane	-	-	n.d.	n.d.	-
Tween 80	+	(+)/v	n.d.	n.d.	n.d.
Valerate	+	-	n.d.	-	-
Xylan	(+)/v	(+)/v	n.d.	n.d.	n.d.
D-Xylose	-	+	n.d.	n.d.	+
Yeast extract	+	+	n.d.	n.d.	n.d.
<b>Electron acceptor utilisation</b>					
Fe-citrate	-	-	n.d.	n.d.	n.d.
Ferrihydrite	+	-	n.d.	n.d.	n.d.
Manganese(IV) oxide	+	+	n.d.	n.d.	n.d.
Nitrate	+	+	n.d.	+	+
Thiosulphate	-	-	n.d.	n.d.	n.d.

**Legend:** +, Growth was scored positive; (+), weak positive; -, negative; +/-, variable results (scored positive and negative in separate experiments); +/- different results obtained from different experiments; n.d., data not determined or not available in publications.



## Appendix

**Table A4.4 (cont.): Phenotypic properties of *Raoultella* sp. isolates and closely related affiliates.**

Characteristic	F17II	F17 VIII	<i>R. planticola</i>	<i>R. ornithinolytica</i>	<i>R. terrigena</i>
<b>Fermentations</b>					
Casamino acids	(+)	+	n.d.	n.d.	n.d.
D-glucose	-	+	n.d.	n.d.	+
Hydrogen production	+	+	n.d.	n.d.	n.d.
<b>Hydrolytic Properties/ Exo-Enzymes</b>					
L-arginine dihydrolase	-	-	-	-	-
Caseinase	+	+	n.d.	n.d.	n.d.
DNase	+	-	n.d.	-	-
Esterase (C4)	-	-	n.d.	n.d.	n.d.
Gelatinase	+	-	-	-	-
Lipase tween 20	+	-	n.d.	n.d.	n.d.
Lipase tween 80	+	-	n.d.	-	-
Lysine decarboxylase	-	(+)	+	+	+
Ornithine decarboxylase	-	(+)	-	+	-
Phosphatase (acid)	+	+	n.d.	n.d.	n.d.
Phosphatase (alkaline)	+	+	n.d.	n.d.	n.d.
Starch hydrolysis	+	-	n.d.	n.d.	n.d.
Urease	-	+	+	+	+
<b>Other Phenotypic Properties</b>					
Indol Production	-	-	+	+	-
Flagella	single polar	not observed	n.d.	n.d.	n.d.
Motility	+	non-motile	non-motile	non-motile	non-motile
<b>Antibiotic MIC [<math>\mu\text{g/ml}</math>]</b>					
Chloramphenicol	2.0	2.0	sensitive (30)	sensitive	n.d.
Erythromycin	8.0	256.0	n.d.	n.d.	n.d.
Gentamycin	8.0	32.0	sensitive (10)	sensitive	n.d.
Kanamycin	64.0	128.0	sensitive (30)	sensitive	n.d.
Penicillin G [IU]	>3200	>3200	n.d.	resistant	n.d.
Streptomycin	64.0	64.0	sensitive (10)	sensitive	n.d.
Tetracycline	64.0	>256	sensitive (30)	sensitive	n.d.
Trimethoprim	16.0	>256	n.d.	n.d.	n.d.

**Legend:** +, Growth was scored positive; (+), weak positive; -, negative; +/-, variable results (scored positive and negative in separate experiments); +/- different results obtained from different experiments; n.d., data not determined or not available in publications.

## Appendix

**Table A4.5: Phenotypic properties of *Pseudomonas* isolates and closely related affiliates.**

Strains: F18II and F18V data from this study; NA101 data from this study and provided by Dr. Henrik Sass; *P. pohangensis* DSM 17875<sup>T</sup>, data from Weon *et al.*, 2006; *P. antarctica* DSM 15318<sup>T</sup>, data from Reddy *et al.*, 2004; *P. proteolytica* DSM 15321<sup>T</sup>, data from Reddy *et al.*, 2004.

Characteristic	F18II	F18V	NA101	<i>P. pohangensis</i>	<i>P. antarctica</i>	<i>P. proteolytica</i>
<b>Isolated from</b>	Deep Sea Sediment (Atlantic Ocean) 4 mbsf	80 mbsf	Tidal Flat Surface Sediment Wadden Sea Germany	Sea shore sand Korea	Cyanobacterial Mats Antarctic Ponds Wright Valley	
<b>Growth Properties</b>						
Temperature Range	< 2°C - 36°C	< 2°C - 39°C	4°C - 40°C	4°C - 35°C	4°C - 30°C	4°C - 30°C
Optimum				20°C - 30°C	22°C	22°C
pH Range	5.9 - 9.4	5.9 - 9.1	n.d.	5 - 9		
Optimum	8.4	8.1		7 - 8	7	7
Salinity Range	3.5% - 7.0%	3.5% - 7.0%	n.d.	up to 3%	up to 3%	up to 3%
Optimum	3.5%	3.5%		1%		
<b>Growth on Substrates</b>						
Acetate	+	+	+	n.d.	+	+
DL-alanine	+	+	n.d.	+	+	+
L-arabinose	-	-	n.d.	n.d.	-	n.d.
L-arginine	+	+	n.d.	n.d.	+	+
Benzoate	-	-	n.d.	n.d.	n.d.	n.d.
Betaine	(+)	+	n.d.	n.d.	n.d.	n.d.
<i>n</i> -Butanol	(+)	(+)	n.d.	n.d.	n.d.	n.d.
Butyrate	(+)	(+)	n.d.	n.d.	n.d.	n.d.
Caproate	(+)	(+)	n.d.	n.d.	n.d.	n.d.
Caprylate	+	+	n.d.	n.d.	n.d.	n.d.
Casamino acids	+	+	n.d.	n.d.	n.d.	n.d.
Cellobiose	-	-	n.d.	n.d.	-	-
Cellulose	-	-	n.d.	n.d.	-	-
Chitin	-	-	n.d.	n.d.	n.d.	n.d.
Citrate	-	-	n.d.	n.d.	+	+
Crotonate	+	+	n.d.	n.d.	n.d.	n.d.
L-cysteine	-	-	n.d.	n.d.	-	-
Ethanol	(+)	(+)	n.d.	n.d.	n.d.	n.d.
Formate	-	+/-	n.d.	n.d.	n.d.	n.d.
D-fructose	-	-	n.d.	n.d.	+	+
Fumarate	+	+	n.d.	n.d.	n.d.	-
D-galactose	-	-	n.d.	n.d.	+	+
D-gluconate	-	+/-	n.d.	n.d.	n.d.	n.d.
D-glucosamine	-	+/-	n.d.	n.d.	n.d.	n.d.
D-glucose	-	-	+	n.d.	+	+
L-glutamic acid	+	+	n.d.	n.d.	+	+
L-glutamine	+	+	n.d.	+	+	+
Glycerol	(+)	(+)	n.d.	n.d.	+	+

**Legend:** +, Growth was scored positive; (+), weak positive; -, negative; +/-, variable results (scored positive and negative in separate experiments); +/- different results obtained from different experiments; n.d., data not determined or not available in publications.

Appendix

Table A4.5 (cont.): Phenotypic properties of *Pseudomonas* isolates and closely related affiliates.

Characteristic	F18II	F18V	NA101	<i>P. pohangensis</i>	<i>P. antarctica</i>	<i>P. proteolytica</i>
<b>Growth on Substrates (continued)</b>						
Glycol	(+)	(+)	n.d.	n.d.	n.d.	n.d.
Glycolate	+	+	n.d.	n.d.	n.d.	n.d.
Isoleucine	-	+	n.d.	n.d.	-	-
α-Ketoglutarate	+	+	n.d.	n.d.	n.d.	n.d.
DL-lactate	+	+	+	+	+	+
Lactose	-	-	n.d.	n.d.	-	-
Laminarin	-	-	n.d.	n.d.	n.d.	n.d.
DL-malate	+	+	n.d.	n.d.	n.d.	n.d.
Malonate	-	-	n.d.	n.d.	n.d.	n.d.
Maltose	-	-	n.d.	n.d.	-	-
Mannitol	-	-	n.d.	n.d.	+	+
D-mannose	-	-	n.d.	n.d.	+	+
Methanol	(+)	(+)	n.d.	n.d.	n.d.	n.d.
Methylamine	-	-	n.d.	n.d.	n.d.	n.d.
Peptone	+	+	n.d.	n.d.	n.d.	n.d.
L-phenylalanine	+	+	n.d.	n.d.	n.d.	+
n-Propanol	(+)	(+)	n.d.	n.d.	n.d.	n.d.
Propionate	(+)	(+)	n.d.	n.d.	n.d.	n.d.
L-rhamnose	-	-	n.d.	n.d.	-	-
Salicylate	-	-	n.d.	n.d.	n.d.	n.d.
Serine	-	-	n.d.	n.d.	+	+
D-sorbitol	-	-	n.d.	n.d.	+	+
Starch	-	-	n.d.	n.d.	n.d.	n.d.
Succinate	+	+	n.d.	n.d.	-	-
Sucrose	-	-	n.d.	n.d.	-	-
Trehalose	-	-	n.d.	n.d.	+	+
L-tryptophane	+/-	+	n.d.	n.d.	+	n.d.
Tween 80	+	+	n.d.	+	n.d.	n.d.
Valerate	(+)	(+)	n.d.	n.d.	n.d.	n.d.
Xylan	-	(+)/v	n.d.	n.d.	n.d.	n.d.
D-xylose	-	-	n.d.	n.d.	-	-
Yeast extract	+	+	n.d.	n.d.	n.d.	n.d.
<b>Electron acceptor utilisation</b>						
Fe-citrate	-	-	-	n.d.	n.d.	n.d.
Ferrihydrite	-	-	-	n.d.	n.d.	n.d.
Manganese(IV)	-	-	-	n.d.	n.d.	n.d.
Nitrate	-	-	+	+	+	+
Thiosulphate	-	+/-	-	n.d.	n.d.	n.d.
<b>Fermentations</b>						
Casamino acids	-	+/-	n.d.	n.d.	n.d.	n.d.
D-glucose	+/-	+/-	n.d.	-	n.d.	n.d.
Hydrogen production	-	-	n.d.	n.d.	n.d.	n.d.

**Legend:** +, Growth was scored positive; (+), weak positive; -, negative; +/-, variable results (scored positive and negative in separate experiments); +/- different results obtained from different experiments; n.d., data not determined or not available in publications.

Appendix

**Table A4.5 (cont.): Phenotypic properties of *Pseudomonas* isolates and closely related affiliates.**

<b>Characteristic</b>	<b>F18II</b>	<b>F18V</b>	<b>NA101</b>	<b><i>P. pohangensis</i></b>	<b><i>P. antarctica</i></b>	<b><i>P. proteolytica</i></b>
<b>Hydrolytic Properties/ Exo-Enzymes</b>						
L-arginine dihydrolase	(+)	(+)	n.d.	+	-	n.d.
Caseinase	-	-	n.d.	-	n.d.	n.d.
DNase	-	-	n.d.	-	n.d.	n.d.
Esterase (C4)	-	-	n.d.	+	n.d.	n.d.
Gelatinase	(+)	(+)	n.d.	-	-	+
Lipase tween 20	+	+	n.d.	n.d.	n.d.	n.d.
Lipase tween 80	-	-	n.d.	n.d.	n.d.	n.d.
Lysine decarboxylase	-	-	n.d.	v	-	-
Ornithine decarboxylase	-	(+)	n.d.	n.d.	n.d.	n.d.
Phosphatase (acid)	-	(+)	n.d.	+	n.d.	n.d.
Phosphatase (alkaline)	-	+	n.d.	-	n.d.	n.d.
Starch hydrolysis	-	-	n.d.	-	-	-
Urease	-	-	n.d.	-	+	-
<b>Other Phenotypic Properties</b>						
Indol production	-	-	-	-	-	-
Flagella	Single polar	Single polar	1-2 peritrich	Not observed	Single polar	Single polar
Motility	Not observed	+	+	-	+	+
<b>Antibiotic MIC [µg/ml]</b>						
Chloramphenicol	32.0	128.0	n.d.	n.d.	Sensitive	Resistant
Erythromycin	64.0	128.0	n.d.	n.d.	Sensitive	Resistant
Gentamycin	4.0	8.0	n.d.	n.d.	Sensitive	Sensitive
Kanamycin	8.0	16.0	n.d.	n.d.	Sensitive	Sensitive
Penicillin G [IU]	12.5	400.0	n.d.	n.d.	Sensitive	Resistant
Streptomycin	32.0	4.0	n.d.	n.d.	n.d.	n.d.
Tetracycline	64.0	128.0	n.d.	n.d.	Sensitive	Sensitive
Trimethoprim	32.0	>256	n.d.	n.d.	Resistant	Resistant

**Legend:** +, Growth was scored positive; (+), weak positive; -, negative-; +/v, variable results (scored positive and negative in separate experiments); +/- different results obtained from different experiments; n.d., data not determined or not available in publications.

## Appendix

**Table A4.6: Phenotypic properties of *Photobacterium* sp. F18I and closely related affiliates.**

Strains: F18I, data from this study; NA42, data from this study and provided by Dr. Henrik Sass; SAMA2, data from Freese *et al.*, 2009; *P. profundum* DSM 21095<sup>T</sup>, data from Nogi *et al.*, 1998b; S10, S11, and 67TD, data from Süß *et al.*, 2008 and provided by Dr. Henrik Sass.

Characteristic	F18I	<i>P. profundum</i>	SAMA2	NA42	67TD	S10	S11
	Deep Sea Sediment (Atlantic Ocean) 4 mbsf	Deep Sea Sediment Ryukyu Trench 80 mbsf	Tidal Flat Sed. Wadden Sea Germany	Tidal Flat Sed. Wadden Sea Germany	Mediterranean Sea		
Isolated from	Cluster I (Sediment) 0.19 mbsf	Cluster I (Sapropel) 2.63 mbsf	Cluster II (Sediment) 2.35 mbsf				
<b>Growth Properties</b>							
Temperature Range	< 2°C - 33°C	4°C - 18°C	4°C - 30°C	4°C - 30°C	4°C - 33°C	4°C - 33°C	4°C - 33°C
Optimum		8°C - 12°C					
pH Range	5.9 - 9.4	n.d.	n.d.	n.d.	5.8 - 9.2	5.8 - 9.2	5.5 - 9.3
Optimum	8.4						
Salinity Range	3.5% - 7.5%	3%	n.d.	3.5% - 10%	1% - 7.5%	1% - 7.5%	1% - 7.5%
Optimum	5%			3.5% - 7%			
<b>Growth on Substrates</b>							
Acetate	+	n.d.	+	-	+	+	-
DL-alanine	+	n.d.	+	-	+	+	-
L-arabinose	-	-	+	-	-	-	-
L-arginine	+	n.d.	-	-	+	+	-
Benzoate	-	n.d.	-	-	-	-	-
Betaine	-	n.d.	+	-	-	-	-
<i>n</i> -Butanol	-	n.d.	+	-	-	-	-
Butyrate	-	n.d.	-	-	-	-	-
Caproate	-	n.d.	+	-	-	+	-
Caprylate	-	n.d.	n.d.	-	-	+	-
Casamino acids	+	n.d.	+	+	-	+	-
Cellobiose	-	-	+	-	-	+	-
Cellulose	-	n.d.	-	-	-	-	-
Chitin	-	n.d.	+	-	-	+	-
Citrate	+	n.d.	+	-	+	+	-
Crotonate	-	n.d.	(+)	((+))	-	-	-
L-cysteine	-	n.d.	+	-	-	-	-
Ethanol	-	n.d.	+	-	-	-	-
Formate	-	n.d.	-	-	-	-	-
D-fructose	+	-	+	+v	-	+	-
Fumarate	+	n.d.	+	((+))	+	+	-
D-galactose	+	+	n.d.	-	n.d.	n.d.	n.d.
D-gluconate	-	n.d.	(+)	-	-	-	-
D-glucosamine	+	n.d.	+	-	-	+	-
D-glucose	+	+	+	+v	+	+	-
L-glutamic acid	+	n.d.	n.d.	-	n.d.	n.d.	n.d.
L-glutamine	+	n.d.	+	+	-	+	-
Glycerol	+	+	+	+	-	+	-
Glycol	-	n.d.	+	-	-	-	-

**Legend:** +, Growth was scored positive; (+), weak positive; -, negative; +/v, variable results (scored positive and negative in separate experiments); +/- different results obtained from different experiments; n.d., data not determined or not available in publications.

Appendix

Table A4.6 (cont.): Phenotypic properties of *Photobacterium* sp. F18I and closely related affiliates.

Characteristic	F18I	<i>P. profundum</i>	SAMA2	NA42	67TD	S10	S11
<b>Growth on Substrates (continued)</b>							
Glycolate	-	n.d.	-	-	-	-	-
L-isoleucine	-	n.d.	+	-	-	-	-
α-Ketoglutarate	+	n.d.	+	-	-	+	+
DL-lactate	+	n.d.	+	-	+	+	-
Lactose	+/v	-	n.d.	+/v	n.d.	n.d.	n.d.
Laminarin	-	n.d.	+	-	-	-	-
DL-malate	+	n.d.	+	((+))	+	+	-
Malonate	-	n.d.	(+)	-	-	-	+
Maltose	+	+	+	+/v	+	+	-
Mannitol	+	+	(+)	+/v	-	+	-
D-mannose	+	+	-	-	+	+	-
Methanol	-	n.d.	+	-	-	-	+
Methylamine	-	n.d.	n.d.	-	n.d.	n.d.	n.d.
Peptone	+	n.d.	+	+	-	+	-
L-Phenylalanine	-	n.d.	+	-	-	-	-
n-Propanol	-	n.d.	+	-	-	-	-
Propionate	-	n.d.	-	-	-	+	-
L-rhamnose	-	-	-	-	-	-	-
Salicylate	-	n.d.	-	-	-	-	-
L-serine	-	n.d.	n.d.	-	n.d.	n.d.	n.d.
D-sorbitol	-	-	n.d.	(+)	n.d.	n.d.	n.d.
Starch	+	n.d.	+	-	+	+	-
Succinate	+	n.d.	+	((+))	-	+	+
Sucrose	+	-	+	-	-	+	-
Trehalose	n.d.	+	+	n.d.	-	+	-
L-tryptophane	-	n.d.	+	-	n.d.	n.d.	n.d.
Tween 80	(+)	+	n.d.	+	-	+	-
Valerate	-	n.d.	+	-	+	+	-
Xylan	-	n.d.	+	(+)	-	-	-
D-Xylose	-	n.d.	n.d.	+/v	-	-	-
Yeast Extract	+	n.d.	+	+	+	+	-
<b>Electron acceptor utilisation</b>							
Fe-citrate	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Ferrihydrite	-	n.d.	-	n.d.	-	-	-
Manganese(IV)	-	n.d.	-	n.d.	-	-	-
Nitrate	-	+	(+)	-	+	+	+
Thiosulphate	-	n.d.	+/v	+/v	-	-	-
<b>Fermentations</b>							
Casamino acids	-	n.d.	n.d.	-	(+)	+	+
D-glucose	+	n.d.	+	+	+	+	+
Hydrogen production	-	n.d.	-	-	-	-	-

**Legend:** +, Growth was scored positive; (+), weak positive; -, negative; +/v, variable results (scored positive and negative in separate experiments); +/- different results obtained from different experiments; n.d., data not determined or not available in publications.

## Appendix

**Table A4.6 (cont.): Phenotypic properties of *Photobacterium* sp. F181 and closely related affiliates.**

Characteristic	F181	<i>P. profundum</i>	SAMA2	NA42	67TD	S10	S11
<b>Other Phenotypic Properties</b>							
Indol production	+	+	n.d.	n.d.	n.d.	n.d.	n.d.
Flagella	not observed	single polar	n.d.	n.d.	n.d.	n.d.	n.d.
Motility	not observed	+	not observed	not observed	+	not observed	not observed
PHB presence	+	not observed	not observed	not observed	+	+	+
<b>Antibiotic MIC [<math>\mu\text{g/ml}</math>]</b>							
Chloramphenicol	n.d.	n.d.	1	n.d.	n.d.	2	2
Erythromycin	n.d.	n.d.	2	n.d.	n.d.	64	0.125
Gentamycin	n.d.	n.d.	8	n.d.	n.d.	256	64
Kanamycin	n.d.	n.d.	16	n.d.	n.d.	>256	128
Penicillin G [IU]	n.d.	n.d.	50	n.d.	n.d.	1.56	< 0.02
Streptomycin	n.d.	n.d.	32	n.d.	n.d.	128	128
Tetracycline	n.d.	n.d.	32	n.d.	n.d.	64	8
Trimethoprim	n.d.	n.d.	>256	n.d.	n.d.	>256	32

**Legend:** +, Growth was scored positive; (+), weak positive; -, negative; +/-, variable results (scored positive and negative in separate experiments); +/- different results obtained from different experiments; n.d., data not determined or not available in publications.

## Appendix

**Table A4.7: Phenotypic properties of *Desulfovibrio* sp. F161 and closely related affiliates.**

Strains: F161 data from this study; *D. oceani* subsp. *galatea* DSM 21391<sup>T</sup>, data from Finster and Kjeldsen 2010; *D. acrylicus* DSM 10141<sup>T</sup>, data from van der Maarel *et al.*, 1997; *D. profundus* DSM 11384<sup>T</sup>, data from Bale *et al.*, 1997; *D. marinisediminis* DSM 17456<sup>T</sup>, data from Takii *et al.*, 2008; *D. desulfuricans* subsp. *desulfuricans* DSM 642<sup>T</sup>, data from Bale *et al.*, 1997. Additional data from 1, Freese *et al.* (2008b); 2, Postgate and Campbell (1966); 3, Fröhlich *et al.* (1999); 4, Bak and Pfennig (1987); 5, Tsu *et al.* (1998)

Characteristic	F161	<i>D. oceani</i> subsp. <i>galatea</i>	<i>D. acrylicus</i>	<i>D. profundus</i>	<i>D. marinisediminis</i>	<i>D. desulfuricans</i> subsp. <i>desulfuricans</i>
<b>Isolated from</b>	Deep Sea Sediment (Atlantic Ocean) 4 mbsf	Suboxic Water Pacific Peru 500 m	Tidal Flat Sediment Wadden Sea Netherlands 10 cmbsf	Deep Sea Sediment Japan Sea 500 mbsf	Coastal Marine Sediment Japan	tar and sand mix around corroded gas main
<b>Growth Properties</b>						
Temperature Range	4°C - 30°C	4°C - 30°C	30°C - 37°C	15°C - 65°C	> 10°C - < 42°C	28°C - 44°C <sup>2</sup>
Optimum		20°C	(10°C - 37°C) <sup>1</sup>	25°C	37°C	
pH Range		6 - 8.5		4.5 - 9	6.3 - 8.6	4.5 - 8.7 <sup>2</sup>
Optimum	n.d.	7 - 8	7.4	7	7.5	
Salinity Range		0.1% - 4%	0.6% - 4%	0.6% - 10%	0.05% - 3.5%	0% - 2.4% <sup>3</sup>
Optimum	n.d.	2.5% - 3.5%	1.8%	0.6% - 8%	2.0% - 2.5%	
<b>Electron acceptor utilisation</b>						
Acrylate	n.d.	-	+	n.d.	-	n.d.
DMSO	(+)	-	n.d.	-	n.d.	-
Ferrihydrite	-	n.d.	n.d.	n.d.	n.d.	n.d.
Fumarate	n.d.	n.d.	n.d.	(+)	-	(+)
Nitrate	(+)	-	-	+	-	+
Thiosulphate	+	+	+	+	+	+
Thiosulphate disp.	-	-	n.d.	n.d.	-	- <sup>4</sup>
Sulphite	+	+	-	+	+	+
Sulphate	+	+	+	+	+	+
Sulphur	-	-	-	-	-	-
<b>Fermentations</b>						
Casamino acids	n.d.	-	n.d.	n.d.	+	n.d.
Fumarate	n.d.	+	n.d.	n.d.	+	n.d.
Lactate	n.d.	+	n.d.	+	n.d.	-
Peptone	n.d.	-	n.d.	n.d.	+	n.d.
Pyruvate	+	+	n.d.	+	+	+ <sup>2</sup>
Yeast extract	n.d.	-	n.d.	n.d.	+	n.d.
Hydrogen production	(+)	n.d.	-	-	n.d.	-
<b>Other Phenotypic Properties</b>						
Flagella	Single, polar	n.d.	multiple polar	Polar <sup>5</sup>	Single, polar	Single, polar <sup>5</sup>
Motility	Not observed	+	+	+	+	n.d.
Desulfoviridin	n.d.	n.d.	+	+	+	+ <sup>2</sup>

**Legend:** +, Growth was scored positive; (+), weak positive; -, negative; +/-, variable results (scored positive and negative in separate experiments); +/- different results obtained from different experiments; n.d., data not determined or not available in publications.



Appendix

Table A4.7 (cont.): Phenotypic properties of *Desulfovibrio* sp. F16I and closely related affiliates.

Characteristic	F16I	<i>D. oceani</i> subsp. <i>galatea</i>	<i>D. acrylicus</i>	<i>D. profundus</i>	<i>D. marinesedimentis</i>	<i>D. desulfuricans</i> subsp. <i>desulfuricans</i>
<b>Growth on Substrates</b>						
Acetate	-	-	-	+	n.d.	-
L-alanine	-	-	+	-	+	-
Aspartate	n.d.	-	-	n.d.	+	n.d.
Casamino acids	(+)	+	n.d.	n.d.	+	n.d.
Choline	+	n.d.	-	-	n.d.	+
L-cysteine	-	n.d.	+	-	n.d.	-
Ethanol	(+)	-	+	-	+	+
Formate	(+)	+	+	-	+	+
D-fructose	n.d.	-	-	-	n.d.	-
Fumarate	+	+	+	-	+	+
D-glucose	-	-	-	n.d.	n.d.	n.d.
Glycerol	+	+	+	n.d.	+	+ <sup>3</sup>
Glycine	-	+	+	-	+	-
Hydrogen (autotrophic)	n.d.	n.d.	n.d.	(+)	n.d.	-
Hydrogen (heterotrophic)	(+)	+	+	+	+	+
DL-lactate	+	+	+	+	+	+
DL-malate	-	+	+	-	+	+
Methanol	-	-	-	n.d.	n.d.	n.d.
<i>n</i> -Propanol	-	-	+	n.d.	+	+ <sup>3</sup>
Propionate	-	n.d.	-	-	n.d.	-
Pyruvate	+	+	+	+	+	+
L-serine	+	-	+	-	+	-
Succinate	+	-	+	n.d.	+	- <sup>3</sup>
Yeast extract	-	+	n.d.	n.d.	+	n.d.

**Legend:** +, Growth was scored positive; (+), weak positive; -, negative; +/-, variable results (scored positive and negative in separate experiments); +/- different results obtained from different experiments; n.d., data not determined or not available in publications.

**Table A4.8: Phenotypic properties of *Arcobacter* sp. F171X and other species of the genus.** Strains: F171X, this study; NA105, data from Henrik Sass and this study; *A. nitrofigilis* DSM 7299<sup>T</sup>, data from McClung *et al.*, 1983; *A. mytili* DSM 23625<sup>T</sup>, data from Collado *et al.*, 2009; *A. halophilus* DSM 18005<sup>T</sup>, data from Donachie *et al.*, 2005; *A. cryaerophilus* DSM 7289<sup>T</sup>, data from Neill *et al.*, 1985. Additional data: 1, Vandamme *et al.* (1991)

Characteristic	Strain F18IV	Strain NA105	Strain NC101	<i>A. nitrofigilis</i>	<i>A. mytili</i>	<i>A. halophilus</i>	<i>A. cryaerophilus</i>
<b>Isolated from</b>	Deep Sea Sediment (Atlantic Ocean) -107.9 mamb	Tidal Flat Surface Sediment (Wadden Sea)	Tidal Flat Sediment (Wadden Sea)	Salt Marsh Sediment (Nova Scotia, Canada)	Mussel (brackish water, Spain)	Hypersaline Lagoon (Hawaii)	Aborted Bovine Fetus
<b>Growth Properties</b>							
Temperature Range	4°C – 30°C	4°C – 40°C	n.d.	< 6°C – < 37°C	< 18°C – 42°C	< 18°C – < 42°C	5°C – 37°C
Optimum							30°C
Salinity Range	n.d.	n.d.	n.d.	0.5% – 7%	< 2% – >4%	0.5% – 13.5%	< 2%
Optimum						2% – 4%	
<b>Aerobic Growth on Substrates</b>							
Acetate	-	+	+	n.d.	n.d.	n.d.	n.d.
D-glucose	-	+	-	n.d.	n.d.	n.d.	n.d.
DL-lactate	-	+	+	+	n.d.	n.d.	n.d.
Misc. Information	+ YPGL Medium			+ for organic/amino acids			
<b>Electron acceptor utilisation</b>							
DMSO	+	+	+	n.d.	n.d.	n.d.	n.d.
Nitrate	n.d.	+	n.d.	+	-	+	+
TMAO	+	+	+	n.d.	n.d.	n.d.	n.d.
<b>Other Phenotypic Properties</b>							
Flagella	Not observed	1-2, polar	Single, polar <sup>1</sup>	Single, polar <sup>1</sup>	Single, polar <sup>1</sup>	Single, polar <sup>1</sup>	Single, polar <sup>1</sup>
Gram Stain	-	-	-	-	-	-	-
H <sub>2</sub> S production	n.d.	n.d.	n.d.	+ from L-cysteine	-	-	-
Motility	Not observed	+	n.d.	+	+	+	+
Sporulation	-	-	-	-	-	-	-

**Legend:** +, Growth was scored positive; (+), weak positive; -, negative; +/-, variable results (scored positive and negative in separate experiments); +/- different results obtained from different experiments; n.d., data not determined or not available in publications.

## Appendix

### A5. 16S rDNA sequences of archaeal DGGE bands from Tamar sediment

#### A5.1 Band A1-1

anggggagcagcaggcGAGAAAACCTTTGCAATGTGCGAAAGCACGACAAGGTTAATCCGAGTGA  
TTTGTGCTAAACGAATCTTTGTTAGTCCTAGAAACACTAACGAATAAGGGGTGGGCAAG  
TTCTGGTGTGTCAGccgcccggta

#### A5.2 Band A1-2

acggggagcagcaggCGAGAAAACCTTTGCAATGTGCGAAAGCACGACAAGGTTAATCCGAGTGA  
TTTGTGCTAAACGAATCTTTGTTAGTCCTAGAAACACTAACGAATAAGGGGTGGGCAAG  
TTCTGGTGTGTCAGCCGCCCGGTa

#### A5.3 Band A1-3

ACGGGGCGCAGCAGGCGAGAAAACCTTTGCAATGTGCGAAAGCACGACAAGGTTAATCCG  
AGTGATTTGTGCTAAACGAATCTTTGTTAGTCCTAGAAACACTAACGAATAAGGGGTGG  
GCAAGTTCTGGTGTGTCagcccggta

#### A5.4 Band A1-4

TCGGGGCGCaGCCGGCCgCGAAAACCTCCCCATGTCCGAAAGCgCGATGAGGGAATCCCa  
AGTGCCCGCTCGTTAGAGTCAGCTGTTCTTTGTCTAAAAAGCCGAGAGAGTAAGGGCTG  
GGCAAGACGGGTGCCagcccggta

#### A5.5 Band A3-1

TCGGGGCGCCGCCGGCgCGAAACCTCCCCAtTGTgCGAAAGCACGATGAGGGGATCCCaA  
GtgCtCGCtCGTAagagTCAGCTgTTCTTTGTCTAAAAAaCataGAGAgTAAGgGctGggtAAGAC  
GGGTgcCAgCCGCCgCGTAATT

#### A5.6 Band A3-2

ACGGGGCGCAGCAGGCGCGAAAACCTTTACAATGCGGGAAACCGCGATAAGGGGATACC  
GAGTGCCAGCATATTATGTTGGCTGTCCACATGTGTAACGGCATGTGTTAGCAAGGGCC  
GGGCAAGACCGGTgccagcccggta

#### A5.7 Band A3-3

ACGGGGCGCAGCAGGCGCGAAAACCTTTACAATGCGGGAAACCGCGATAAGGGGATACC  
GAGTGCCAGCATATTATGTTGGCTGTCCACATGTGTAACGGCATGTGTTAGCAAGGGCC  
GGGCAAGACCGGTgccagcccggta

## Appendix

### A5.8 Band A3-4

TCGGGGCGCCGCAGGCGCGAAAACCTTTACAATGCGGGAAACCGCGATAAGGGGATCCCC  
AGTGCCCGCCTATTATGTTGGCTGTCCACATGTGTAAACGGCATGTGTTAGCAAGgggccggg  
caagaccggtgccagccgccgtaat

### A5.9 Band A5-1

TCGGGGCGCCGCAGGCGCGAAACCTTCACCATGTGCGAAAGCACGATGAGGGGATCCCA  
AGTGCTTGCTCGTAAGAGTAAGCTGTTTCTATGTCTAAAAAGCATAGAGAGTAAGAGCTG  
GGTAAGACGGGtgccagccgccgtaatt

### A5.10 Band A5-2

TCGGGGCGCCGCCGCCCCGAAACCTTCCCCATGTCCGAAAGCCCGATGAGGGGATCCCC  
AGTGCTCGCTCCTAAGAGTAAGCTGTTTCTTTGTCTAAAAAGCATAGAGAGTAAGGGCTG  
GGTAAGACGGGtgccagccgccgtaatt

### A5.11 Band A5-3

TCCGGGGGCGCCGCCGCCCCGAAACCTCCCCAATTCCGGAAGCCCCGTCGGGGGATCCCC  
AAGTCCCACCTCGTTAGGGTTAGCTGTTTTCATGTCTAAAAACATAGAGAGTAAGGGCTG  
GGTAAGACGGGtgccagccgccgtaata

### A5.12 Band A5-4

ACGGGGCCCAgCCGGCCCCGAAACCTCCCCAATGCCGGAAGCCCCGAGGGGGGATCCCC  
AAGTgCCaGCCCGTAAGAGTTGGCTGTTTCCTTGTCTAAAAAACATAGAGAGTAAGGGCT  
GGGTAAGACGGGtgccagccgccgtaat

### A5.13 Band A5-5

ACGGGGCGCAGCAGGCGCGAAACCTCCTCAATGCaCTAACGTGCGAAGGGGTTACCTCG  
AGTGCCACCTGAAGAAGGTGGCTTTTCCTTGGTCTAAAAAGCCTAGGGAATAAGAGGggg  
ggCAAGACTGgtgtCagccGcCGCGGtaat

### A5.14 Band A5-6

gaaggggctaCCtcGaGtgCCaCCTgatgaagGtggcTtttcCTaGGTctAaaaagCcggtggaatAAGagggGGGCA  
AgactggtgtCAGCcGCCgcGGtaat

### A5.15 Band A5-7

TAGGGGGgCCaCCgGgcgggAAACtTTcccatgtcgggaAAcCggcgggtaagGGGGatccgcAggatgtcgC  
cgCctaTTaagatgtGggcgtcgTtttcCatGtgtataaaaacCcttgtggaCaAAGGgCCGGGCAAGACCGgtgcCAG  
CgcCGCGGtaat

## Appendix

### A5.16 Band A5-8

GGGGCGCCGcNGGcNCGAAAACCTTTaCaATTgCCGGAAACCGCGATAAGGGGATTCCgAGT  
GCCCCCTATTATGTTGGCTGTCCACATGTGTTAACGGCATGTGTTAGCAAGGGCCGGGC  
AAGaccggtgccagccgccgtaatt

### A5.17 Band A5-9

ACGGGGCGCAGCAGGCGCGAAAACCTTTACAATGCGGGAAACCGCGATAAGGGGATACC  
GAGTGCCAGCATAATTATGTTGGCTGTCCACATGTGTAAACGGCATGTGTTAGCAAGGGCC  
GGCAAGACCGGTGCCAGCCGCCgcgtaatt

### A5.18 Band A9-1

ACGGGGCGCAGCAGGCGCGAAACCTTCACAATGTGCGAAAGCACGATGAGGGGATCCCA  
AGTGCTTGCTCGTAAGAGTAAGCTGTTTCTTTGTCTAAAAAGCAGAGAGAGTAAGAGCTG  
GGTAAGACGGGTgccgcccgcgtaatt

### A5.19 Band A9-2

ACGGGGCGCAGCAGGCGCGAAACCTTCACAATGTGCGAAAGCACGATGAGGGGATCCCA  
AGTGCTTGCTCGTAAGAGTAAGCTGTTTCTATGTCTAAAAAGCATAGAGAGTAAGGGCTG  
GGTAAGACGGGTGccagcccgcgtaatt

### A5.20 Band A9-3

ACGGGGCGCAGCAGGCGCGAAAACCTTCACAATGTGCGAAAGCGCGATGAGGGGATCCC  
AAGTGCTTGCTCGTAAGAGTaAGCTGTTTCTTTGTCTAAAACGCAGAGAGAGTAAGGgctggg  
taagacgggtgccagcgg

### A5.21 Band A9-4

ACGGGGCgCAgCAGGCGCGAAAACCTCCtCAATGCaCTAACGtGCGAAGGGGTTACCtCGAGT  
GCCACtGAAGAAGGTGGCTTTTCTTGGTCTAAAAAGCCTAGGGAATAAGAGgGGGCA  
AGactggtgtcag

### A5.22 Band A9-5

aNGGGGcGAGCAGGCGCGaAANCTTcCAATGCggaGAAAGCGTgtAcGGGGgCaCCCCGAGT  
cCcTCCGATGAgGNTgGCTTTTcCtTgGTATAAcTANNcAGGGGAATAAGGGgNggCAAGCCT  
GGTgTCAgcCGN

### A5.23 Band A15-1

ACGGGGCGCAGCAGGCGCGAAAACCTTCACACTGTGCGAAAGCGCGATGAGGGGATCCCA  
AGTGCTTGCTCGTAAGAGTAAGCTGTTTCTTTGTCTAAAAAACAGAGAGAGTAAGGGCTG  
GGTAAGACGGGTgccagcccgcgtaattt

## Appendix

### A5.24 Band A15-2

ACGGGGCGCAGCAGGCGCGAAAACCTTCACACTGTGCGAAAGCACGATGAGGGAATCCCA  
AGTGCTTGCTCGTAAGAGTAAGCTGTTTCTTTGTCTAAAAACAGAGAGAGTAAGAGCTG  
GGTAAGACGGGtgccagccgccggaatt

### A5.25 Band A15-3

ACGGGGCGCAGCAGGCGCGAAAACCTTCACACTGTGCGAAAGCGCGATGAGGGAATCCCA  
AGTGCTTGCTCGTAAGAGTAAGCTGTTTCTTTGTCTAAAAAGCAGAGAGAGTAAGGGCTG  
GGTAAGACGGGtgccagccgccgta

### A5.26 Band A15-4

TCGGGGCGCCGCGAGGCGCGAAACCTTCACCCTGTGCGAGAGCACGGTGAGGGGATCCCA  
AGTGCTGGCTCGTAAGAGTCAGCTGTTTCTTTGTCTAAAAACAGAGAGAGTAAGGGCTG  
GGTaagacgggtgccagccgccgta

### A5.27. Band A19-1

ACGGGGCGCAGCAGGCGCGAAAACCTTCACACTGTGCGAAAGCGCGATGAGGGAATCCCA  
AGTGCTTGCTCGTAAGAGTAAGCTGTTTCTTTGTCTAAAAAGCAGAGAGAGTAAGAGCTG  
GGTAAGACGGGtgccagccgccggaatt

### A5.28. Band A19-2

ACGGGGCGCAGCAGGCGCGAAAACCTTCACACTGTGCGAAAGCGCGATGAGGGAATCCCA  
AGTGCTTGCTCGTAAGAGTAAGCTGTTTCTTTGTCTAAACGCAGAGAGAGTAAGGGCTG  
GGTAAGACGGGtgccagccgccggaatt

### A5.29. Band A19-3

TCGGGGCGCCGCGCCCGGAAACCTCCCCCTGTCCGAAAGCACGGTGAGGGGATCCCC  
AGTGCTCGCTCGTAAGAGTAAGCTGTTTCTCTGTCTAAACGCAGAGAGAGTAAGGGCTG  
GGTAAGACGGGTGCCAGCcgCGCGtaatt

### A5.30. Band A19-4

ACGGGGCGCAGCAGGCGCGAAACCTCCGCAATGCTCGAAAGAGTGACGGGGGGATTCCA  
AGTGCCCTTCgCTAgGCGTaGGCTGTTCaGAtGCCTAAAAAGCaTctGGAGGAAGGACtGGGtA  
Agacgggtgccagccgccg

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### A6. 16S rDNA sequences of bacterial DGGE bands from Tamar sediment

#### A6.1 Band B1-1

TACGGGAGGCAGCAGTGGGGAATATTGGaCAATGGGCGCAAGCcTgacCaGCAAtgCCGcGT  
GNGTGAAGAAGgCcTGCGGGTTgTAAAgCACTTTCAGTAGNGAAGAAAAGCNTACGGTTA  
ATAGCcGTGAGTNTTGACGNTACCTaCAGAAGAAGCACCGGCTAAcTCCGTgccagccagccG  
GGtaa

#### A6.2 Band B1-2

atgNTaANtccaGNTigtgtaagAagAaGAAACGGTTAATNnCGNNGTTTGACGGTACCtcaAGAAA  
AGCACCGGCTAAcTCCGTGCCAGCAGCCGCGGTAA

#### A6.3 Band B1-3

GTAANcctgcTTTNNNNCAGGAAGAATCattNacacgctGTGGGNNTTGACGGTACCnTcaaGAA  
NAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAA

#### A6.4 Band B1-7

GGCACCAGTGGGGAATATTGGCCAATGGGGGAAACCCTGATCCAGCACTGCCGCGTGTG  
TGAAGAAGGCCCTTGGGGTTGTAAAGCACTTTCAGTGGGGAGGAAAAGCTTACGGTTAAT  
AGCCTTGAGTCTTGACGGTACCTACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCC  
G

#### A6.5 Band B1-8

CGGGGGCGTACGGGAGGCAGCAGTGAGGAATATTGGGCAATGGAGGCAAATCTGATCC  
AGCCATGCCCGGTGCAAGAAGACTGCCCTATGGGTTGTAACTGGTTTTATACAGGAAG  
AAACTTAATCGTGTAGGGTGTGACGGTACCTAAGAATAAGCACCGGCTAACTCCGT  
GCCAGCAGCCG

#### A6.6 Band B3-1

CttTTAATAGNGaAGNNtANTGACGGTACCtcaaGAANAAGCAcCGGCTAACTCCGTGCCAGC  
AGCCgCGG

#### A6.7 Band B9-0

GGCAGCAGCAAGGAATATTGGGCAATGGGCGAAAGCCTGACGCAGTGACGCTGCGTGGG  
GGATGAAGGCTTFCGGTTGTAAACCTCTTTTATCAGGGAAGAATAATGACGGTACTTGA  
GGAATAAGTCTCGGCTAACTACGTGCCAGCAGCCG

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### A6.8 Band B9-1

GGAGGCNCGCANCAAGGAATNNNNNCAATGNNNGAAAGCTTGACCCANNNACNCCNCGT  
GAGgGANgAAGgCCTTcGggttNtAAACCTcttTTNttgaGGGAAGAattaatGACGGTaccCaAGGAAT  
AAGCcTCGGCtAaNTACGTGCCAGCAGCCGcgta

### A6.9 Band B9-2

GGCAGCAGTAGGGGATTTTGCCCAATCGGCGAAAGCCAGAAGCAGCCACGCCGCGTGGG  
CGATGAAGGCCTTCGGGTTGTAAACCCCTTTTCTGGGGGAAGAGAAATGACGGTACCCC  
AGGAATAAGCCCCGGCTAACTACGTGCCAGCAGCCGCG

### A6.10 Band B9.-2.5

TCGGGAGGCAGCCGTCGGGGATTTTGCGCAATCGGCGAAAGCCGGAAGCAGCGACGCCG  
CGTGGGCGAAGAAGGCCTTCGGGTTGTAAAACCCCTTTTTCGGGGGAAGAGGCAAGGACG  
GTACCCCAGGAATAAGCCCCGGCTAACTACGTGCC

### A6.10 Band B9-3

GGCGCCGGCAGGGATTTTTGGCCATTGGGGGAACCCGGAGCCACCACCCCGGTTGGG  
GGAGAAAGCCTTTGGGTTTATAAACCCCTTTTCTGGGGGAGGAGCTATGACGGTACCCCA  
GGAATAAGCCTCGGCTAACTACGTGCCA

### A6.10 Band B19-1

CGGGAGGCAGCAGTGGGGAAATNNTGNNCAATGGGGGAANCNNNNNNNNGNGNCGCNG  
NNNGNANNATGAAGGCCTNTNGGTTGTAAAATgCgCTgTaTaTngcggtgGAAGNAaCtatatAcac  
gtgctttctaaTAANNCNNTAANNTGACGGTACCCCAAGANtaAAGcttccGGCtaAaTactGTGCCAG  
CAGCCGCGGTAAT

### A6.10 Band B19-3

TCGGGAAGGCGCCACAGGGGATTTTGCCCATGGGAGGAAGCTTGGATGCAGCACCGCC  
GGTGGGGGGAGAACGCATTCGGTGTAAACCTCTTTTATATGGGAGGATAATGACGGT  
ACCCTATGAATAAGCACCGGCTAACTCCGTGCCAGCAGCCGC

### A6.10 Band B19-6

GGCAGCAGCAAGGAATCTTGGGCAATGAGCGAAAGCTTGACCCAGCGACACCGCGTGAG  
GGATGAAGGCCTTCGGGTTGTAAACCTCTTTTCTTGGGGGAAGAATTCTGACGGTACCCGA  
GGAATAAGCCTCGGCCAACTACGTGCCAGCA

### A6.10 Band B19-7

GGCAGCCGCAAGGAATTTTGGGCAATGGGCGAAAGCTTGACCCAGCGACGCCGCGTGGG  
GGAAGAAGGCCTTCGGGTTGTAAACCTCTTTTCTTGGGGGAAGAATAATGACGGTACCCGA  
GGAATAAGCCTCGGCTAA



## A7. The GASP phenotype, cryptic growth, and the role of endogenous metabolism

An intriguing pattern of culturable cell development during starvation was observed in this study but is indiscernible when data are averaged: a wave-like pattern within the late stability phase. Culturable cell counts in some strains or treatments alternately increased and decreased with time during the late stability phase (Fig. A7.1), hence the earlier addition of the phrase “with a certain degree of variation” when describing this phase. This pattern occurred most pronounced in MPNs of *A. malicum*, “A”, *Arcobacter* sp. F17IX “B”, *Arcobacter* sp. NA105, and *Photobacterium* sp. NA42 (Fig. A7.1).

There is evidence in the literature that suggests a growth advantage in stationary phase (GASP phenotype) brought about by advantageous mutations in subpopulations of starving cultures (Zambrano *et al.*, 1993; Fig. 1.4). Apparently, during the long-term stationary phase (Finkel, 2006) these mutations allow successive rounds of GASP phenotypes to scavenge nutrients released from dead cells more effectively than parental cells. Thus, supposedly population shifts occur in a cryptic dynamic period (Llorens *et al.*, 2010). This implies that viability and thus culturability of a certain subpopulation increases when other cells die. The released cellular components serve as substrate for the otherwise limited survivors. Therefore, bizarrely viability and possibly culturability should be elevated when there are more dead cells present in the culture.

During this project this was observed for some strains and treatments (Fig. A7.1). The development of culturability and the percentages of dead cells are almost parallel for *Photobacterium* sp. NA42 “B” and *Arcobacter* sp. F17IX “B” but also similar trends occur, but are less clear, in the other two strains presented. However, a significant change in AODC total counts only occurred for *Photobacterium* sp. NA42 “B” during starvation (Chapter IV, 2.2.1b). Please note the different scales on the secondary y-axis for percentages of dead cells. It appears that for *A. malicum* an increase of 94% of dead cells between 250 and 365 days of starvation resulted in only an increase of  $3.1 \times 10^4$  culturable cells  $\text{ml}^{-1}$  while an increase of 0.6% dead cells in *Arcobacter* sp. NA105 between 180 and 270 days caused an increase of  $9.9 \times 10^4$  culturable cells  $\text{ml}^{-1}$ . Thus variability is high and could be due to different metabolic

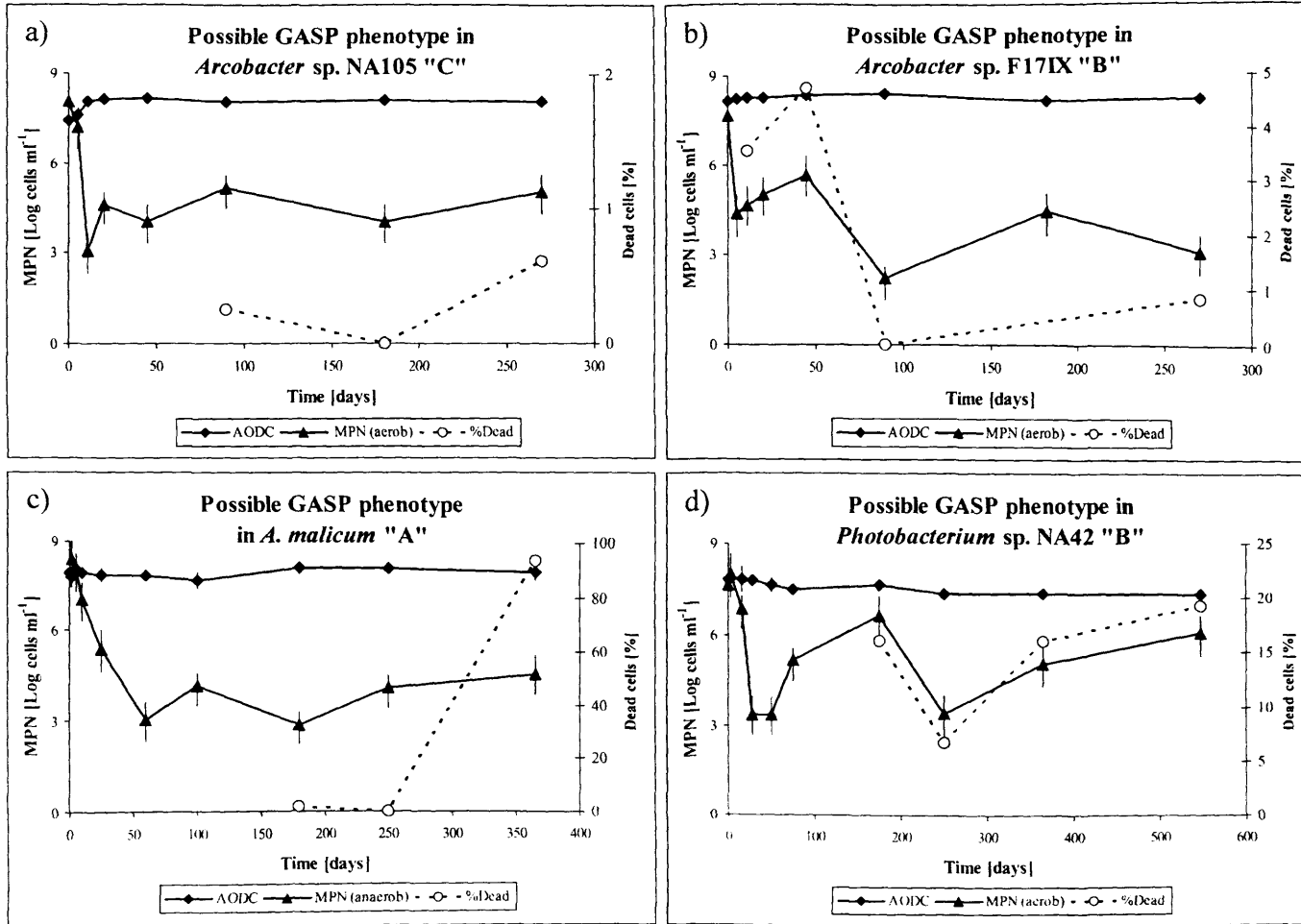
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efficiencies of the different strains under similar incubation conditions. Changes of percentages of dead cells and culturable cells are only significantly correlated for *Arcobacter* sp. F17IX “B” ( $p < 0.05$ ; Spearman’s correlation coefficient  $r = 0.99$ , critical value, 0.95; Fig. 6.2 b) but not in others (e.g. *Photobacterium* sp. NA42 “B”;  $r = 0.87$ ; Fig. 6.2 d). In the only culture where a significant correlation occurred but no significant change in total cell counts, an average change of 1% in dead cells, increasing or decreasing, caused a change in culturable cells by  $1.5 \times 10^5$  cells  $\text{ml}^{-1}$ . With an average total count of  $1.8 \times 10^8$  cells  $\text{ml}^{-1}$  in this culture 1% of dead cells equal  $1.8 \times 10^6$  cells  $\text{ml}^{-1}$ . This would mean that for ~12 cells that die, one culturable cell appears. For *Photobacterium* sp. NA42, the only one of the four cultures where a significant change in total cell numbers occurred but where the correlation between dead and culturable cell numbers was not statistically significant, the ratio of dead to new cells was 2.6:1.

This ratio incidentally is also calculated when using averaged data of all four strains (no significant correlation) and average cell concentrations of all starving strains (averaged AODC total count of all strains; Fig. 6.1) with a range of one new culturable cell from between 1539 and 0.9 dead cells. At least the minimum determined is not feasible for energetic reasons. It indicates, however, that the changes of culturability cannot be influenced by the presence of dead cells alone. However, Postgate and Hunter (1962) calculated that 50 dead cells in the culture supported the division of one cell while Druilhet and Sobek (1976) estimated that one new cell is formed at the expense of 6.7 cells (previously heat-killed and added to the medium instead of glucose). These values are not too dissimilar from the 12:1 ratio in *Arcobacter* sp. F17IX and the average of 2.6:1 ratio calculated here, given the wide range determined.

The concept of cryptic growth (Ryan, 1959) or cannibalism (Harrison, 1960) is not very well studied. However, a number of authors concluded that it did not occur during their starvation experiments (e.g. Thomas and Batt, 1968; Boylen and Ensign, 1970; Kurath and Morita, 1983). Kaprelyants and Kell (1993) expressed some reservations about this phenomenon, which is also the case for some of the results presented here: “given the constancy of the total counts, it is implausible that lysis could be so exactly balanced by growth” (Fig. 6.1).

Fig. A7.1: Possible occurrence of GASP phenotype in some of the starving cultures. Note the differing scales on the secondary y-axis for percentage of dead cells



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Once more there is an energetic problem. It has not been shown yet how much energy can possibly be obtained from cell remains, especially under anaerobic conditions. Aerobic respiration would allow maximum energy gain from the substrate. In the absence of oxygen, however, fermentation of endogenous substrates is most likely the only way to gain energy under pure culture conditions. No alternative electron acceptors would be available as it most likely would have been depleted during growth (note this situation also occurs in the deep biosphere, general absence of oxygen and, for example, in sulphate-free sediment layers). Thus only little energy can potentially be obtained from macromolecules of lysed cells under anaerobic culture conditions to support growth and subsequently division of the advantaged phenotype and would therefore require the turnover of a lot of cell debris. Even if this occurred, it could not be continued indefinitely. As with all biochemical reactions a certain amount of energy is “lost” from the system in the form of thermal energy (heat). Hence there must be a finite amount of GASP cycles within a culture and additionally a decrease in total cell counts must accompany each cycle. This only occurred in one of the strains presented above (*Photobacterium* sp. NA42; Fig. A7.1d)

Furthermore, for several GASP rounds to occur a new and advantageous mutation needs to arise each time. Ryan (1955) reported of beneficial mutations at a rate of  $10^{-9}$  mutations per bacterium per hour during stationary phase (from histidine requiring mutants to prototrophic phenotype), which “occur as a constant function of physical time”. Mutations could possibly be caused either during DNA repair (Tang *et al.*, 2000) or cryptic growth during starvation (Bridges, 1996). However, Ryan (1955) already stated that one in fifty cells would need to lyse (same number as Postgate and Hunter, 1962) and be replaced per hour to enable this mutation rate and that with very efficient utilisation of released substrate. A similar rate of beneficial mutations ( $4 \times 10^{-9}$  per cell and generation) was determined by Imhof and Schlötterer (2001) for growing *E. coli* cells. Thus the estimated mutation rate in stationary phase cells observed by Ryan (1955) might be too high, although he reported it to be on fortieth of the rate occurring during growth. These adaptive mutations to environmental challenges might be stress-specific (Hall, 1998) and have even been called starvation-associated mutations (Bridges, 1994).

During the relatively short incubation times (270 to 548 days) approximately  $10^2$  and  $10^3$  beneficial mutations could have occurred during starvation (Fig. A7.1)

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using the mutation rate of Ryan (1955). Even if this rate was 100 times lower, theoretically at least one beneficial mutation that might have conferred the GASP phenotype could have arisen. However, for reasons given above (Chapter VI 3.3), it seems unlikely that propidium iodide reliably accounts for the number of dead cells. Thus the correlation between changes in culturability and “dead” cells in the cultures might be spurious given that significant changes in AODC total counts and dead cells only occurred concomitantly in one strain (*Photobacterium* sp. NA42; Fig. A7.1d) during the possibly observed GASP cycle.

Mutation rates and thus adaptive mutation to starvation might, however, play a role in marine sediments. Although division times are low in this environment (from 0.2 to 22 years, Schippers *et al.* 2005 up to 1,000 years, Whitman *et al.*, 1998) and adaptive mutations have been shown to decrease in frequency with time (after 2,000 generations; Lenski and Travisano, 1994) the timescales that they could occur in are geological (millions of years), thus cells might slowly adapt to substrate limitation. During this project, however, significant differences between near-surface and deep subsurface strains were not revealed, but then all strains were “only” starved for up to three years, not millions.

To conclude this section, there is some indication for the GASP phenotype activity in some of the anaerobically starved cultured studied (Fig. A7.1) but the underlying principles and energy balances are far from understood in pure cultures let alone the transferability of this phenomenon to the natural environment.

### **A8. Effect of environmental parameters on starvation-survival**

#### A8.1 Effect of oxygen on starving *Photobacterium* strains

During starvation-survival experiments with *Photobacteria*, oxygen was detected in some cultures. There are two explanations for this. Firstly, *Photobacterium* sp. SAMA2 was the first strain used for starvation experiments. Rubber stoppers used for this strain were much thinner than those used in later experiments. Maybe due to long-term use combined with repeated piercing during sampling these rubber stoppers allowed oxygen to penetrate. This is supported by the fact that the peak for

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oxygen in the headspace was of similar size compared to the peak of oxygen in air. Secondly, oxygen was only present in the headspace for treatments “A” and “B” in the other strains (NA42, S10, S11, and 67TD) not in “C”. This leads to the conclusion that the headspace gas was not sufficiently flushed when setting up the experiments. Treatment “A” contained no substrate, “B” contained only small amounts (15  $\mu\text{mol}$ ) and either only small amounts of VFAs ( $\mu\text{mol}$  range), probably not sufficient to aide complete removal of oxygen from the headspace. In treatment “C”, however, initial VFA concentrations were much higher (mM range) and with oxygen being present strains utilised these, which they could not have under anaerobic condition, (Fig. 4.6, and 4.9 to 4.11) thereby removing oxygen from the headspace and gaining energy.

No oxygen was detected in any other starving culture including *Photobacterium* sp. F18I, which was set up later than the other *Photobacterium* strains.

It has been reported that reactive oxygen species (ROS) such as  $\text{O}_2^-$  and  $\text{H}_2\text{O}_2$ , which are mainly produced during aerobic activity, can cause damage to cell constituents including nucleic acids, proteins, and lipids (Halliwell and Gutteridge, 1984; Davies *et al.*, 1987). Dukan and Nyström (1998) reported that with continued starvation intracellular levels of oxidised proteins increased. They showed that as a response to oxygen stress the enzyme superoxide dismutase was expressed, which catalyses the conversion of superoxide to oxygen and hydrogen peroxide, which is then converted by catalases or peroxidases to oxygen and/or water thus disarming potentially toxic ROS (Fridovich, 1978). Furthermore, cells produced heat shock proteins, which are known for their chaperone-like function (Hendrick and Hartl, 1993) to maintain a pool of functional proteins. When oxygen was removed from glucose-starved *E. coli* cultures (Dukan and Nyström, 1999) no significant decrease in cell numbers occurred while in aerobically incubated cultures ~98% of cells died within 10 days of starvation. The authors concluded that this was caused by the endogenously generated oxygen damage. Similar to these findings are results by Roslev and King (1994) who observed better survival in anaerobically starved methanotrophic bacteria compared to aerobic parallels (based on higher metabolic activity and protein production after 42 days of starvation). Furthermore, oxidative stress has been linked

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to the formation of VBNC cells (Cuny *et al.*, 2005; Noor *et al.*, 2009) and even to cell lysis during stationary phase of *E. coli* cultures (Noor *et al.*, 2009).

However, Doyle and Oliver (Poster at ISME-13, 2010, Abstract number: PS.13.005) showed active gene expression and culturability in long-term aerobically starved *Vibrio vulnificus*. Culturability initially decreased from  $10^7$  to  $\sim 10^4$  cells ml<sup>-1</sup> where it remained for 20 years. If oxygen or reactive oxygen species had a continuous effect on cellular components and therefore survivability surely all cells in a culture should have suffered irreparable damage after 20 years of exposure. Hence the negative effect of oxygen might be more important during active growth and early starvation than in long-term starved cultures where reactive oxygen species have been removed by enzymes.

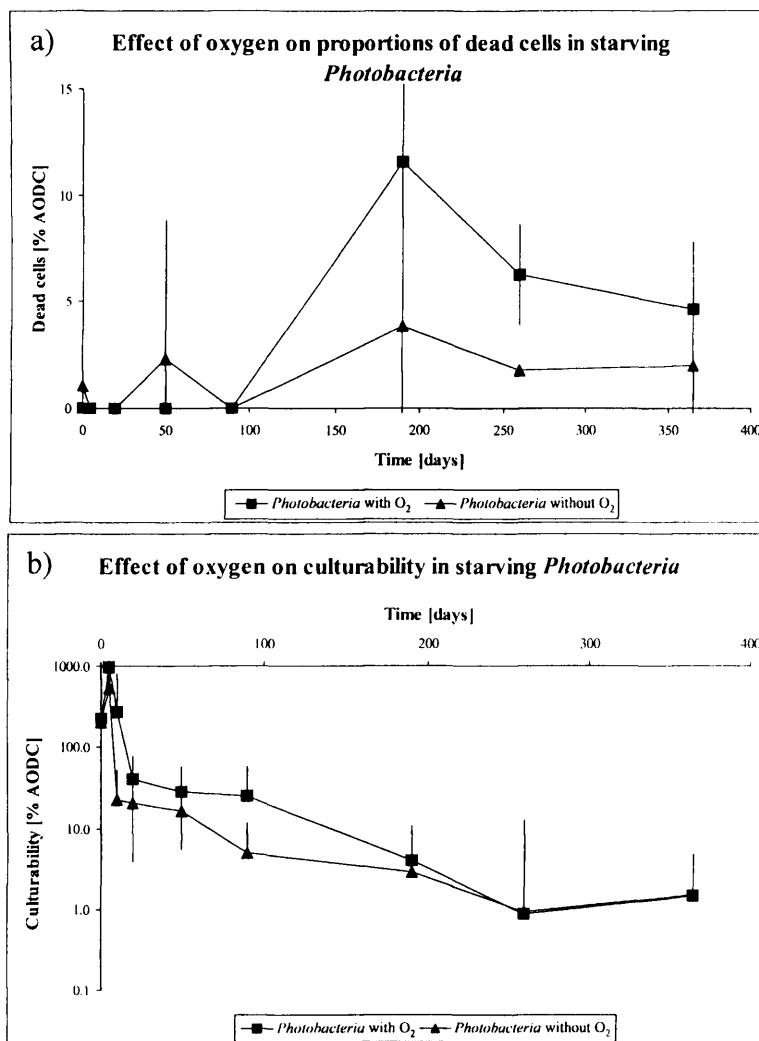


Fig. A8.1: Effect of oxygen on proportion of dead cells and cultivation efficiencies in starving *Photobacterium* cultures. a) Proportion of dead cells; b) Cultivation efficiencies.

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During this project, a general increase in the percentages of dead cells occurred with continued starvation (Fig. 6.1). However, if *Photobacterium* cultures are grouped in regard to the presence of oxygen in the headspace, there are higher proportions of dead cells in oxygen-containing cultures between 190 days and 365 days of starvation than in anaerobic cultures (on average between 2% and 7%, respectively; Fig. A8.1a; Table A8.1). In contrast to this, cultivation efficiencies are elevated in aerobic cultures between  $t_0$  and 190 days of starvation compared to anaerobic cultures (between 1% and ~440%; Fig. A8.1b; Table A8.1). This possibly indicates a bilateral effect of oxygen. On the one side it is advantageous allowing cells to gain more energy from endogenous metabolism or extracellular VFAs thus aiding the maintenance of viability and culturability (up to 190 days). From a certain timepoint onwards, however, the damage caused by ROS to essential cellular biomolecules outweighs the earlier advantages and cells start to lose viability, culturability (Fig. A8.1b), and a portion subsequently die (190 days onwards, Fig. A8.1a). Dead cells will serve as substrate for still viable cells, which might even be able to divide as a consequence. This could explain why no significant changes in AODC total counts occurred (cryptic growth). The presence of oxygen, however, seems to have detrimental effects on starving microorganisms (elevated percentages of dead cells after 190 days of starvation) but only after an initial stimulus, represented by higher cultivation efficiencies at the beginning of starvation.

**Table 8.1: Effect of oxygen on proportion of dead cells and cultivation efficiencies in starving *Photobacterium* cultures.** Legend: Cult. Eff., cultivation efficiencies; n, sample number.

Starvation Time [days]	Cultures with oxygen				Cultures without oxygen			
	Proportion of Dead cells [%AODC]	n	Cult. Eff. [%AODC]	n	Proportion of Dead cells [%AODC]	n	Cult. Eff. [%AODC]	n
0	0	2	224	18	1	4	203	11
5	0	2	977	9	0	1	540	9
10	n.d.	n.d.	271	7	n.d.	n.d.	23	8
20	0	2	41	14	0	1	21	9
50	0	2	28	8	2	4	16	8
90	0	2	25	10	0	1	5	9
190	12	11	4	7	4	6	3	8
260	6	8	1	8	2	1	1	2
365	5	5	1	6	2	7	20	7



## A8.2 Effect of temperature on starving *Gammaproteobacteria*

During starvation experiments *Photobacterium* strains were incubated at 10°C while all other strains were starved at 25°C. To investigate the effects of temperature on survivability averaged data for *Photobacteria* (without oxygen) are now compared to data of their close phylogenetic relatives *Vibrio* spp. (Farmer and Hickman-Brenner, 2006) and other *Gammaproteobacteria* (excluding *Vibrio* spp.; Fig. 6.4; Table 6.4).

Lower incubation temperatures (10°C) did not seem to affect proportions of dead cells in *Photobacteria* compared with other *Gammaproteobacteria*. Confidence limits, however, are large and overlapping for most timepoints thus indicating a relatively large variance in the data between individual strains within the groups and no definite conclusions can be drawn. Interestingly, the relatively closely related genera *Vibrio* and *Photobacterium* had different proportions of dead cells in the cultures at 190 days of starvation with 0.2% and 4%, respectively thus possibly indicating that lower temperature might have a disadvantageous effect on the proportion of dead cells in starving cultures. However, this difference was not significant and other factors such as growth conditions prior to starvation might have had an impact on subsequent starvation survivability.

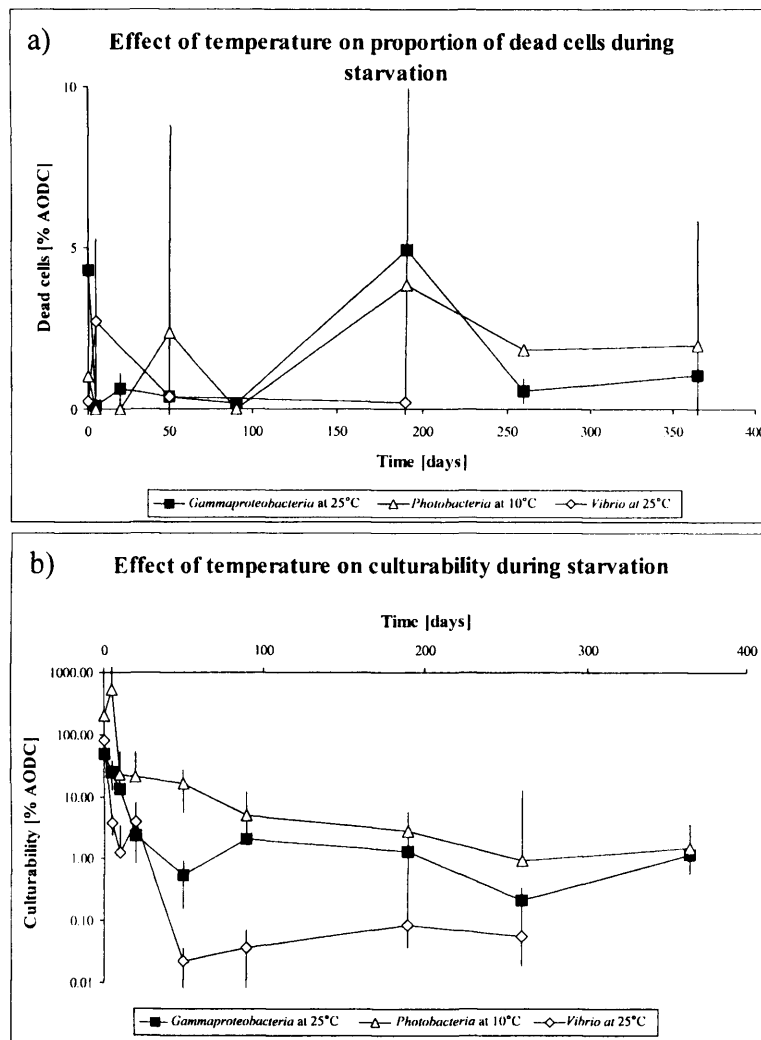
In contrast, elevated temperature seems to have a significant negative effect on culturability (Fig. 6.4b; Table 6.4) as confidence limits rarely overlap. Cultivation efficiencies of *Photobacteria* are on average one order of magnitude above values of other *Gammaproteobacteria* and two orders of magnitude higher than in *Vibrio* spp. Thus, higher temperature might present an additional stress to starvation causing lower culturability in substrate limited cultures.

However, differences in viability (percent dead cells) and culturability between broader phylogenetic groups were shown in Chapter IV (section 4.4.3). It cannot be completely ruled out that genetic disposition rather than temperature caused the differences observed between starving *Photobacterium* cultures at 10°C compared to *Vibrio* spp. and other *Gammaproteobacteria* at 25°C

It has been reported in the literature that higher temperature has a negative impact on culturability of starving cultures (Postgate and Hunter, 1962; Thomas and Batt, 1968). Marteinsson *et al.* (1997) starved the hyperthermophilic *Pyrococcus abyssi* at

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4°C and 95°C. No changes in culturability occurred at the lower incubation temperature over a 30 day period while at 95°C culturability decreased by one order of magnitude from  $2 \times 10^8$  cells ml<sup>-1</sup> to  $3 \times 10^7$  cells ml<sup>-1</sup> within seven days of starvation (Table 6.1). Joux *et al.* (1997) starved cultures of non-psychrophilic *Deleya aquamarina* at 5°C and 20°C and observed approximately half the culturable cell numbers between 42 and 95 days for the warmer incubation ( $1 \times 10^6$  cells ml<sup>-1</sup> compared to  $2 \times 10^6$  cells ml<sup>-1</sup>, Table 6.1).



**Fig. A8.2:** Effect of temperature on proportion of dead cells and cultivation efficiencies in starving *Photobacterium* cultures compared with other *Gammaproteobacteria* and *Vibrio* spp. a) Proportion of dead cells; b) Cultivation efficiencies.

A final example is a report by Vattakaven *et al.* (2006) who investigated the induction of the VBNC state via starvation in two *Vibrio* species isolated from diseased corals. *V. shiloi* was isolated from warm waters (16°C to 30°C) and no

Table 8.2: Effect of temperature on proportion of dead cells and cultivation efficiencies in starving *Photobacterium* cultures compared to other *Gammaproteobacteria* and *Vibrio* spp. Legend: Cult. Eff., cultivation efficiencies; n, sample number.

Starvation Time [days]	<i>Photobacteria</i> at 10°C				Other <i>Gammaproteobacteria</i> at 25°C				<i>Vibrio</i> spp. at 25°C			
	Proportion of Dead cells	n	Cult. Eff.	n	Proportion of Dead cells	n	Cult. Eff.	n	Proportion of Dead cells	n	Cult. Eff.	n
	[%AODC]		[%AODC]		[%AODC]		[%AODC]		[%AODC]		[%AODC]	
0	1	4	216	29	4	6	48	27	0.2	6	82	12
5	0	1	758	18	0.1	12	25	24	3	6	4	12
10	n.d.	n.d.	139	15	n.d.	n.d.	14	25	n.d.	n.d.	1	12
20	0	1	33	23	0.6	11	2	30	n.d.	n.d.	4	12
50	2	4	22	16	0.4	9	0.6	30	0.4	6	0.02	12
90	0	1	16	19	0.2	14	2	30	n.d.	n.d.	0.04	12
190	4	6	3	15	5	18	1	30	0.2	6	0.08	12
260	2	1	1	10	0.6	9	0.2	12	n.d.	n.d.	0.06	12
365	2	7	1.5	13	1	9	1.2	18	n.d.	n.d.	n.d.	n.d.

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culturable cells were detected in either 4°C or 20°C incubations after 125 days of starvation (Table 6.1). In contrast, *V. tasmaniensis* originated from temperate waters (7°C to 17°C) and in this strain VBNC was not induced when this strain was starved at 4°C for 157 days. However, culturability decreased by six orders of magnitude (from  $10^9$  to  $10^3$  cells ml<sup>-1</sup>) when incubated at 20°C.

Within the different temperature clades growth and enzymatic activity generally increase with increasing temperature until the optimum temperature for growth is reached (Madigan *et al.*, 2003). Thus if starvation occurs at sub-optimal temperatures physiological activity is slowed. Therefore, energy required for maintenance is decreased (Joux *et al.* 1997), which could aid survival under substrate limitation.

### A8.3 Effect of pressure on a starving mixed microbial community

Gas-hydrate containing sediment samples from 227 mbsf (water depth ~1300 m) from the Gulf of Mexico were obtained without depressurisation during the Gulf of Mexico Hydrates Joint Industry Project (Parkes *et al.*, 2009). These sediments were used for enrichments in nutrient broth medium and incubated at different pressures (0.1 MPa, 14 MPa, and 78 MPa). Parkes *et al.* (2009) reported that cell concentrations were generally highest in enrichment cultures close to *in situ* conditions (14 MPa). After initial enrichment cultures had been incubated for one year they were analysed under atmospheric pressure in regard to their total cell counts, percentages of dead cells, and culturability (anaerobic, nutrient broth medium). Indeed, highest AODC total counts occurred in the two parallels incubated at 14 MPa ( $\sim 1.6 \times 10^8$  cells ml<sup>-1</sup>) while total cell counts in incubations at 0.1 MPa and 78 MPa were significantly lower ( $6.5 \times 10^5$  cells ml<sup>-1</sup> and  $5.8 \times 10^7$  cells ml<sup>-1</sup>, respectively; Fig. 6.5). Once more total cell counts obtained with the LIVE/DEAD® Kit were up to one order of magnitude below AODC total counts but the same variation with pressure occurred. The increased total cell counts in close to *in situ* pressure incubations compared to the other two incubations (0.1 MPa and 780 MPa) were the same as occurred in initial growth (Parkes *et al.*, 2009).

However, it is interesting that after less than one year of starvation the lowest percentages of dead cells were also present in the 14 MPa incubations (between 4%

and 7%; Fig. 6.5) while higher percentages occurred in incubations at other pressures (17% for 0.1 MPa and 19% for 78 MPa; Fig. 6.5). Additionally, culturable cell numbers were only obtained from MPN dilution series of the 14 MPa incubations ( $1.4$  to  $2.8 \times 10^7$  cells  $\text{ml}^{-1}$ ; incubated under atmospheric pressure; Fig. 6.5) but not from other pressures incubations (visible bars in Fig. 6.5 represent detection limit, less than 2 cells  $\text{ml}^{-1}$ ; Fig. 6.5). These findings indicate the pressure might be a factor in the starvation-survival of deep biosphere microbial communities.

Depressurisation of the original sediment sample occurred for the first time during subsampling for this investigation. The LIVE/DEAD<sup>®</sup> counts were conducted as quickly as possible. However, it cannot be ruled out that some of the cells were affected by the relatively sudden loss of pressure (e.g. damage to cell membrane causing a change in its permeability or bursting of cells) and were subsequently scored as “dead” although they might have been “live” before sampling. This effect was possibly elevated for samples incubated at the highest pressure (76 MPa) but would have been completely absent from the atmospheric incubation (0.1 MPa). However, percentages of dead cells from the latter were almost as high as in the former (17% and 19%, respectively; Fig. 6.5). The same consideration applies to culturability. However the complete absence of culturable cells from 0.1 MPa and 78 MPa subsamples is puzzling.

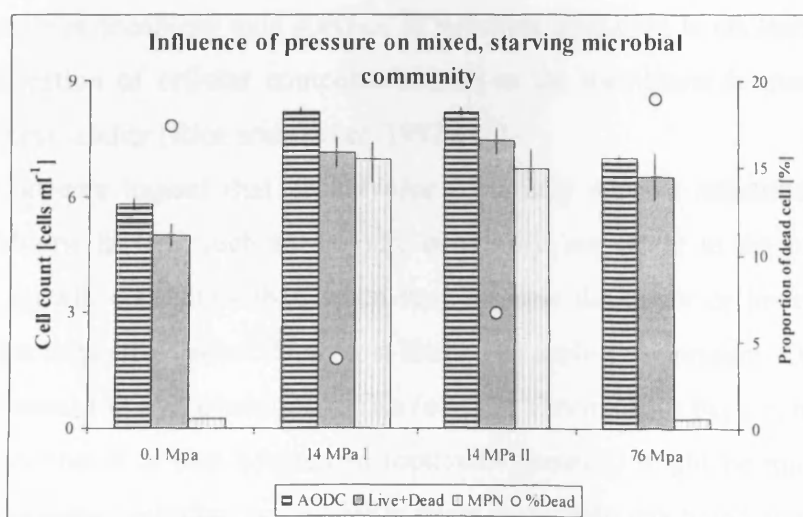


Fig. A8.3: Influence of pressure on a mixed, starving microbial community.

During starvation experiments with pure cultures a lack of culturability after less than one year was rarely observed (e.g. *Photobacterium* sp. NA42, S10, S11, 67TD,

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*D. desulfuricans*, and *D. profundus*). However, for *Photobacteria* culturability subsequently increased again. *Marinilactibacillus* sp. G8a3 investigated in regard to its starvation-survival (Chapter IV) was isolated from enrichments using sediment from the same sampling site and depth horizon. This strain showed considerable culturability ( $10^4$  to  $10^5$  cells ml<sup>-1</sup>) after one year of starvation under atmospheric pressure yet was apparently not able to grow in MPNs after one year of starvation in a mixed microbial community at 0.1 MPa.

What advantages could pressure impart on starvation survival? DeLong and Yayanos (1985) observed that pressure influences the fatty acid composition of the bacterial membrane. They reported that higher pressure lead to higher concentrations of unsaturated fatty acids (especially C<sub>16:0</sub> and C<sub>18:0</sub>) in a barophilic marine bacterium (*Vibrio* sp. CNPT3), which allows a higher membrane fluidity (Guckert *et al.*, 1986) and is the converse of changes that increasing temperature has on the membrane composition but both are homoviscous responses. However, fatty acids of this strain were also analysed during starvation by Rice and Oliver (1992) and an opposing trend was observed. It appears that cells decreased membrane fluidity by increasing the concentrations of saturated fatty acids (mainly C<sub>16:0</sub> and C<sub>18:0</sub>) but might have counteracted this simultaneously by increasing proportions of short-chain fatty acids (Rice and Oliver, 1992). Despite these contradictory results, changes of membrane composition during starvation may be facilitated by elevated pressure. How changes in membrane permeability aide survival of substrate limitation is unclear. However, any modification of cellular components such as the membrane is most likely to increase survivability (Rice and Oliver, 1992).

It appears logical that prokaryotes potentially survive substrate limitation better if abiotic factors, such as pressure or salinity, are closer to the prokaryotes' optimum growth conditions than when they are near the upper or lower tolerance limit for the respective factor. This most likely also applies to pressure. Temperature, however, seems to be an exception to this (see 4.2). Potentially a bacterium starved at the pressure that it is best adapted to (optimum pressure) might be more resistant towards substrate limitation or any other stress than when incubated at atmospheric pressure. Although the majority of deep biosphere isolates obtained so far are piezotolerant (Parkes *et al.*, 2009), meaning they are able to grow at atmospheric and at elevated pressures, it cannot be ruled out that the majority of strains *in situ* might actually better adapted to higher pressures (piezophilic). However, this trait is rarely

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included in current species descriptions but Bale *et al.* (1997) and Toffin *et al.* (2004a) observed highest rates of activity at elevated pressures for *Desulfovibrio profundus* (optimum 15 MPa, based on H<sub>2</sub>S production) and *Shewanella profunda* (optimum 10 MPa, based on growth rate), respectively. *D. profundus* was investigated in regard to its ability to withstand starvation at 0.1 MPa (Chapter IV). Of all strains investigated culturability and FISH detectability decreased most rapidly in this isolate supporting the here suggested link between incubation at sub-optimal pressure regimes and poor starvation survivability.

### A7.4 Summary of environmental effects on starvation survivability

In the comparisons above brief insights into the importance of environmental factors have been gained. In subsurface marine sediments, oxygen is generally absent, temperature is generally low (between 1°C to 3°C at surface up to 15°C assuming an average sediment depth of 500 mbsf with a temperature gradient of 30°C km<sup>-1</sup>), and pressure is elevated (on average 38MPa at the sediment surface; Abe and Horikoshi, 2001). These three *in situ* parameters all had a positive effect on the starvation-survival of marine, sedimentary microorganisms.

The presence of oxygen had a positive effect on the culturability of *Photobacterium* strains up to 190 days of starvation (Fig. A8.1b). At the same time the negative impact of ROS on cellular structures such as the membrane increased with continued incubation (at 190 days and beyond; Fig. A8.1a) as indicated by increasing proportions of dead cells. Furthermore, it appears that higher temperature (25°C, equivalent to 833 mbsf assuming a temperature gradient of 30°C km<sup>-1</sup>) may have caused continuously lower culturability in *Gammaproteobacteria* and *Vibrio* strains compared with *Photobacterium* strains starved at 10°C (equivalent to 333 mbsf; (Fig. A8.2b). Elevated pressure (14 MPa, equivalent to 1,400 m water depth) had a positive effect on growth in enrichment cultures and also on the viability and culturability than lower (0.1 MPa) or higher pressures (76 MPa; Fig. A8.3).

Thus, it can be concluded that the *in situ* conditions present in deep marine sediments most likely support the starvation-survival of deep biosphere prokaryotes. Future physiological experiments with isolates or mixed populations from this environment should therefore not exclude these parameters.

