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A review of prokaryotic populations and processes in sub-seafloor sediments, including biosphere:geosphere interactions

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ABSTRACT

A general review of the sub-seafloor biosphere is presented. This includes an update and assessment of prokaryotic cell distributions within marine sediments, current deepest 1922 m, and the impact of this on global sub-seafloor biomass estimates. These global estimates appear relatively robust to different calculation approaches and our updated estimate is 5.39×10^{29} cells, taking into consideration new data from very low organic matter South Pacific Gyre sediments. This is higher than other recent estimates, which is justified as several sediments, such as gas hydrate deposits and oil reservoirs, can have elevated cell concentrations. The proposed relationship between elevated cell concentrations and Milankovitch Cycles in sequential diatom rich layers at some sites, demonstrates not only a dynamic deep biosphere, but also that the deep biosphere is an integral part of Earth System Processes over geological time scales. Cell depth distributions vary in different oceanographic provinces and this is also reflected in contrasting biodiversity. Despite this there are some clear common, sub-seafloor prokaryotes, for *Bacteria* these are the phyla *Chloroflexi*, *Gammaproteobacteria*, *Planctomycetes* and the candidate phylum JS1, and for *Archaea* uncultivated lineages within the phylum *Crenarchaeota* (Miscellaneous Crenarchaeotal Group and Marine Benthic Group B), *Euryarchaeota* (SAGMEG, Marine Benthic Group-D/Thermoplasmatales associated groups) and *Thaumarchaeota* (Marine Group I). In addition, spores, viruses and fungi have been detected, but their importance is not yet clear. Consistent with the direct demonstration of active prokaryotic cells, prokaryotes have been enriched and isolated from deep sediments and these reflect a subset of the total diversity, including spore formers that are rarely detected in DNA analyses.

Activities are generally low in deep marine sediments (~10,000 times lower than in near-surface sediments), however, depth integrated activity calculations demonstrate that sub-surface sediments can be responsible for the majority of sediment activity (up to 90%), and hence, are biogeochemically important. Unlike near-surface sediments, competitive metabolisms can occur together and metabolism per cell can be 1000 times lower than in culture, and below the lowest known maintenance energies. Consistent with this, cell turnover times approach geological time-scales (100–1000s of years). Prokaryotic necromass may be an important energy and carbon source, but this is largely produced in near-surface sediments as cell numbers rapidly decrease. However, this and deposited organic matter may be activated at depth as temperatures increase. At thermogenic temperatures methane and other hydrocarbons, plus H_2, acetate and CO_2 may be produced and diffuse upwards to feed the base of the biosphere (e.g. Nankai Trough and Newfoundland Margin). Temperature activation of minerals may also result in oxidation of sulphides and the formation of electron acceptors, plus H_2 from low temperature (~55 °C) serpentenisation and water radiolysis. New mineral surface formation from fracturing, weathering and subduction etc. can also mechanochemically split water producing both substrates (H_2) and oxidants (O_2 and H_2O_2) for prokaryotes. These and other biosphere:geosphere interactions may be important for sustaining a globally significant sub-seafloor biosphere.

1. Introduction

Approximately 70% of the Earth is covered by seawater and most of this area also has sediments, which accumulate over geological time scales and now they contain the largest reservoir of organic carbon (~15,000 × 10^{18} g, Hedges and Keil, 1995). In addition, there are contrasting habitats within these sediments (Fig. 1), ranging from organic rich shelf/margin sediments to Mud Volcanoes and Carbonate Mounds, and organic poor Pacific Ocean Gyre sediments. However, intense degradation of sedimenting organic matter in the water column and near surface sediments, resulting in recalcitrant organic matter in subsurface layers, combined with characteristically low temperatures and elevated pressures led to the consideration that deep marine sediments were too

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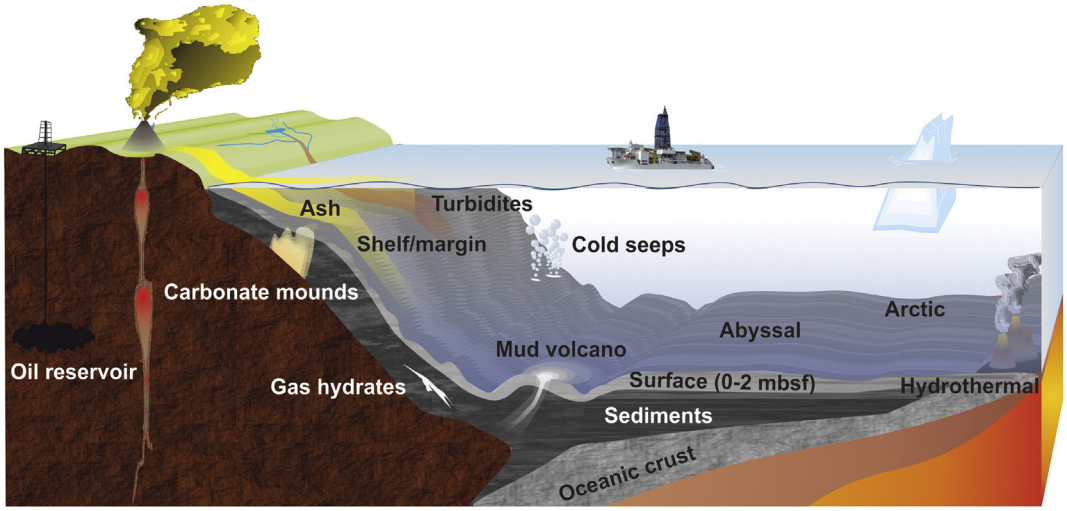

Fig. 1. Diagram of some of the major sub-seafloor biosphere habitats.

extreme for life (Morita and Zobell, 1955). Therefore, when in 1994 Parkes et al. proposed the presence of a globally significant prokaryotic deep biosphere in sub-seafloor sediments (Parkes et al., 1994), with an estimated global biomass of 10% of total biosphere carbon, it was rather contentious. The perceived low energy supply coupled with geological time scales resulted in the view that most microorganisms in sub-seafloor sediments must be either inactive or adapted for extraordinarily low metabolic activity (D'Hondt et al., 2002). However, as was originally suggested (Parkes et al., 1994), most cells were subsequently shown to be active (Schippers et al., 2005; Biddle et al., 2006; Schippers et al., 2010; Lloyd et al., 2013a), hence, these subsurface prokaryotes (*Archaea* and *Bacteria*) are indeed able to survive on very limited energy flux (~1000 times lower than required by laboratory cultures, Hoehler and Jorgensen, 2013). These results also suggest that laboratory "live fast die young" microbial cultures are inadequate for understanding the energy requirements and survival of sub-seafloor prokaryotes, and also probably most *Bacteria* and *Archaea* in the natural environment. Hence, we have to re-consider our understanding of some fundamental principles of microbiology, such as minimum cell energy requirements, cell survival, dormancy, minimum metabolic rates, as well as biosphere:geosphere interactions. The first global census of prokaryotic biomass (Whitman et al., 1998), suggested that subsurface prokaryotes (terrestrial and sub-seafloor) might even account for the majority of prokaryotic cells on Earth and with ~70% of total prokaryotic biomass residing in sub-seafloor sediments. This further increased the concern about the energy sources available to support such an enormous biomass and the basis for such estimates, including whether detected intact cells were indeed living or just fossils. In this review we address these questions along with other aspects of the sub-seafloor biosphere. In addition, we summarise recent sub-seafloor biosphere research results, which further reinforce the presence of a surprisingly large prokaryotic habitat in ocean sediments, with some unique biodiversity, and which functions on "geological" time scales.

2. Global biomass estimates of the sub-seafloor biosphere

Additional sites (1738 counts, from our published and unpublished results) including from the Atlantic Ocean and Mediterranean Sea (Fig. 2) have been added to the original data on prokaryotic cell distributions with depth in marine sediments published by Parkes et al. (1994, 299 counts), which was based solely on Pacific Ocean sites. Intact prokaryotic cells were present in all samples analysed, even including deep sourced mud volcano breccia and hydrothermal samples (estimated upper temperature 160 °C, Parkes et al., 2000) and this reinforces the ubiquitous presence of prokaryotes in sub-seafloor sediments (total 2037 cell counts). However, despite the approximate factor of 7 increase in numbers of cell counts, the resulting cell depth regression is little changed (Log cells = 8.05 − 0.68Log depth. R^2 = 0.70, compared to

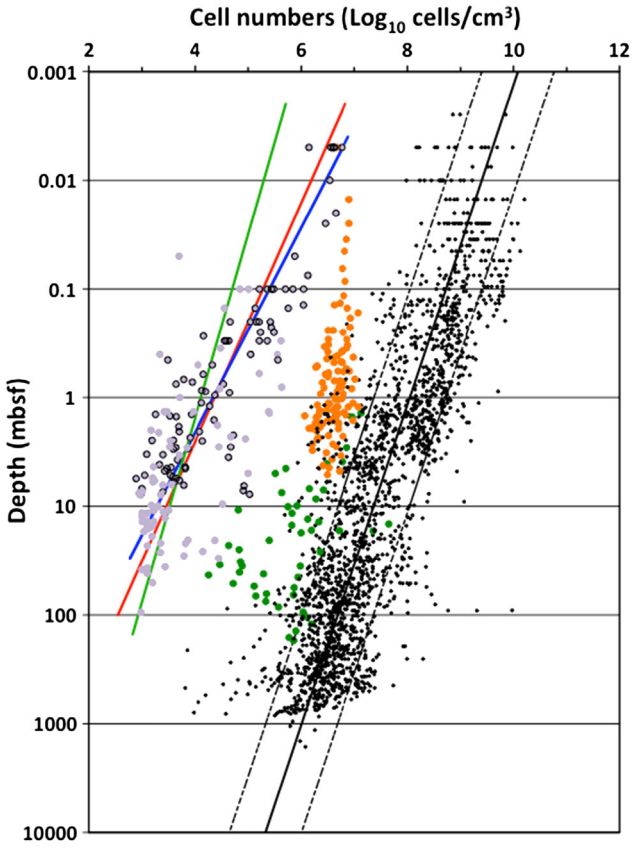

Fig. 2. Depth (metres below sea floor) distribution of prokaryotic cells in sub-surface sediments at 106 locations, including 17 ODP/IODP Legs, black dots. Bold black regression line is Log cells = 8.05 − 0.68 Log depth. (R-sq = 0.70 & n = 2037) and light dashed lines are 95% lower and upper prediction limits. Orange circles are mud volcano breccia samples and green circles are hydrothermal samples. Mauve circles with a black outline are South Pacific Gyre samples, presented by Kallmeyer et al., 2012, with blue regression line through samples, the red regression is for these same samples plus additional data from an IODP Cruise (mauve circles) to the same sites (Expedition 329 Scientists, 2011), and the green regression line is only through the later IODP cruise data. There is no significant difference (F = 0.79; d.f. = 1, 2105) in slope between this IODP cruise data regression (green line) and the regression (black line) through cell-depth distributions in other marine sediments (black dots).

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the original regression Log cells = 8.06 − 0.72 Log depth. $R^2 = 0.49$), so neither has the global cell number estimate of 9.3×10^{29} cells, compared to the original estimate of 8.65×10^{29} cells. These data include samples from the Newfoundland Margin which are one of the deepest and oldest sub-seafloor sediments which have been systematically analysed for prokaryotes: 1626 mbsf, 111 My and 60–100 °C (Fig. 3, Roussel et al., 2008). At this Newfoundland Margin site, cell numbers fluctuate around 1.5×10^6 cells/cm^3, with no indication of an accelerating decrease in deeper layers, suggesting that it is likely that prokaryotic cells will be present much deeper, and may only be limited at higher temperatures (~122 °C, current prokaryotic upper temperature limit, Takai et al., 2008). Similar cell depth regressions have been obtained by other research groups, either by also using microscopic techniques (Morono et al., 2009) or with an independent assessment based on quantification of intact polar membrane lipids and prokaryotic DNA(Lipp et al., 2008). Recently, the presence of diverse microorganisms has been documented in sediments down to 1922 mbsf in the Canterbury Basin (Ciobanu et al., 2014).

The mud volcano breccia samples (Gulf of Cadiz, Black Sea Dvurechensky MV) are clearly distinct from the cell depth distributions of all other sites (Fig. 2) being both lower and having little variation with sediment depth. This may reflect their deep origin (Milkov, 2000). In fact comparing these mud volcano counts to normal marine sediment cell depth distributions, shows that the mud volcano counts are equivalent to cell concentrations expected at ~322 mbsf (276–376 mbsf, 95% confidence limits), providing an estimate of their depth of recent origin. These cells may be trapped in the low porosity mud breccia along with ancient, matured organic matter, and are now at low near-surface temperatures and have little opportunity for growth. Even lower cell numbers occur in deep, hydrothermal samples, which probably reflect the high temperature conditions (up to 155 °C and above, Parkes et al., 2000). Also, Fig. 2 shows the cell counts from Pacific Gyre sites (Expedition 329 Scientists, 2011; Kallmeyer et al., 2012), which have the greatest distance from continents, the lowest phytoplankton productivity, and lowest sedimentation rates anywhere in the world's ocean; hence, it is not surprising that these sediments have cell concentrations much lower than other ocean sediments. Kallmeyer et al. (2012) included the 42% global area of these low cell containing oceanic gyres, together with the depth decrease from 1 mbsf in cell counts and the actual sediment depth at other locations (1° by 1° grid), to provide a new estimate of the total global number of prokaryotic cells in marine sediments. This estimate was 2.9×10^{29}, which is similar to the global cell numbers in seawater and soil. They also estimated global marine sediment prokaryotic biomass to be 0.18–3.6% of global total biomass.

However, this low value is a known underestimate of sub-seafloor prokaryotic cells, as the sensitive counting technique required for low cell concentration sediments misses some 10 to 30% of cells (Kallmeyer et al., 2008) or greater (Schippers et al., 2010), and subsurface increases in cell concentrations at some 40% of sites (e.g. Fig. 4) were excluded from the estimate (considered noise or erratic cell depth distributions by Kallmeyer et al., 2012). Sites without cell counts both above and below 1 mbsf were also excluded. Despite this, the Kallmeyer et al. estimate is still within the 95% confidence limits of our updated global cell estimate of between 1.95×10^{29} and 4.35×10^{30} cells, using a simple average sediment depth calculation (Parkes et al., 1994). If we include the 42% of Ocean Gyre sites, that have particularly low cell counts, in our calculations the estimated global total cell numbers for marine sediments becomes 5.39×10^{29} cells; even closer to the Kallmeyer et al. estimate of 2.9×10^{29} cells. Interestingly, although the South Pacific Gyre data presented in the Kallmeyer et al. paper has much lower cell numbers which decrease more rapidly with depth than our data (Fig. 2, regression slopes significantly different $P \ll 0.001$), cell depth distributions in the much deeper samples from a subsequent IODP Cruise to this area (Expedition 329 Scientists, 2011) had decreases identical to our data (Fig. 2). This suggests that there are similar controls on changing prokaryotic cell concentrations with depth in all marine sediment locations so far studied, excluding extreme conditions, such as hydrothermal and deep sourced mud volcano breccia. The general cell-depth relationship also surprisingly covers a subsurface, actively biodegrading, heavy oil reservoir in Canada (Bennett et al., 2013). These comparisons suggest that the global cell number estimates for marine sediments are rather robust despite the still limited data available, and this reinforces marine sediments as a major prokaryotic habitat that extends to kilometre depths.

Our slightly higher global cell estimate compared to Kallmeyer et al. (2012), seems reasonable considering areas with elevated

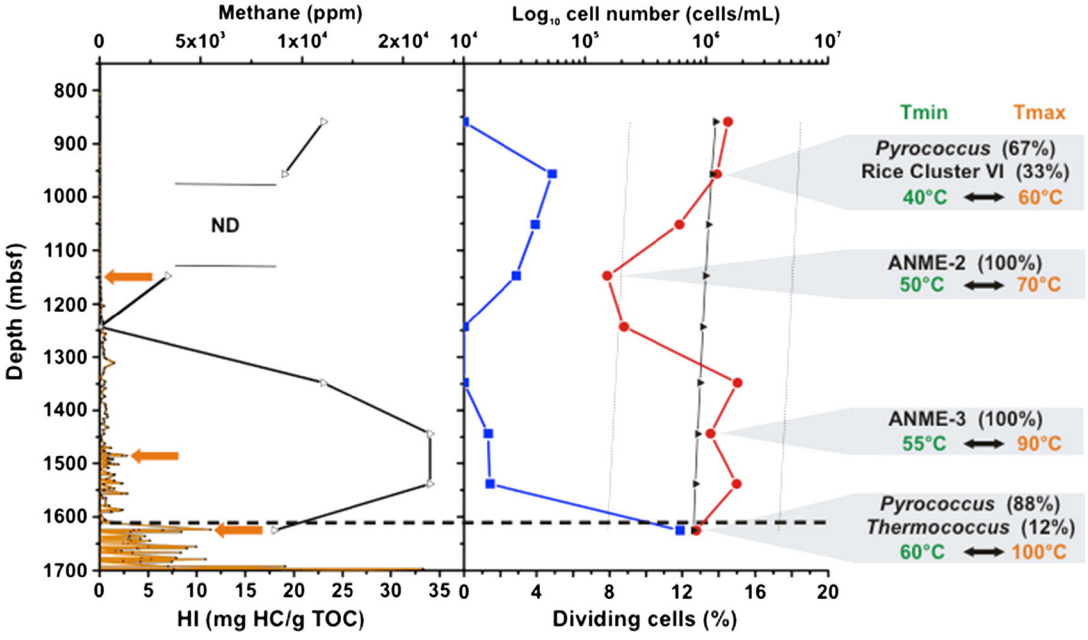

Fig. 3. Newfoundland Margin, Leg 210 (Roussel et al., 2008). Depth profiles of methane (black dots with orange line), prokaryotic cells (red circles), and percentage dividing cells (blue squares). Regression line for prokaryotic cells in other marine deep sediments (solid triangles), prediction limits (…). Orange arrows show local increases in methane. HI (open triangles) measured as mg of hydrocarbon (HC) per g of total organic carbon (TOC). ND, no corresponding data. Dominant archaeal 16S rRNA gene sequences and in situ temperature range are on the right at the depths obtained. The diabase sill is shown as a bold horizontal dashed line.

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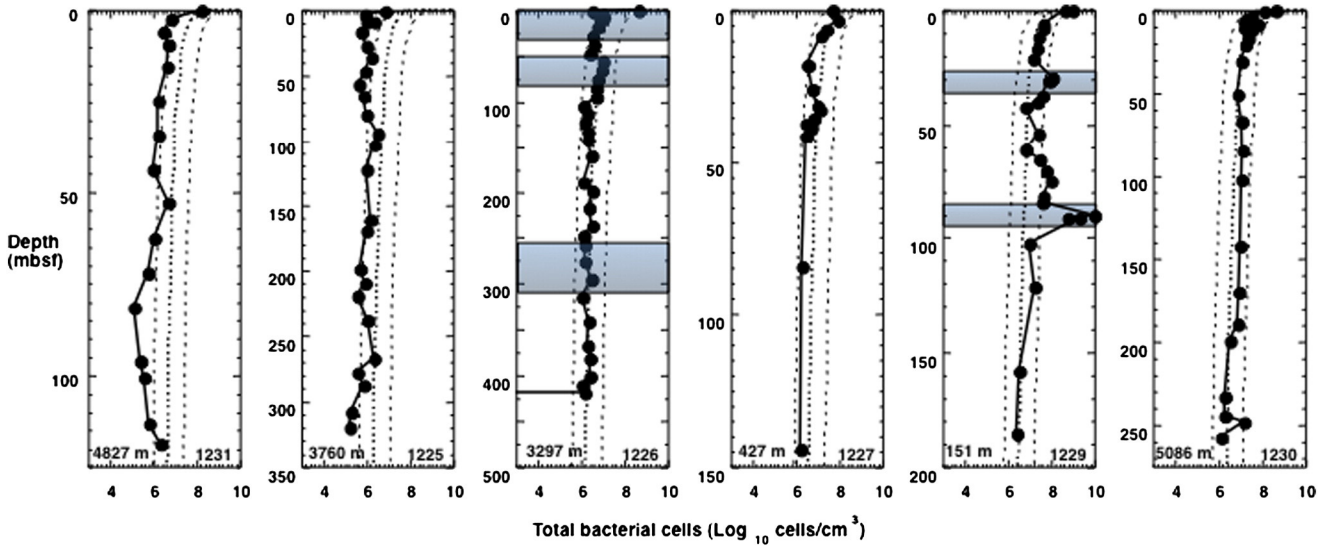

Fig. 4. Eastern Equatorial Pacific and Peru Margin Sites, Leg 201. Total cell numbers compared to cell depth profiles at other sites (Parkes et al., 2000). Cell populations increase as water column depth decreases, except for the deep-water gas hydrate Site 1230. Subsurface increases in cell numbers are highlighted by shaded areas in Sites 1226 and 1229.

deep sediment prokaryotic activities and populations (e.g. Fig. 4): in subsurface gas hydrate formations (Wellsbury et al., 2000); at sulphate: methane interfaces (Parkes et al., 2005); in organic rich layers (see below); with products of deep thermogenic processes diffusing upwards to fuel the base of the biosphere (Horsfield et al., 2006) and in oil and gas reservoirs (Head et al., 2003; Bennett et al., 2013). All of which would be excluded in the Kallmeyer et al. estimate. Stimulation of deep prokaryotic activity and populations in the subsurface can also occur in repeating deep diatom layers/interfaces in some open ocean sites (Fig. 4, ODP Leg 201, Site 1226). This shows, surprisingly, that viable prokaryotes can survive on buried organic matter in deep ancient deposits over geological time scales (7–11 My, deepest ~250 to 320 mbsf, Parkes et al., 2005). This situation may paradoxically be due to diatomaceous organic matter being particularly recalcitrant to degradation, and thus, it survives to fuel stimulated prokaryotic populations in ancient deposits. However, whatever the mechanism of stimulation, prokaryotes in buried diatom layers must be metabolising extremely slowly. The diatom layers and associated prokaryotes correlate with orbital forcing (Milankovitch Cycles, Aiello and Bekins, 2010), via enhanced ocean productivity, which elegantly shows how the deep biosphere is an integral part of Earth System Processes over geological time scales. Similar cell stimulation occurs in repeating layers of organic-rich sapropel layers (max 4.7 My) in the Mediterranean Sea (Parkes et al., 2000) which are the product of major oceanographic changes. Sapropel layers up to 217,000 years old have been shown to also have increased activities of hydrolytic exoenzymes, and increased anaerobic glucose degradation rates, as well as elevated cell numbers, directly demonstrating that organic substrates remain bioavailable on these "geological" time scales (Coolen et al., 2002).

Water column depth, and hence, presumably, the supply of degradable organic matter influences the cell distributions at different sites. For example, within ODP Leg 201 Sites, a deep water Eastern Equatorial Pacific Site at 4827 m water depth (Site 1231) has cell depth distributions at or below the lower 95% prediction limits of the global subseafloor sediment counts (Fig. 4), and as the water depth of Leg 201 sites decreases the cell counts progressively increase, such that by Site 1229 in 151 m water depth many cell counts are at or above the upper 95% confidence limits. The only exception to this trend is the deep-water Site 1230 (5086 m), which has cell depth distributions very close to the global average (Fig. 2). This is a site which has gas hydrates, and it has previously been shown that sub-seafloor sediments containing gas hydrates are more biogeochemically active compared to non-gas hydrate sites (Wellsbury et al., 2000). Gas hydrate sites in general are also one of the locations which seem to have distinct prokaryotic communities (Section 3) and their cell depth distributions are very similar to the global average, but with some depth zones with much higher cell numbers (Fig. 5). Some of the other locations with distinct communities (Fig. 7) also have distinct cell depth distributions (Fig. 5), for example, organic-rich shelf/margin sites are similar to gas hydrate sediments, except that overall cell concentrations and subsurface increases are higher. These subsurface increases generally are due to deep geochemical interfaces (Parkes et al., 2005). In contrast, abyssal sites have much lower cell concentrations than the global average, especially in the top few hundred metres (Fig. 5), which is even more extreme in gyre sediments (Fig. 2). Near-surface gyre sediments (0–2 m) cell concentrations are also consistently lower than in other near surface sediments, which overall have distinct prokaryotic communities (Fig. 7). Deep carbonate sediments have cell depth distributions which are also rather lower than the global average, but these decrease less with increasing depth. Hence, there are distinct cell distributions in a number of sub-seafloor sediment provinces (Fig. 5) and many of these also have distinct prokaryotic populations (Fig. 7, Section 3).

It has been suggested that bacterial endospores are as abundant as intact prokaryotic cells in sub-seafloor sediments, and hence, they could increase current biomass estimates (Lomstein et al., 2012). However, this is only if spores are not stained by dyes such as acridine orange, used in cell counting, and hence, not already detected. There is evidence that some cultured prokaryotes with endospores can be stained (Fichtel et al., 2008, see also Fig. 6) and thus, some spores might already be included in prokaryotic biomass estimates. Interestingly, deep marine sediments and subsurface petroleum reservoirs have been suggested as possible sources of spore-forming thermophiles to cold Arctic and other sediments, via seeps and mud volcanoes etc. (Hubert et al., 2009). If these survive burial, then they might germinate when temperatures rise with increasing depth. Conversely, spores might be drawn down with seawater as part of the basin-wide hydrothermal circulation (marine aquifer, Fig. 9) and travel through porous basement (Fisher and Becker, 2000), with the potential to inoculate warm sediments above. Ultimately via seeps, the spore cycle could continue. Certainly spores can survive for long periods of time to enable dispersal, for example, the half-life of thermophilic, sulphate-reducing bacterial spores in sediments has been estimated to be ~300 years (de Rezende et al., 2013), and spores have been detected in ancient samples (millions of years old, Parkes, 2000).

Finally, viruses (Middelboe et al., 2011; Engelhardt et al., 2013) and fungi have been detected in deep marine sediments (Edgcomb et al.,

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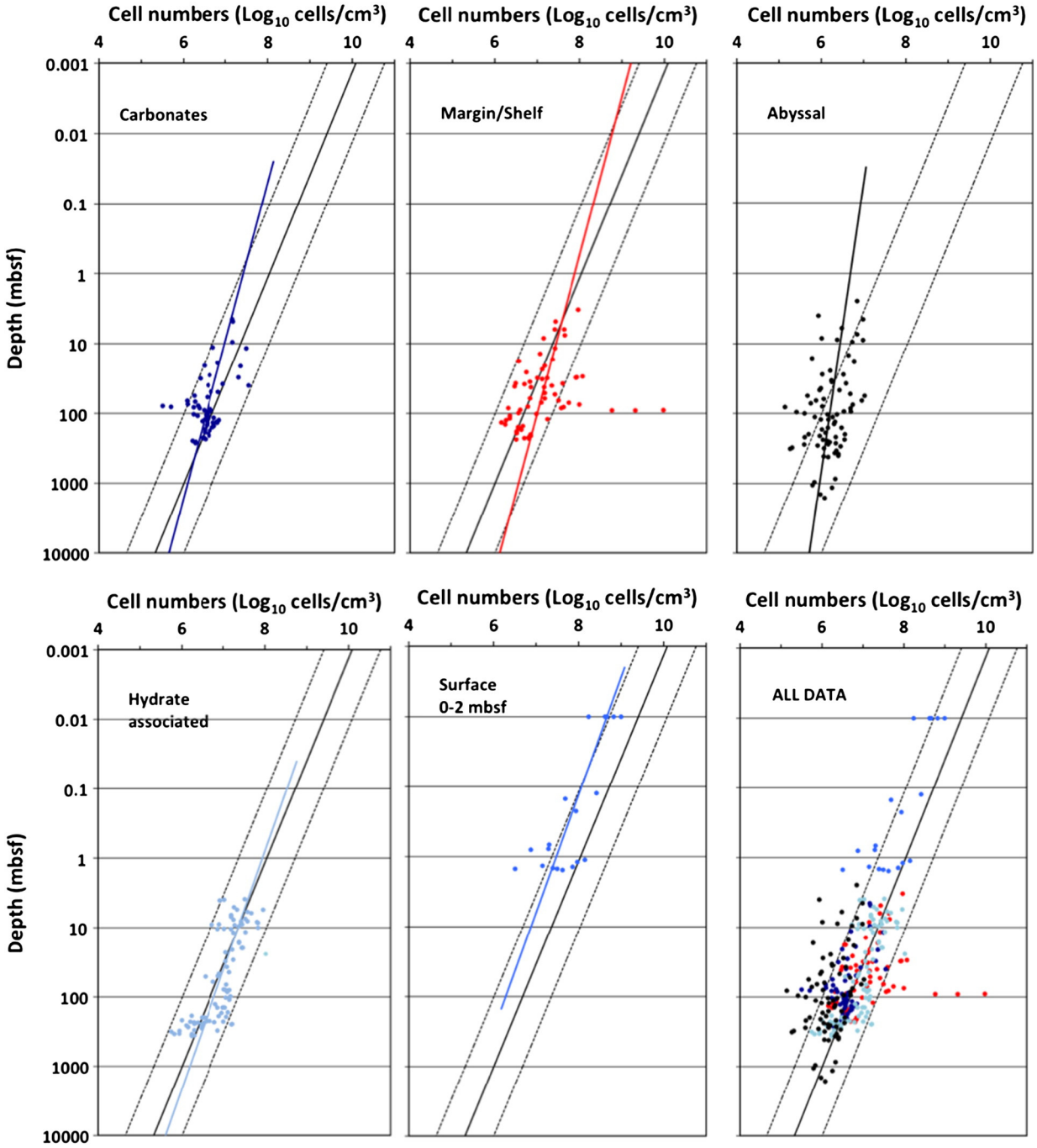

Fig. 5. Prokaryotic cell distributions in sub-seafloor sediment locations that have distinct diversities shown in Fig. 7.

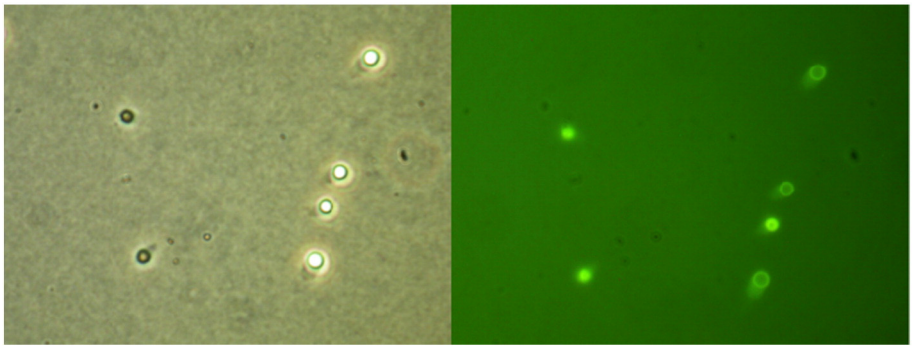

Fig. 6. *Desulfotomaculum acetoxidans* with spores of different stages under phase contrast (left) and acridine orange stained epifluorescence (right) microscopy.

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2011; Orsi et al., 2013a,b), but their biomass and role in sub-seafloor sediments is unclear.

3. Microbial diversity in sub-seafloor sediments by molecular approaches

The earliest comprehensive microbiological investigations of sub-seafloor sediments showed that metabolically diverse culturable microbial populations were present to at least 80 mbsf (deepest samples, Parkes et al., 1990), however, culturability was quite low (~0.0003%). This reflects the low culturability from environmental samples in general and motivated the first molecular studies of microbial communities in sub-seafloor sediments (Rochelle et al., 1992). These initial studies were beset by major technical problems that were mainly the result of the low prokaryotic biomass in deep subsurface samples, presence of compounds that interfered with the analysis (e.g. PCR inhibitors; Webster et al., 2003), and biases, now well recognised, associated with nucleic acid extraction and PCR amplification. As discussed later in this section, these problems have not been entirely overcome; however, our understanding of the diversity of prokaryotic communities inhabiting the deep sub-seafloor biosphere has advanced greatly, and some clear indications of key bacterial and archaeal taxonomic groups have emerged from the studies of sediments from ODP, IODP and other expeditions by various research groups. For example: Inagaki et al. (2006) presented an integrated analysis of prokaryotic community diversity in Pacific Ocean sediments from 6 ODP sites as deep as 330 mbsf; Fry et al. (2008) reviewed the results from 13 independent (mainly ODP) molecular studies of deep sub-seafloor sediment prokaryotic communities; Durbin and Teske (2012) contrasted archaeal distributions in organic-lean marine sediments in deep marine basins and oligotrophic open ocean locations with the more frequently studied, organic-rich continental margin sediments and found that the organic-lean sediments are inhabited by distinct lineages of *Archaea*, and Orcutt et al. (2011) summarised the prokaryotic diversity of the dark ocean above, at, and below the seafloor. In this review we have expanded the analysis of Fry et al. (2008) to include 32 independent nucleic acid-based studies (see Supplementary Figs. 1A and 1B), mostly using first generation sequencing of PCR generated 16S rRNA gene libraries, but also including some more recent diversity studies using next generation sequencing methods (Suppl Figs. 1A and 1B). The comparative results of relative abundance of prokaryotic taxa, however, must be interpreted with caution, since they are derived from numbers of clones in 16S rRNA gene libraries generated mostly by non-quantitative PCR. Nevertheless, some interesting and consistent patterns are evident. This meta-analysis has been extended by principal components analysis (PCA) and canonical analysis to consider potential associations between prokaryotic taxa and sediment type/habitat (Fig. 7).

Collectively, the studies reviewed here (see Supplementary Figures) clearly show that novel *Archaea* and *Bacteria* predominate in sub-seafloor sediments. Analysis of 205 prokaryotic 16S rRNA gene libraries from a range of subsurface sediments and depths (Fig. 7) revealed that for *Bacteria* the dominant phyla are *Chloroflexi*, *Gammaproteobacteria*, *Planctomycetes* and the candidate phylum JS1 (Webster et al., 2004), with 25.5%, 10.3%, 5.6% and 22.2% (sum = 63.6%; present in 66% of libraries from subsurface sediments deeper than 2 mbsf) of 16S rRNA gene clones, respectively (Suppl Fig. 1A). The *Alpha-*, *Beta-*, *Delta-* and *Epsilonproteobacteria* are less common, averaging 4.1%, 2.4%, 4.6% and 1% of clones, respectively. Of the remaining 24.5% of clones, the novel group NT-B6, originally found in the Nankai Forearc Basin (Reed et al., 2002) are the most abundant (an average of 5.7% clones in 21% of libraries), and are also present in sediments from the Cascadia Margin (Inagaki et al., 2006; Nunoura et al., 2008), Gulf of Mexico (Nunoura et al., 2009) and the Peru Margin (Webster et al., 2006). Other phyla with on average >1% of clones are *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, *Spirochaetes* and novel phyla/groups OP8, OP11 and NT-B2.

For *Archaea* (Suppl Fig. 1B), the majority of sequences in subsurface sediments (below 2 mbsf) belong to uncultivated lineages within the phylum *Crenarchaeota* (4 groups), representing 66.1% of all 16S rRNA gene clones, whilst only 23.1% of sequences belong to the *Euryarchaeota* (10 groups) and 8.9% to *Thaumarchaeota* (Marine Group I). The most abundant *Crenarchaeota* groups are the Miscellaneous Crenarchaeotal Group (MCG) and Marine Benthic Group B (MBG-B; alternatively named the Deep-Sea Archaeal Group, DSAG; Inagaki et al., 2003) comprising 32% and 29.1% of clones, respectively. The next most abundant groups are the South African Gold Mine Euryarchaeotal Group (SAGMEG; 7.3%) and the Marine Benthic Group-D (MBG-D)/Thermoplasmatales associated groups (7.5%). Methanogens (*Methanosarcinales*, *Methanomicrobiales* and *Methanobacteriales*) and anaerobic methane-oxidising Archaea (ANME) are less abundant, only representing 2.6% of clones overall, whilst thermophilic *Archaea* (*Thermococcales*, *Methanococcales* and *Archaeoglobales*) account for 5.3% of clones and were mainly found in deeper subsurface sediments (e.g. Cascadia Margin, Nankai Trough, Newfoundland Margin, Kormas et al., 2003; Inagaki et al., 2006; Nunoura et al., 2008; Roussel et al., 2008).

Canonical analysis suggests that prokaryotic composition may be linked to sediment type or oceanographic province (Fig. 1), presumably reflecting site-specific geochemical and physical conditions, such as oxygen, sulphate, methane hydrate, organic and inorganic carbon content, mineralogy, water and sediment depth (Fig. 7). Near-surface sediments (above 2 mbsf) contain relatively diverse bacterial and archaeal communities (at the phylum level), with a significant correlation to MG1/*Thaumarchaeota* ($P < 0.001$), *Epsilonproteobacteria* ($P < 0.05$) and *Planctomycetes* ($P < 0.001$), as well as a high percentage of *Chloroflexi*. Cultured members of both *Thaumarchaeaota* and *Planctomycetes* are known to oxidise ammonia aerobically or anaerobically, respectively (Junier et al., 2010), suggesting nitrification maybe an important metabolic process in near-surface sediment prokaryotic communities. However, surface sediments from cold seeps with high rates of activity and strongly reducing conditions contain noticeably different microbial communities linked to sulphur and methane cycling, with bacterial communities being dominated by *Deltaproteobacteria* (contains major genera of sulphate reducers; Muyzer and Stams, 2008), and dominant *Archaea* belonging to ANME and methanogens (*Methanosarcinales*). Surface seeps are positively correlated with the presence of ANME ($P < 0.001$), as well as *Epsilonproteobacteria* ($P < 0.005$), which includes several known lithotrophic sulphur-oxidising species (Hubert et al., 2012).

In contrast, subsurface sediment 16S rRNA gene libraries from organic-rich shelf/margin sites (Fig. 7, mainly Peru Margin ODP Leg 201) significantly correlate with a high average percentage of uncultivated members of the *Chloroflexi* (41.3%, $P < 0.01$) and MCG (62.7%, $P < 0.001$). These are prokaryotes with unknown metabolism, although it has been suggested that MCG are heterotrophic (Biddle et al., 2006) and associated with lower respiration rates (Kubo et al., 2012). In addition, single cell genomics have shown that some MCG *Archaea* have the ability to degrade detrital proteins (Lloyd et al., 2013b), whilst other studies have shown that they can incorporate ^{13}C-labelled glucose and acetate (Takano et al., 2010; Webster et al., 2010). They also have high diversity and are widely distributed in subsurface sediments, suggesting that this group is globally important in sedimentary processes (Kubo et al., 2012; Lloyd et al., 2013b). With regard to *Chloroflexi*, sub-seafloor communities often contain several *Chloroflexi* subgroups, with some representatives distantly related to *Anaerolineae* (subphylum I), whose cultured representatives are organoheterotrophs (Yamada et al., 2006), and the *Dehalococcoidia* (subphylum II; Hugenholtz and Stackebrandt, 2004), which includes the obligate anaerobic reductive dehalogenator, *Dehalococcoides mccartyi* (Loffler et al., 2013), as well as subphylum IV which exclusively comprises of environmental sequences. Recently, analysis of a single cell genome from an uncultured member of the *Dehalococcoidia* from Aarhus Bay (Denmark) sediments suggests the presence of greater metabolic diversity than related

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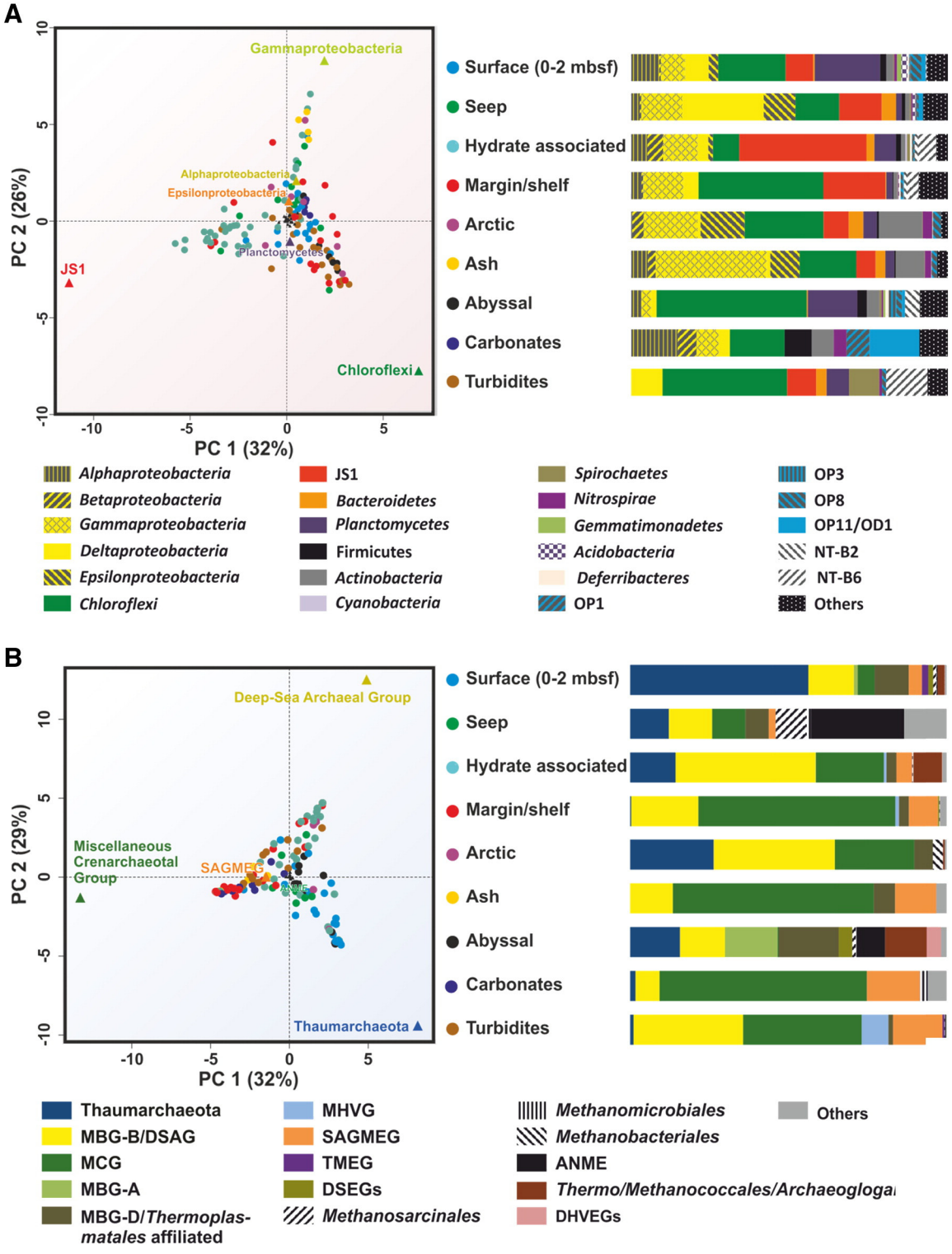

Fig. 7. Principle Component Analysis of prokaryotic phylogenetic groups in specific sub-seafloor locations: A *Bacteria*, B *Archaea*. See Fig. 5 for corresponding cell depth distributions and supplementary data.

cultured members, with identification of numerous genes encoding enzymes involved in the oxidation of fatty acids, plus aromatic compounds, but interestingly, no evidence for reductive dehalogenation (Wasmund et al., 2013).

Deep sediments with gas hydrates significantly correlate ($P < 0.001$), with high percentages of candidate division JS1 (43.6%) and MBG-B (DSAG; 44.3%) phylotypes (Fig. 7), which supports previous reports that hydrate bearing sediments from the Pacific Ocean Margin are dominated by these specific microbial communities (Inagaki et al., 2006). In addition, re-investigation of Cascadia Margin sediments from ODP Leg 146 site 889/890, which, in contrast to other hydrate sites was originally reported to be dominated by *Proteobacteria* and *Bacteroidetes* (Marchesi et al., 2001, Suppl Fig. 1A), using methods developed subsequently for ODP Leg 201 sediments (Webster et al., 2006), also showed that these sediments were dominated by JS1 and novel *Archaea* (Webster, Weightman, Parkes, unpublished data; Suppl Fig. 2A and 2B). These results underline the effect that specific methodologies can have on diversity studies using molecular methods, particularly with respect to earlier studies.

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Hydrate sediments down to 77 mbsf from the Ulleung Basin (Japan Sea) are also dominated by members of JS1 and MBG-B (Lee et al., 2013), and JS1 were also a significant proportion of bacterial phylotypes from near surface Gulf of Mexico and Chilean Margin hydrate sediments (Lanoil et al., 2001; Mills et al., 2005; Hamdan et al., 2012). In contrast, however, some studies have shown that gas hydrate sediments from locations within the Indian Ocean have very low cell numbers and are dominated by *Firmicutes* with no detectable *Archaea* (Parkes et al., 2009; Briggs et al., 2012).

Although studies on subsurface sediments from oligotrophic, open oceans and abyssal basins are relatively few, especially for *Bacteria*, initial results suggest that they have a relatively diverse microbial community dominated by *Chloroflexi* and *Planctomycetes* with site- and depth-specific populations of *Archaea* (Fig. 7). For example, sediments from the South Pacific Gyre and Equatorial Pacific are dominated by MBG-A *Crenarchaeota*, whilst some sediments from the Fairway Basin (site MD06-3028) are dominated by MG1 *Thaumarchaeota* (Roussel et al., 2009), others (site MD06-3022) are dominated by novel *Euryarchaeota*, and specific sediment depths of the Fairway Basin site MD06-3027 (4.5 mbsf) are dominated by MBG-B, along with Peru Basin at 9 mbsf (Sørensen et al., 2004; Roussel et al., 2009). The abyssal sites included in this survey positively correlate with MG1 ($P < 0.01$) and negatively correlate with MBG-B ($P < 0.01$) and MCG ($P < 0.001$). *Proteobacteria* are particularly prevalent in sediments from the Arctic Ocean at the Lomonosov Ridge and the Arctic Mid-Ocean Ridge along with a high percentage of MCG, MG1 and MBG-B *Archaea* (Forschner et al., 2009; Jorgensen et al., 2012). Whereas, archaeal communities in other organic-poor deep sediment sites are dominated by MCG, for example, volcanic ash layers of the Okhotsk Sea; (Inagaki et al., 2003); carbonate mounds from the Porcupine Seabight (Webster et al., 2009; Hoshino et al., 2011), and turbidite layers from the Gulf of Mexico (IODP Exp. 308), plus *Chloroflexi* (Nunoura et al., 2009).

The efficient extraction and subsequent analysis of nucleic acids from low biomass deep marine sediments, are essential for the fidelity of resulting diversity estimates. However, to achieve this with challenging and variable deep sub-seafloor samples (e.g. low biomass, co-extraction of humic substances, carbonate and clay matrix), has resulted in the use of a wide variety of techniques in different laboratories (Webster et al., 2003; Luna et al., 2006; Lloyd et al., 2010; Alain et al., 2011; Lloyd et al., 2013a), including clean-up procedures to remove PCR inhibitors (e.g. Kallmeyer and Smith, 2009; Lloyd et al., 2010), which can result in different biases (Lipp et al., 2008; Lloyd et al., 2010). Extraction of DNA and RNA from subsurface sediments has mostly involved direct methods whereby microbial cells are lysed or physically disrupted within the sediment matrix (e.g. Webster et al., 2003), but some studies have used indirect methods which require cell separation from the sediment before breakage (e.g. Luna et al., 2006; Lloyd et al., 2013b). Cell separation is always incomplete and often quite variable (Schippers et al., 2010), and it is unclear what effect this has on diversity estimates. In addition, the choice of "universal" bacterial or archaeal-specific PCR primer/probe varies greatly between laboratories (e.g. Inagaki et al., 2006; Webster et al., 2006; Roussel et al., 2008), especially for 16S rRNA genes, and can add further biases. Teske and Sorensen (2008) reviewed and highlighted this problem with respect to PCR primers and probes that have been used routinely to study archaeal diversity in marine subsurface sediments. With the increased use of high-throughput next generation technologies this issue may need to be addressed further. It has already been suggested that the deep biosphere scientific community should discuss and standardise their methodologies to determine the best target gene, region and primers, so that future studies can be compared and provide robust datasets for a better understanding of global trends in subsurface microbial diversity (Orcutt et al., 2013).

To date, the few deep marine sediment studies that have used next generation pyrosequencing have shown that overall the same groups of *Bacteria* and *Archaea* predominate (e.g. Hoshino et al., 2011; Jorgensen et al., 2012). For example, in a survey of sediments and deeply buried coral carbonates (IODP Expedition 307), approximately 16,000–28,000 bacterial and archaeal 16S rRNA-tagged sequences per sample, demonstrated that the dominant prokaryotic phylotypes were MCG, SAGMEG and MBG-B Archaea, as well as *Chloroflexi*, *Proteobacteria* and bacterial candidate divisions OP1 and OP11/OD1 (Hoshino et al., 2011, Suppl Figs. 1A and 1B). These were similar to the dominant groups found by Webster et al. (2009) using PCR-DGGE analysis (MCG, SAGMEG, *Chloroflexi*, *Proteobacteria* and candidate division JS1). Likewise, V6-tag pyrosequencing of bacterial and archaeal 16S rRNA genes from ODP Leg 201 Peru Margin site 1228 also showed good agreement with 16S rRNA gene libraries and PCR-DGGE analysis previously described (Webster et al., 2006), with the prokaryotic communities being dominated by *Chloroflexi*, *Gammaproteobacteria* and MCG (Suppl Figs. 1A and 1B). In addition to good agreement with previous data and providing confidence that the main deep biosphere prokaryotic groups have been well sampled, next generation sequencing also greatly increases the overall microbial diversity found and has allowed the detection of many microbial groups that occur at low-frequency and were missed by Sanger sequencing of clone libraries, giving microbial ecologists an insight into what has been termed the 'rare biosphere' (Sogin et al., 2006). In the deep biosphere it has been proposed that these rare groups include *Bacteria* and *Archaea* involved in sulphate reduction and methanogenesis, groups that are often missing from first generation 16S rRNA gene surveys of deep marine sediments (Fry et al., 2008). Pyrosequencing data from Peru Margin site 1228 demonstrates that such sequences are present in low abundance, with retrieved sequences being related to sulphate-reducing *Deltaproteobacteria*, methanogenic *Euryarchaeota* (orders *Methanomicrobiales* and *Methanobacteriales*), and anaerobic methanotrophic Archaea (ANME) groups (Suppl Figs. 1A and 1B).

Contrasting biodiversities have often been obtained when DNA- or lipid-biomarkers have been used, even to the Domain level, where there is disagreement whether sub-seafloor sediments are dominated by *Bacteria* or *Archaea*. For example, CARD-FISH and Q-PCR analysis of Peru Margin (ODP Leg 201) deep sediments concluded that *Bacteria* were the dominant prokaryotes in deeply buried marine sediments (Schippers et al., 2005), whilst another Peru Margin sediment study using FISH combined with intact polar membrane lipids (IPL) and rRNA analysis, suggested that the active portion of the microbial community was dominated by *Archaea* (Biddle et al., 2006). In addition, analysis of subsurface sediments from a broad range of oceanographic settings indicated that at least 87% of IPL biomarkers were from archaeal membranes, and thus *Archaea* were a major fraction of the active prokaryotic biomass (Lipp et al., 2008). These conclusions were supported by data obtained from modified Q-PCR and slot-blot hybridisation techniques, however, even with these improved techniques archaeal 16S rRNA genes were only 50% of total prokaryotic DNA. A likely explanation for differences between DNA and lipid biomarker approaches may be that archaeal glycosidic ether lipids seem to degrade more slowly than bacterial phospholipids in sediments (Schouten et al., 2010; Logemann et al., 2011; Xie et al., 2013), and therefore, archaeal lipids could be more representative of fossil biomarkers rather than living *Archaea*. This is consistent with distinct bacterial community changes at deep methane-sulphate interfaces in Peru Margin sediments (30 and 90 mbsf) demonstrating an active community responding to changing environmental conditions, whereas, in contrast, archaeal diversity at this site was more limited, did not change with depth, and hence, was less active (Parkes et al., 2005). However, at another Peru Margin site archaeal distributions determined by 16S rRNA, which may directly represent more active prokaryotes, showed these communities did change over short distances in geochemically distinct zones of deep sub-seafloor sediments (Sorensen and Teske, 2006). The reasons for these differences are unclear and there are similar inconsistencies between other approaches.

The first metagenomic analysis of deep biosphere sediments suggests that whilst *Bacteria* dominate the near-surface sediments

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(1 mbsf), *Archaea* may become more important in deeper layers (50 mbsf, but with large errors in Q-PCR data, Biddle et al., 2008). However, the first metatranscriptome study on the same Peru Margin sediments (5 to 159 mbsf, Site 1229, Orsi et al., 2013b) found that *Archaea* were in noticeably low abundance, despite their previous detection at this site (Biddle et al., 2006), whilst the dominant transcripts were all from *Bacteria* (*Firmicutes*, *Actinobacteria*, *Alphaproteobacteria* and *Gammaproteobacteria*, consistent with previous phylogenetic surveys e.g. Webster et al., 2006). In addition, this metatranscriptome study suggests that fungi are an active component of the sub-seafloor biosphere, representing between 3 and 20% of the transcripts.

Other considerations regarding biodiversity in the deep biosphere are the presence of bacterial spores (Lomstein et al., 2012) and bacteriophages (Engelhardt et al., 2011). The presence of spores in sub-seafloor sediments is consistent with the detection of *Firmicutes* in both cultivation and molecular diversity surveys. The abundance of viruses, like prokaryotic cells (Fig. 2), has been shown to decrease exponentially with sediment depth in sub-seafloor sediments from the Porcupine Seabight (IODP Expedition 307, Mound Site, Middelboe et al., 2011). However, it has also been suggested that most of these viruses are in microenvironments where they are protected from decay, as even mixing the sediment resulted in a rapid decrease in viral numbers, hence, these protected viruses may persist for hundreds of thousands of years, (Middelboe et al., 2011). The impact of viruses on the sub-seafloor biosphere remains unclear as prevailing conditions of spatial separation/isolation of host cells, low activity and cell division etc. are particularly challenging for viruses.

4. Cultured prokaryotes from sub-seafloor sediments

As previously noted, only a very small proportion of total cells in sub-seafloor sediments can be detected by cultivation. One of the many reasons for this is the inability of severely energy limited sub-seafloor prokaryotes (Hoehler and Jorgensen, 2013) to grow in rich laboratory media, including damage ('substrate-accelerated death', Postgate and Hunter, 1963) when suddenly exposed to high substrate concentrations in normal media. Indeed, the use of substrates at sub-millimolar concentrations in subsurface sediment enrichments have shown increased culturability by up to four orders of magnitude, compared to use of standard rich microbiological media (Sub et al., 2004). In addition, in many cases colonies formed in agar shakes or on plates are extremely small, often only detectable using a stereomicroscope and easily overlooked (Sub et al., 2004). However, there are some deep sediments sites/depths that have elevated subsurface viable counts, such as the sapropel layers of the Eastern Mediterranean Sea (Sub et al., 2004), sediments with deep hydrocarbons (Japan Sea, Parkes et al., 1994) or deep brines (Parkes et al., 1990) and presence of methane hydrate (Blake Ridge, Cascadia Margin, Cragg et al., 1996; Wellsbury et al., 2000, Table 1). This clearly shows the presence of significant numbers of culturable, and hence, viable cells in deep sediment layers, and that this viability does not necessarily decrease with increasing depth in sediments millions of years old. Similarly, porous sediments seem to have higher numbers of culturable bacteria, whilst clayey sediments, despite having similar total cell counts and microbial activities, have lower viable counts (Inagaki et al., 2003). At some sites, this can lead to an increase in culturable cells with depth, with very low viable cells in the upper layers and higher viable cells in very deep layers (Table 1, Kobayashi et al., 2008; Webster et al., 2009).

Bacterial and archaeal isolates from sub-seafloor sediments, mostly belong to the *Proteobacteria*, *Firmicutes*, *Actinobacteria* and *Bacteroidetes* or the euryarchaeotal genera *Methanoculleus* and *Methanococcus* (Bale et al., 1997; Barnes et al., 1998; Inagaki et al., 2003; Mikucki et al., 2003; Sub et al., 2004; Toffin et al., 2004; Lee et al., 2005; Takai et al., 2005; Kendall et al., 2006; Batzke et al., 2007; Kobayashi et al., 2008; Parkes et al., 2009), which are mostly typical inhabitants of near-surface environments. The presence of some of these genera, has also been confirmed by cultivation-independent molecular biological techniques (e.g. *Pseudomonas*, *Halomonas*, *Marinobacter*, *Acinetobacter* and *Rhizobium* spp., Reed et al., 2002; Inagaki et al., 2003; Kormas et al., 2003; Parkes et al., 2005; Inagaki et al., 2006; Sub et al., 2006), indicating that they do contribute significantly to the in situ microbial communities. In a number of studies (Sub et al., 2004; Batzke et al., 2007; Kobayashi et al., 2008; Parkes et al., 2009) these bacterial genera were enriched despite using 'selective' media targeting other physiological groups like fermenters, sulphate-reducing bacteria (SRB) or methanogens (Fig. 8). One explanation may be the low concentration of the target prokaryotes (Parkes et al., 2005), and hence, lack of competition.

However, the spore forming *Actinobacteria* and *Firmicutes* are rarely detected using molecular methods (~1–2% of all clone libraries, Fig. 7) despite often constituting a major proportion of deep subsurface isolates (e.g. Batzke et al., 2007; Kobayashi et al., 2008). Endospores formed by certain *Firmicutes* are specifically adapted to respond very quickly to favourable growth conditions, therefore, it can be expected that they easily outgrow any other organisms after inoculation into culture media. This also implies that if numbers of viable spores are high, it will be relatively difficult to isolate slow growing non-spore formers, and one study has suggested that endospores are as abundant as vegetative prokaryotic cells in the deep marine biosphere (Lomstein et al., 2012). Another study has suggested that the number of spores relative to vegetative cells actually increases with sediment depth (Fichtel et al., 2007), hence, further increasing their probability of cultivation. However, it is not known how many of these spores are still viable.

Table 1
Total and maximum viable cell counts of anaerobes in various deep-sea sediments using mineral media containing a variety of different substrates (shaded depths have higher percentage culturability than near surface sediments).

Deep-sea sediment	Total cell count	Viable cell count	% cultivated
Japan Sea, ODP Leg 128			
Surface	2.7×10^8 cm^{-3}	5×10^4 cm^{-3}	0.019
0.8 mbsf	7.9×10^7 cm^{-3}	3.1×10^3 cm^{-3}	0.004
500 mbsf	6.8×10^6 cm^{-3}	1.2×10^4 cm^{-3}	0.18
Blake Ridge, ODP Leg 164			
Surface	2.5×10^9 cm^{-3}	2.8×10^6 cm^{-3}	0.11
1 mbsf	4.4×10^7 cm^{-3}	1×10^6 cm^{-3}	2.27
365 mbsf	3.2×10^6 cm^{-3}	1.2×10^5 cm^{-3}	3.75
Woodlark Basin, ODP Leg 180			
Surface	3.3×10^8 ml^{-1}	9.4×10^4 ml^{-1}	0.028
10 mbsf	1.6×10^7 ml^{-1}	100 ml^{-1}	0.001
366 mbsf	4.2×10^6 ml^{-1}	1.3×10^6 ml^{-1}	30.9
Cascadia Margin, ODP Leg 146			
Surface	3.7×10^8 cm^{-3}	6.3×10^4 cm^{-3}	0.017
10 mbsf	1×10^7 cm^{-3}	8.4×10^3 cm^{-3}	0.084
200 mbsf	1×10^7 cm^{-3}	1.6×10^4 cm^{-3}	0.16
Eastern Mediterranean Sea			
Surface	7.5×10^8 cm^{-3}	2.2×10^7 cm^{-3}	2.9
Sapropel S5, 4 mbsf	6.6×10^8 cm^{-3}	2.2×10^7 cm^{-3}	3.3
Juan de Fuca Ridge			
9 mbsf	6.8×10^7 cm^{-3}	40 cm^{-3}	5×10^{-5}
141 mbsf	3.1×10^7 cm^{-3}	1.1×10^6 cm^{-3}	3.5
260 mbsf	2.9×10^7 cm^{-3}	2.1×10^5 cm^{-3}	0.7
Challenger Mound, IODP Leg 307			
4.3 mbsf	1.5×10^7 cm^{-3}	0	0
77 mbsf	2.2×10^6 cm^{-3}	300 cm^{-3}	0.014
129 mbsf	3.7×10^6 cm^{-3}	2.1×10^4 cm^{-3}	0.57
254 mbsf	1.9×10^6 cm^{-3}	330 cm^{-3}	0.018

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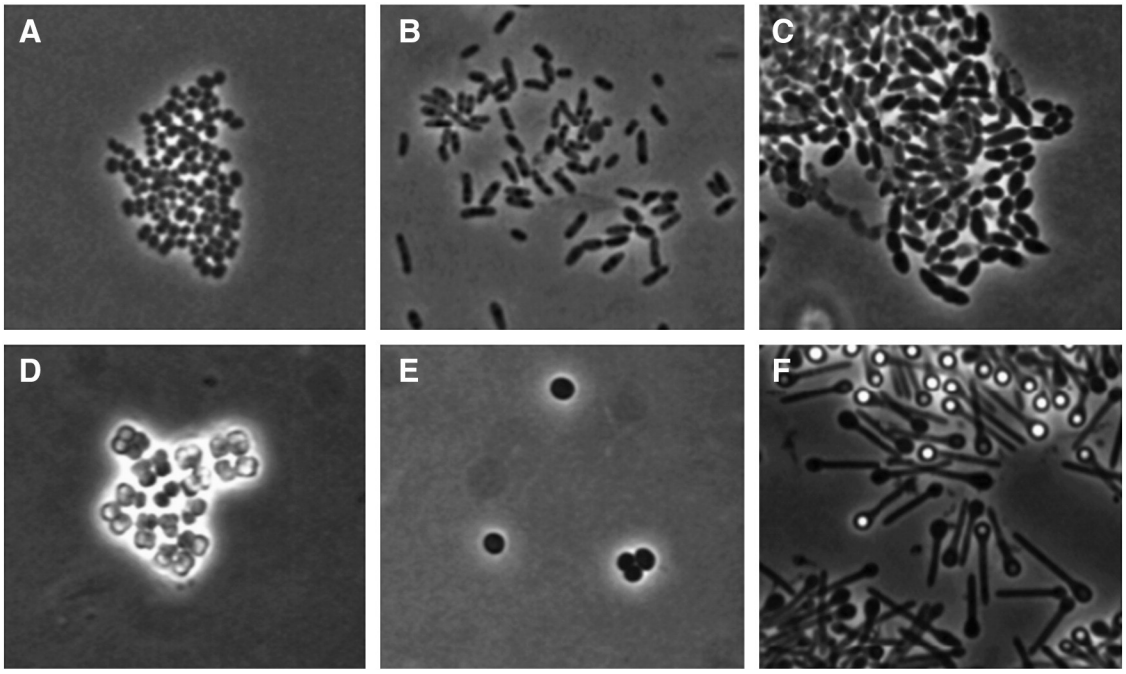

Fig. 8. Bacterial isolates from Eastern Mediterranean sapropels and deep hemipelagic sediments isolated on anoxic media targeting sulphate-reducing bacteria. A: *Alteromonas* sp. S8FS1 isolated with short-chain fatty acids; B: *Halomonas* sp. S7A isolated with lactate; C: *Bacillus* sp. S6BB isolated with thiosulphate and acetate; D: *Micrococcus* sp. Z1A isolated with thiosulphate and acetate; E: *Acinetobacter* sp. Z7TS1 isolated with thiosulphate and acetate; and F: *Clostridium* sp. SO1 isolated with lactate.

Similar to surface sediments, there is a general discrepancy between the dominant prokaryotes determined by molecular biology and pure cultures. Therefore, it has been questioned whether the enrichment and isolation of pure cultures are an effective approach to understand deep biosphere prokaryotes in situ, especially given the considerable time required for cultivation and isolation. Recent technical advances (metagenomics, single-cell genome analysis, and metatranscriptomics) have allowed an increase in knowledge of the genetic capacities of (uncultured) microorganisms. However, some critical physiological characteristics, such as temperature and pressure characteristics, substrate range and preference, are currently only definitively obtained by cultivation. For example, members of the genus *Photobacterium* from Mediterranean surface sediments and sapropels differ with respect to their metabolic capacities, despite belonging to the same genus (Sub et al., 2008). Important sub-seafloor biosphere phylotypes, such as, the *Chloroflexi* and the candidate division JS1 are not unculturable, as they have been enriched from pillow lavas and coastal shallow subsurface sediments (Lysnes et al., 2004; Kopke et al., 2005; Webster et al., 2011). Pure culture isolation may have failed for various reasons, including the lack of effective selective growth conditions, that they are outgrown by faster microorganisms like *Firmicutes* or *Proteobacteria*, or they do not form visible colonies on solid media. Rarefaction analysis of culturable diversity has shown that in Mediterranean and Peru Margin sediments that this diversity is well represented by the isolates obtained, and that it would be highly unlikely that just by conducting more enrichments that additional diversity would be obtained (Sub et al., 2004; Batzke et al., 2007). Hence, new approaches are required, such as: continuous-flow-type bioreactors (Girguis et al., 2003; Imachi et al., 2011); sub-nanomolar H_2 concentration microbial culture apparatus capable of separating syntrophs (Valentine et al., 2000); and enrichment/isolation under elevated pressure, which is a major feature of most sub-seafloor sediments. Recently, the pressure-retaining sampling (HYACINTH) and processing/growth (DeepIsoBug) equipment was used to culture prokaryotes without depressurisation from subsurface gas hydrate sediments (Parkes et al., 2009). Although the range of isolates (*Acetobacterium*, *Carnobacterium*, *Clostridium*, *Marinilactibacillus*, and *Pseudomonas*) obtained was different from other studies using similar media (Sub et al., 2004; Batzke et al., 2007), similar bacteria were obtained under both undepressurised and depressurised conditions. This may have been due to the sediments not being deep enough (total depth 1126–1527 m) to recover obligate piezophiles.

5. Metabolism and activity

In near-surface coastal sediments oxygen is usually rapidly removed due to aerobic respiration and this then facilitates anaerobic respiration which utilises a sequential series of electron acceptors providing decreasing energy yield (NO_3^-, Mn^{4+}, Fe^{3+}, SO_4^{2-}, and CO_2), and the formation of characteristic zones of NH_4^+, Mn^{2+}, Fe^{2+}, S^{2-} (often as metal sulphides) and CH_4 with increasing depth (e.g. Fig. 9, Parkes et al., 2007b). The most highly energy yielding electron acceptor is used before the next electron acceptor in the series, as there is often competition from terminal oxidising prokaryotes for common substrates, thus, there are also sequential changes in prokaryotic groups (Fig. 9). Similar changes occur in many deep, sub-seafloor sediments. For example, often there is a sulphate–methane transition zone (SMTZ), but this is much deeper (e.g. Fig. 4, Site 1229, 151 m water depth, 2–8% organic carbon, SMTZ ~35 mbsf, Parkes et al., 2005) than in high organic matter shallow water sites (e.g. Skagerrak, Denmark SMTZ ~0.7 mbsf, Parkes et al., 2007b). In deeper water sites with low organic carbon, for example, Woodlark Basin in the Pacific Ocean, (1150–2303 m water depth, ~0.4% organic carbon, Wellsbury et al., 2002) sulphate removal is much slower and SMTZs are much deeper, 107 to 199 mbsf at various sites. Active prokaryotic sulphate reduction can be measured using radiotracers (^{35}S–SO_4^{2-}) in deep layers, but activity is low, some 10,000 times lower than near surface rates. Despite this, depth integration of sulphate reduction activity shows that deeper layers, below 20 mbsf, are responsible for a significant amount of the total sediment sulphate reduction activity: between 35 and 72% for Woodlark Basin Sites (Wellsbury et al., 2002). Interestingly, the site with higher rates of sulphate reduction had most sulphate reduction in the top 20 mbsf, due to more rapid sulphate removal and resulting sulphate limitation. For methanogenesis and acetate oxidation, without

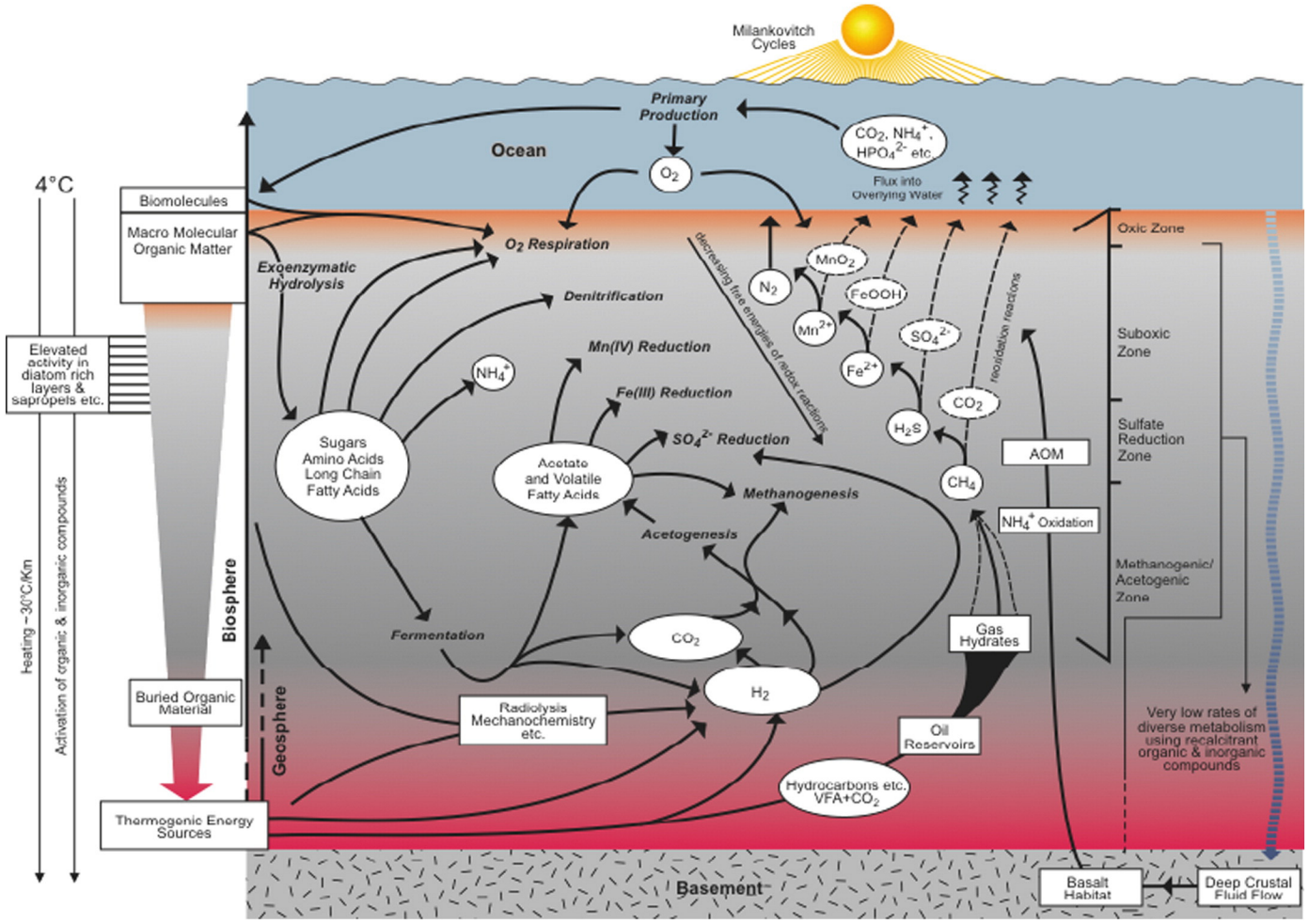

Fig. 9. Schematic deep biosphere diagram.

this restriction, ~90% of activity occurred below 20 mbsf, which correlated with 78% of total prokaryotic cells and 93% of total cell production (Wellsbury et al., 2002). This demonstrates the biogeochemical importance of sub-seafloor sediments.

Substantial methanogenesis from H_2/CO_2, acetate and methanol has been detected in deep sediments (Newberry et al., 2004). However, although $\delta^{13}C$–CH_4 analysis suggests that H_2/CO_2 methanogenesis should be dominant in marine sediments (Whiticar, 1999), this is not always the case, and at some sites acetate methanogenesis dominates at depth (Parkes et al., 2005). However, the situation may be further complicated by the presence of methane production by syntrophic acetate oxidation coupled to hydrogenotrophic methanogenesis in some subsurface environments (e.g. a high-temperature petroleum reservoir, Mayumi et al., 2011). Such syntrophic coupling may also involve direct intercellular electron transport (Rotaru et al., 2014). Acetogenesis may be an additionally important metabolism in sub-seafloor sediments (Lever et al., 2010) and measurable hydrogenase activity (Soffientino et al., 2009) supports the importance of H_2 as a metabolic intermediate and/or a prime substrate (see Section 6). Some novel reactions, such as ethanogenesis, propanogenesis (Hinrichs et al., 2006) and anaerobic sulphate reducing ammonia oxidation (Schrum et al., 2009) may also be occurring in deep sediments.

Although CH_4 concentrations only increase when sulphate is depleted with depth in many sub-seafloor sediments, active methanogenesis is often detected in the presence of sulphate (e.g. Woodlark Basin, Wellsbury et al., 2002 and Peru Margin, Parkes et al., 2005). The absence of CH_4 in these zones likely indicates active anaerobic oxidation of methane (AOM) is occurring. In Izu–Bonin Trench sediments, Western Pacific (Cragg et al., 2003), there is geochemical evidence for simultaneous low levels of sulphate reduction, methanogenesis and manganese reduction over ~80 m intervals. This also occurs at other sites, including re-occurring zones of manganese reduction and other activities at a Pacific Open Ocean site down to ~400 mbsf (D'Hondt et al., 2004; Parkes et al., 2005). This surprisingly shows not only that, reactive manganese oxides can survive in deep sediments over millions of years, but also the existence of co-occurring metabolic processes in these sediments which would be clearly separated within discrete depth zones in active, near-surface sediments. It may be that severe energy limitation means that no one metabolism/prokaryotic population can dominate, and this is probably linked to the recalcitrance of both buried organic and inorganic compounds, plus changes in both their type and quantity during deposition over time. Interestingly, this explanation would be consistent with recognised terminal oxidising prokaryotes often not being detected in molecular genetic surveys at depths where geochemical or other evidence suggests they are active (Parkes et al., 2005; Biddle et al., 2006). Conversely, in deep sediments some of the many common phylotypes with no closely related cultured representatives may be catalysing terminal oxidising and other metabolic activities, and hence, metabolically active deep sediment communities may be different from those in high activity, near-surface marine sediments. For example, it has been suggested that anaerobic oxidation of methane in deep sediments may be conducted by Marine Benthic Group B and the Miscellaneous Crenarchaeotal Group (Sorensen and Teske, 2006), rather than the expected ANME groups, however, they may not assimilate methane-carbon into their cells (Biddle et al., 2006). In contrast, in Newfoundland Margin deep sediments ANME

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sequences were detected and at depths where CH_4 was present (Roussel et al., 2008).

Despite the above, in subtropical gyre sediments, the most oligotrophic regions of the oceans, prokaryotic metabolism is so low that O_2 can penetrate to greater than 30 mbsf and probably for the whole sediment column at some sites (Roy et al., 2012). Consistent with this, total cell numbers are low (D'Hondt et al., 2009), but interestingly the per-cell respiration rate (~10^{-3} fmol/cell/day, Roy et al., 2012) is within the range for cell specific rates of anaerobic respiration in other subsurface sediments. Hence, irrespective of the mode of energy generation, subseafloor prokaryotes seem to survive on similarly small amounts of energy per cell. As the amount of energy used per cell is about 1000 times lower than used in anaerobic cultures (estimated maintenance energy for anaerobic cultures at 25 °C is 3.3×10^{-13} kJ/cell/day, Hoehler and Jorgensen, 2013), and below the lowest known maintenance energy requirements, this indicates that there is a lot to learn about the metabolism of sub-seafloor cells. Aerobic metabolism and/or use of more oxidised electron acceptors in deep sediments may also be possible due to fluids flowing through oceanic basement rock as part of the marine hydrothermal (aquifer) system (Fisher and Becker, 2000). Such electron acceptors could diffuse into the reduced sediments above the basement rock providing a "mirror image" of the cascade of respiratory reactions occurring in near-surface sediments (Fig. 9), including anaerobic oxidation of methane with sulphate (Mather and Parkes, 2000) and ammonium oxidation (D'Hondt et al., 2004).

Heterotrophy, utilising photosynthetically derived organic matter, seems to be the main form of catabolism in sub-seafloor sediments (Biddle et al., 2006). Proteins/amino acids (Lomstein et al., 2012; Lloyd et al., 2013b) and lipid membranes (Takano et al., 2010) are degraded and carbon incorporated into new prokaryotic cells, with cell turnover times of the order of 100–2000 years (Biddle et al., 2006), or longer (Parkes et al., 2000). This is astonishing considering the turnover time of laboratory cultured prokaryotic cells can be less than 20 min. However, organic matter deposited on the surface sediments rapidly forms macromolecules (Parkes et al., 1993, 79% of TOC at ~23 mbsf) which must slow degradation considerably and facilitate sedimentary organic matter to last for millions of years (Parkes et al., 2005; Lomstein et al., 2012). Consistent with this, research suggests that most sub-seafloor microbes are alive (Schippers et al., 2005; Morono et al., 2011; Lloyd et al., 2013a), but are energy, rather than C or N limited (Morono et al., 2011). Dead prokaryotic cells (necromass) may add to biomolecules available for growth of sub-seafloor cells (Lomstein et al., 2012) and/or add to the uncharacterised organic matter (Parkes et al., 1993). Calculating prokaryotic necromass from the global decrease in cells with increasing depth (Fig. 10), shows that most necromass is produced in the top 10 cm of sediment and that below 1 m there is very little further increase in dead cells. Thus, the number of dead cells decreases rapidly with depth, such that at 1 cm they are 368% of live cells and are very rapidly recycled, but by 100 m they are only 0.007% of live cells, which gives a limited energy and carbon supply. That is unless near-surface necromass is still, or becomes, bioavailable at depth.

6. Biosphere:geosphere interactions

The energy available to fuel the large biomass within sub-seafloor sediments is quite limited (e.g. Hoehler and Jorgensen, 2013), when considering the often low supply of photosynthetically derived organic matter, which has been highly degraded in both the water column and near-surface sediments. Hence, it is surprising that energy can be obtained by significant populations of prokaryotes in kilometre deep sediments (Roussel et al., 2008; Ciobanu et al., 2014) with ~100 My old organic matter (Roussel et al., 2008). However, accumulation over geological time scales has resulted in marine sediments being the largest global reservoir of organic carbon (Hedges and Keil, 1995). In addition, sediment slurry heating experiments demonstrate that warming of sediments during burial can activate both buried organic and inorganic compounds (Parkes et al., 2011), thus slowly and continuously supplying energy sources for subsurface prokaryotes. For example in Nankai Trough, below ~300 mbsf, there is a broad match between modelled increasing organic matter reactivity and rates of prokaryotic activity (Horsfield et al., 2006), plus there is H_2 and acetate formation at depth and relative increases in prokaryotic cell numbers (Fig. 11). Both H_2 and acetate can be products of thermal activation/maturation and aromatisation of buried organic matter which may be facilitated by prokaryotic activity below ~120 °C (Wellsbury et al., 1997; Parkes et al., 2007a). A similar situation occurred at Newfoundland Margin sediments, but here methane increases were associated with the occurrence of ANME sequences and presumably utilisation of CH_4 as a deep energy source (Fig. 9). However, at higher temperatures (60–100 °C),

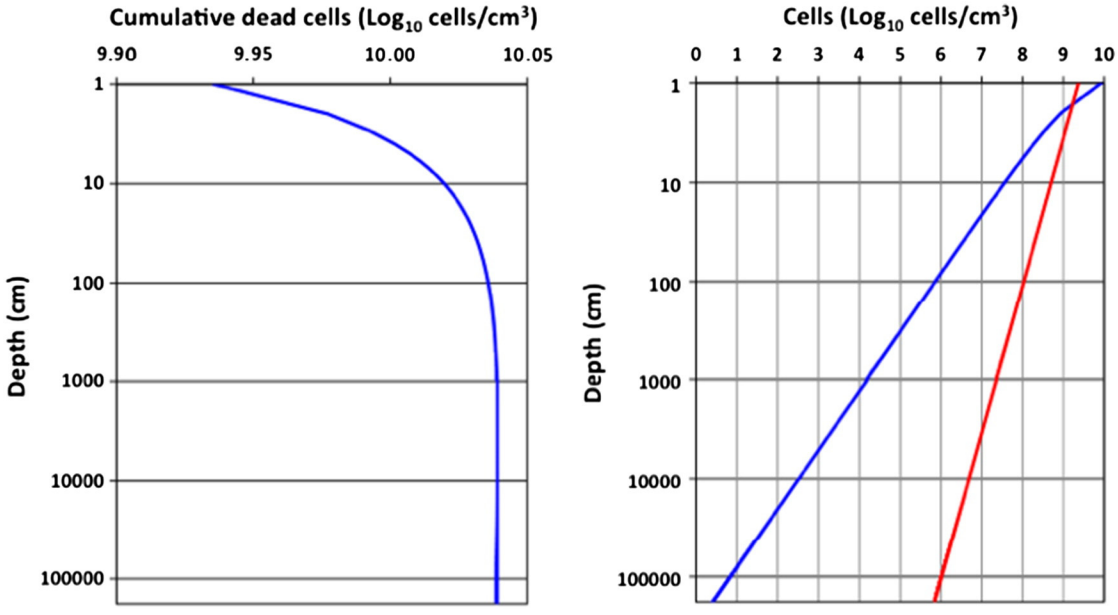

Fig. 10. Prokaryotic necromass cell distributions in sub-seafloor sediments calculated from the global decrease in cells with depth (Fig. 2), plus estimate of live (red) and dead cell (blue) concentrations.

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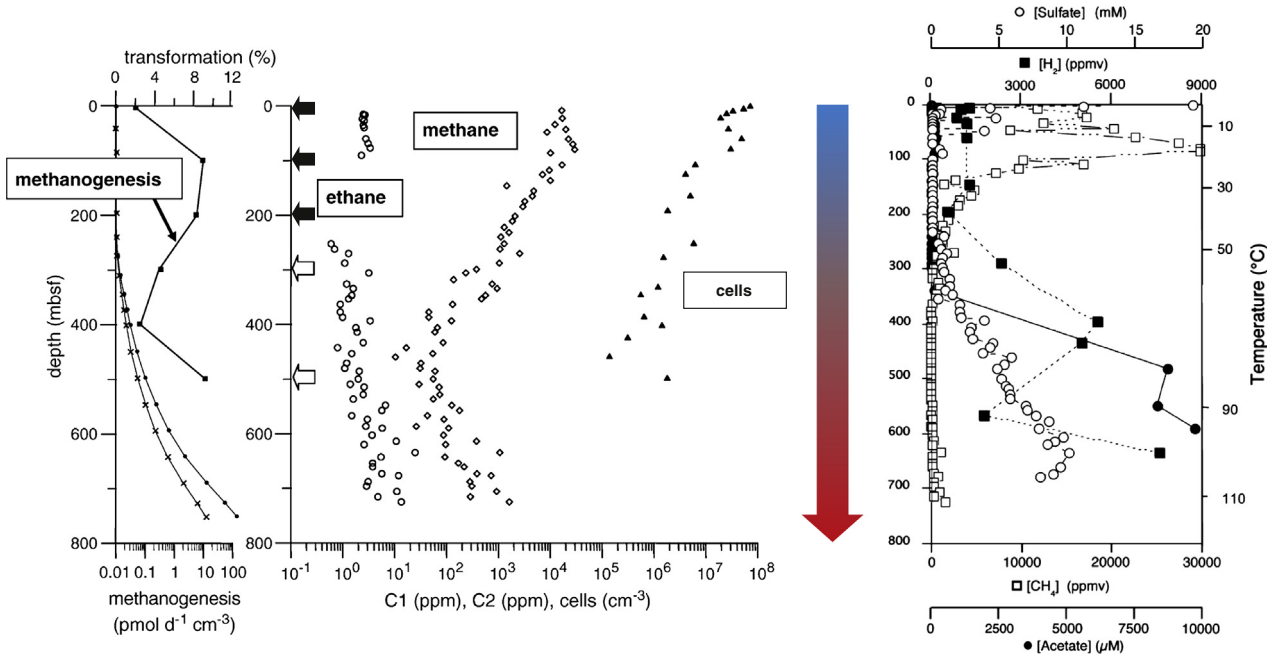

Fig. 11. Nankai Trough Site 1173 geomicrobiology and biogeochemistry summary. Left panel: Generation curves from kinetic modelling and experimentally determined rates of potential methanogenesis. Middle panel: gas concentrations in ppm (Moore et al., 2001) — methane (diamonds) and ethane (circles), and total cell counts in $\log_{10}$ cm^{-3} (triangles), light arrows mark depths where intact phospholipids (PL) were detected (Zink et al., 2003) and dark arrows the depths where amplifiable DNA was obtained (Newberry et al., 2004). Right panel: Sediment geochemistry against depth and temperature (Parkes et al., 2007a). Gas concentrations in ppmv of hydrogen (filled squares) and methane (open squares), and pore water concentrations of sulphate (mmol/L, open circles) and acetate (μmol/L, closed circles).

archaeal thermophiles/hyperthermophiles dominated in one of the deepest (1626 mbsf) and oldest (111 My) sub-seafloor sediments that prokaryotic cells have currently been detected (Roussel et al., 2008). These prokaryotes might be utilising thermogenic higher hydrocarbons diffusing from below. A similar situation occurs in many oil reservoirs, where there are significant prokaryotic populations (e.g. 10^4–10^5 bacterial cells/g,

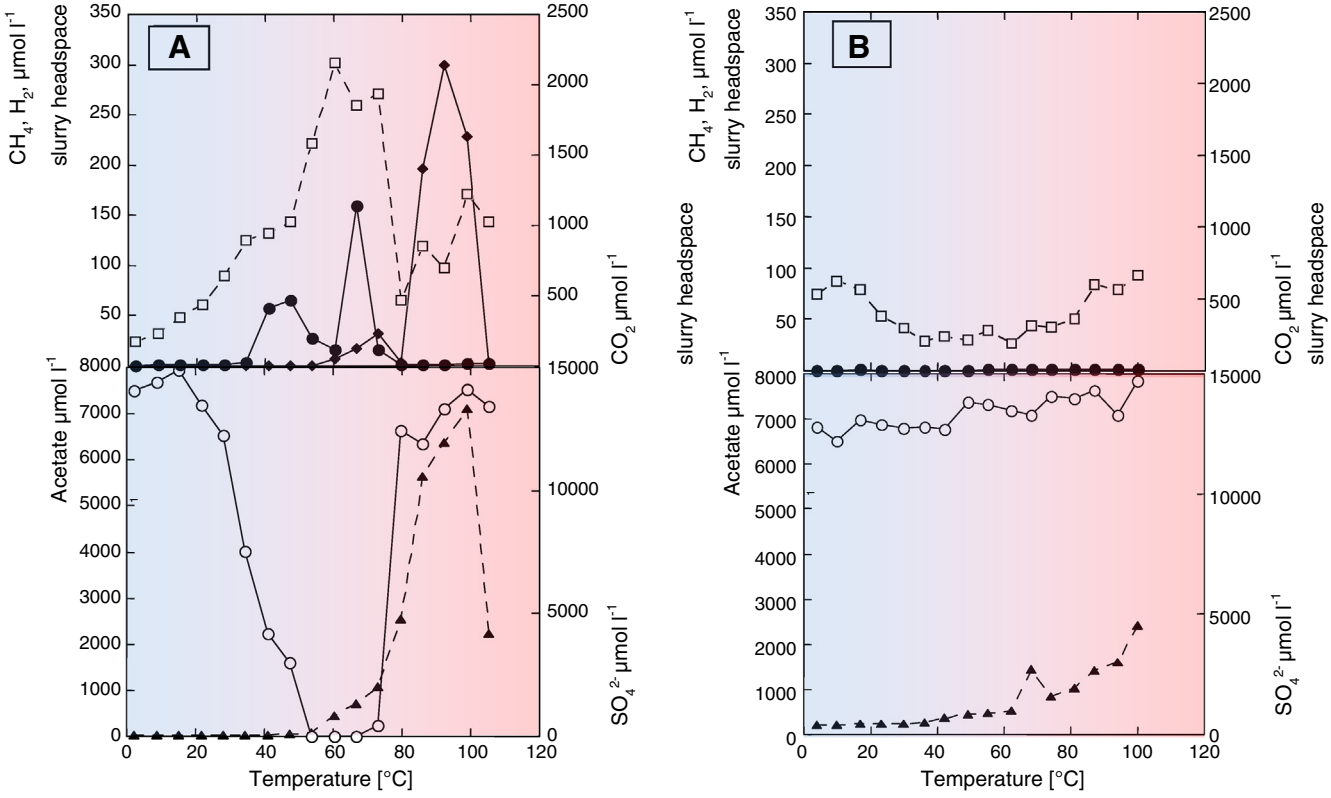

Fig. 12. Headspace gas and sulphate and acetate changes in A. Basalt amended sediment slurries heated in a Thermal Gradient (83 days) compared to sterile controls (130 days) B. □ CO_2, ● CH_4, ◆ H_2, ○ sulphate, ▲ acetate (Parkes et al., 2011).

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and at geochemical interfaces elevated bacterial concentrations of 10^6–10^7 cells/g) and in-reservoir petroleum biodegradation, (Bennett et al., 2013). There may be an even closer interaction between deep biogenic and thermogenic processes, as sediment slurry sequential heating experiments (Fig. 12, Parkes et al., 2011) demonstrate that previous biogenic alteration of organic matter greatly stimulates hydrocarbon production at thermogenic temperatures. Also in these experiments at thermogenic temperatures, SO_4^{2-} concentrations were shown to increase. This situation provides the potential for stimulation of hydrocarbon degradation via sulphate reduction (Stetter et al., 1993; Rueter et al., 1994), in addition to methanogenesis in deep oil reservoirs (Jones et al., 2008) and sediments (Newberry et al., 2004). Stable isotopic changes in SO_4^{2-} in deep sediments (Bottrell et al., 2000) demonstrates that sulphide oxidation is also occurring in situ and this may be due to metal oxides stored within the mineral fraction of marine sediments (Bottrell et al., 2008; Holmkvist et al., 2011). Thus, there can be production of both substrates and electron acceptors in some subsurface environments, which enables continuing energy generation for prokaryotic activity, albeit usually at very low rates. Importantly, in the sediment heating experiments previously described, prokaryotic populations of both *Bacteria* and *Archaea*, representative of many deep sediment types developed (Parkes et al., 2011), including archaeal thermophiles at high temperatures (65–90 °C), which can dominate deep hot sediments. This suggests that the sediment slurry heating experiments are reasonable models for reactions in deep, sub-seafloor sediments.

The energy available to sub-seafloor prokaryotes, however, might not be restricted to the utilisation of organic matter ultimately derived from photosynthesis, but inorganic, "dark energy", from a wide variety of geological sources could also provide fuel for deep microbial communities (e.g. Pedersen, 2000; Orcutt et al., 2011). This is exemplified by the anaerobic Subsurface Lithoautotrophic Microbial Ecosystems (SLIMEs, Stevens and McKinley, 1995) where weathering and oxidation of ferrous-bearing silicates (such as olivine and pyroxene) in basalts were thought to drive the formation of H_2 from water, along with the precipitation of magnetite (Fe_3O_4) and other secondary phases. This H_2 could then be used by chemolithoautotrophic prokaryotic communities, making them independent of photosynthesis. However, further experimental studies questioned the feasibility of such a process being important in situ in basaltic aquifers (Anderson et al., 1998), as H_2 was not produced from basalt at an environmentally relevant, alkaline pH, and even at a lower pH only small, transitory amounts of H_2 were produced. In addition, geochemical considerations suggested that previously reported rates of H_2 production could not be sustained over geologically significant time frames. Despite this, H_2 based prokaryotic communities have been documented in other environments, such as terrestrial hot springs (Chapelle et al., 2002; Brazelton et al., 2013) and hydrothermal systems (Takai et al., 2004; Kelley et al., 2005). Also, more recent experiments have demonstrated H_2 formation from these serpentinisation type reactions at temperatures as low as 55 °C (Mayhew et al., 2013), which is prevalent at relatively shallow sediment depths (with an average thermal gradient of ~30 °C/km this equates to ~1.8 km depths) and is well within the temperature range of prokaryotes (up to ~122 °C, Takai et al., 2008). This overlap provides the prospect that prokaryotes might also facilitate, in addition to benefiting from, these oxidation/weathering reactions (Parkes et al., 2011; Mayhew et al., 2013), as they do in lower temperature near-surface environments (e.g. Montross et al., 2012). In addition, reactions generating H_2 from basaltic minerals could possibly fuel microbial communities across the sediment/basement interface in deep sediments. Pyrite formation from products of prokaryotic sulphate reduction (H_2S and FeS) also produces H_2 under anoxic conditions (Drobner et al., 1990), however, it remains unclear if the rate of the reaction under in situ environmental conditions would be sufficient to sustain chemolithoautotrophic prokaryotic communities (Rickard, 1997).

The Earth is tectonically active, so deep sediments and rocks are constantly subjected to stress, fracturing and faulting, and this is considered to be of global significance for the deep biosphere in providing a conduit for deep energy sources to reach subsurface prokaryotic communities (Sleep and Zoback, 2007). However, this activity also produces reactive surfaces and this results in H_2 formation, for example, during earthquakes (Kita et al., 1982) and crushing rocks (Freund et al., 2002), with H_2 production increasing with temperature (to ~200 °C, Kita et al., 1982). Recently, it has been shown that addition of a range of ground, common rocks (both iron and non-iron containing) to sediment slurries at a range of temperatures (0–100 °C) stimulates both H_2 production and prokaryotic activity, which increased with incubation time (83 days for basalt) and temperature (Fig. 12, Parkes et al., 2011). In contrast, sterile controls showed negligible H_2 formation and other geochemical changes, even at temperatures as high as 100 °C (e.g. basalt incubation for 130 days). These results are, therefore, in agreement with previous experiments showing limited H_2 formation from basalt under sterile conditions (Anderson et al., 1998), but they also demonstrate that in the presence of prokaryotes there is considerable H_2 formation, which significantly stimulates prokaryotic activity. The ubiquitous presence of prokaryotes in deep, subsurface environments (Whitman et al., 1998) coupled with H_2 formation from a range of common minerals (Parkes et al., 2011), suggests that mineral H_2 formation may be an environmentally significant process. The mechanism of this H_2 formation, however, has to be different from the ferrous oxidation originally proposed for the SLIMEs (Stevens and McKinley, 1995). It has been suggested that mechanochemistry could be the mechanism involved in general mineral H_2 formation (Parkes et al., 2011), whereby free radical reactions on fresh mineral surfaces result in water hydrolysis and H_2 formation (Kita et al., 1982; Saruwatari et al., 2004). This explains H_2 production during earthquakes (Ito et al., 1998), however in addition, even stressed rocks produce H_2 by a similar mechanism (Balk et al., 2009). As the Earth's crust is critically stressed so that it is near frictional failure down to at least 8 km which results in reoccurring faults and fractures, plus there is: a) subduction stresses and faulting affecting most of the world's convergent margins (von Huene and Ranero, 2003); b) friction and shearing due to landslides; and c) turbidite flows and glacial abrasion etc.; there is great potential for reactive mineral surface formation and H_2 production in the subsurface.

Mechanochemical geosphere H_2 formation would be independent of surface photosynthetic activity, and potentially also to would be the prokaryotes that use this H_2. However, if the electron acceptors used to obtain energy from this H_2, actually are derived directly or indirectly from photosynthesis, then this would both limit deep biosphere prokaryotes and make them still dependent on photosynthesis. For example, oxidised fluids flowing through oceanic basement providing oxidants to the sediment above and the rock basement (Mather and Parkes, 1999; D'Hondt et al., 2004). However, CO_2 has geological sources, is relatively ubiquitous in the subsurface and can be utilized as an electron acceptor for autotrophic H_2 ultilisation, such as hydrogenotrophic methanogenesis and acetogenesis, and potentially ethanogenesis and propanogenesis (Hinrichs et al., 2006), producing at the same time compounds that are common in many deep, subsurface environments. For example, biogenic CH_4 has been shown to be associated with active earthquake periods in a granite-enclosed aquifer (Brauer et al., 2005). In addition, mineral derived H_2 from water hydrolysis will also produce O_2 (Kita et al., 1982) or related compounds (e.g. H_2O_2, Balk et al., 2009), which could be used directly as an electron acceptor by prokaryotes, or could oxidise reduced inorganic compounds in sediments forming other electron acceptors, such as NO_3^-, SO_4^{2-} and S°. Interestingly, in this context, in sediment slurry incubation experiments with added minerals (Parkes et al., 2011) CH_4 concentration decreased and CO_2 concentrations increased compared to sediment slurries without minerals. This could be explained by the oxidised products of water hydrolysis being used for oxidation reactions/respiration

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by prokaryotes. A direct analogue for this is the radiolytic decay of pyrite in a deep fracture (3–4 km) in the Mponeng gold mine South Africa (Lin et al., 2006), which produces both H_2 and SO_4^{2-} and could enable continuing prokaryotic sulphate reduction over millions of years with no apparent reliance on photosynthetically derived compounds. Radiolysis of water in deep marine sediments by natural radioactive elements (e.g. K, Th and U) will also occur and it has been estimated that radiolytic production of H_2 might fuel up to 10% of the estimated respiration in the deep sediments of a Peru Basin site (4827 m water depth) and might be even more important in sediments with even lower rates of organic-fueled respiration (Blair et al., 2007).

In a final twist, abiotic synthesis of hydrocarbons may occur in the presence of ultramafic rocks, water and moderate amounts of heat via the Fischer–Tropsch Type reaction involving H_2 (Proskurowski et al., 2008), thus any deep prokaryotes utilising these hydrocarbons would be heterotrophs utilising abiotically produced organic matter: one of the scenarios for the origin of life!

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.margeo.2014.02.009.

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