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Life under extreme energy limitation: a synthesis of laboratory- and field-based investigations

Mark A. Lever[1,*], Karyn L. Rogers[2], Karen G. Lloyd[3], Jörg Overmann[4], Bernhard Schink[5], Rudolf K. Thauer[6], Tori M. Hoehler[7] and Bo Barker Jørgensen[1]

[1]Center for Geomicrobiology, Institute of Bioscience, Aarhus University, Ny Munkegade 114, 8000 Aarhus C, Denmark, [2]Rensselaer Polytechnic Institute, Earth and Environmental Sciences, Jonsson-Rowland Science Center, 1W19, 110 8th Street, Troy, NY 12180, USA, [3]Department of Microbiology, University of Tennessee at Knoxville, M409 Walters Life Sciences, Knoxville, TN 37996-0845, USA, [4]Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Inhoffenstraße 7B, D-38124 Braunschweig, Germany, [5]Microbial Ecology, Department of Biology, University of Konstanz, P.O. Box 55 60, D-78457 Konstanz, Germany, [6]Max Planck Institut für terrestrische Mikrobiologie, Karl-von-Frisch-Straße, D-35043 Marburg, Germany and [7]NASA Ames Research Center, Mail Stop 239-4, Moffett Field, CA 94035-1000, USA

*__Corresponding author:__ ETH Zürich, Department of Environmental Systems Sciences, Institute of Biogeochemistry and Pollutant Dynamics, Universitätsstrasse 16, CH-8092 Zürich, Switzerland. Tel: (+41) 044-251-31-82; Fax: (+41) 44-633-11-22; E-mail: mark.lever@usys.ethz.ch
One sentence summary: This is a synthesis of laboratory- and field-based investigations on microbial life under energy limitation.

ABSTRACT

The ability of microorganisms to withstand long periods with extremely low energy input has gained increasing scientific attention in recent years. Starvation experiments in the laboratory have shown that a phylogenetically wide range of microorganisms evolve fitness-enhancing genetic traits within weeks of incubation under low-energy stress. Studies on natural environments that are cut off from new energy supplies over geologic time scales, such as deeply buried sediments, suggest that similar adaptations might mediate survival under energy limitation in the environment. Yet, the extent to which laboratory-based evidence of starvation survival in pure or mixed cultures can be extrapolated to sustained microbial ecosystems in nature remains unclear. In this review, we discuss past investigations on microbial energy requirements and adaptations to energy limitation, identify gaps in our current knowledge, and outline possible future foci of research on life under extreme energy limitation.

Keywords: energy limitation; microbial life; laboratory; starvation; subseafloor; adaptation

INTRODUCTION

Over the past two decades, our notions of microbial adaptation to energy limitation and persistence under low-energy conditions have expanded dramatically. Laboratory incubations have shown that bacterial populations in cultures deprived of fresh energy supplies maintain nearly stable populations and actively turn over biomass for months to years (Zambrano *et al.* 1993; Finkel and Kolter 1999). Cell counts and measurements of geochemical gradients in deeply buried marine sediments have revealed a vast, metabolically active biosphere that has been cut

off from new detrital energy inputs for thousands to millions of years (Parkes *et al*. 1994; Whitman, Coleman and Wiebe 1998; D'Hondt *et al*. 2004; Lomstein *et al*. 2012). The insights gained and extrapolations made from these studies suggest that energy limitation is the prevailing physiological state among microorganisms on Earth (Morita 1997). Yet, little is known about how microbial communities live under and adapt to long-term subsistence at very low energy turnover. To address this, the strategies of microbes to cope with energy limitation, the theoretical energy minima required by microbial cells to live and the energy sources available to microbes in the environment need to be better understood.

Here we review published data on pure cultures and natural communities in the context of energy availability, assess the state of knowledge and identify gaps in our understanding of microbial energy requirements, and name priorities for future research, including methodological and technological developments. The knowledge reviewed and approaches suggested draw from a wide range of disciplines, including biochemistry, geochemistry, geology, molecular biology, microbiology, ecology, biotechnology and nanotechnology.

DEFINITION OF ENERGY LIMITATION

Even though the two terms are often used interchangeably in the microbiological literature, we differentiate between 'energy limitation' and 'nutrient limitation'.

'Energy limitation' describes the limitation of energy production (catabolism) of an organism by the total flux of energy available to the organism. Energy sources include light (photons) for phototrophic organisms, and exergonic reactions involving chemical compounds for chemotrophic organisms. Chemotrophic organisms fall into chemolithotrophs, which obtain energy from oxidation of inorganic compounds (e.g. hydrogen, iron(II), sulfide, ammonium), and chemoorganotrophs, which obtain energy from oxidation of organic compounds (e.g. fatty acids, alcohols, alkanes, monomeric and oligomeric constituents of macromolecules, such as carbohydrates or proteins). Energy availability is possibly the main factor limiting growth of chemotrophic microorganisms on Earth (Morita 1997; Hoehler 2004).

By contrast, 'nutrient limitation' describes the limitation of biomass synthesis (anabolism) by the availability of an essential element or compound. Growth is neither limited by energy nor the total pool of nutrients but by the availability of one or a few essential element(s) or compound(s). Nutrient limitation often controls biomass production in phototrophic organisms in surface habitats of the Earth where light is abundant. Cases are also known where nutrient availability limits growth in chemotrophic organisms, such as heterotrophic bacterioplankton or microbes in deep aquifers (Le, Wehr and Campbell 1994; Kieft *et al*. 2005). Common limiting nutrients in terrestrial, freshwater and marine ecosystems are nitrogen, phosphorus and iron (e.g. Martin and Fitzwater 1988; Vitousek and Howarth 1991; Le, Wehr and Campbell 1994; Lever and Valiela 2005; Elser *et al*. 2007).

The distinction between energy and nutrient limitation is fundamental to understanding how organisms respond differently to resource limitation in nature. Yet, distinctions are not always obvious. For instance, Fe(III), CO_2, nitrate or acetate are energy sources to some microbes in their role as electron acceptors or electron donors. However, they also serve as nutrients, with Fe, C and N being essential elements, and acetate being required as a precursor for biomass synthesis by many microbes. As a consequence, microbes can be simultaneously energy and nutrient limited. Moreover, certain microbial guilds in a given habitat may be energy limited, while others are nutrient-limited, e.g. organoheterotrophic compared to photoautotrophic bacterioplankton in many aquatic systems. Furthermore, nutrient and energy limitation are often linked, e.g. the commonly limiting nutrient nitrogen is a necessary component of enzymes, which are in turn required for energy conservation. Nonetheless, effects of energy and nutrient limitation differ fundamentally on a biochemical and physiological level and are often experimentally distinguishable. Thus, the distinction between energy and nutrient limitation is not only theoretically relevant.

Since it is the aim of this review to discuss energy limitation, all following sections focus on responses and coping mechanisms of microbes to energy limitation. Nutrient limitation is discussed only where comparisons to energy limitation are deemed important and interesting. Moreover, though light limitation is an important and widespread form of energy limitation in nature, we here—except where noted—present the larger body of literature on chemical energy limitation. Future comparisons will provide fascinating insights to the strategies employed by microorganisms to cope with these very different forms of energy limitation.

MORPHOLOGICAL AND BEHAVIORAL CHANGES DURING ENERGY LIMITATION

Morphological and behavioral responses of microbial cells to energy limitation have been studied for decades. Commonly observed responses include changes in cell size, cell shape, cell motility and cell adhesion, and will be discussed in the following sections.

Cell size and volume

The accurate assessment of the size and volume of microbial cells is challenging. Different methods of fixation, staining and measurement result in divergent estimates of both cell size and cell volume (reviewed in Bölter *et al*. 2002; Romanova and Sazhin 2010). The same is true for indirect estimation methods, e.g. based on cell C content, which are at least in part due to variations in intracellular compositions between microbial species, or microbes of the same species that have been grown under different energy, nutrient and redox conditions (also see section 'Cell compositional indicators of energy limitation'). Despite these methodological challenges, distinct patterns of cell size can be seen in relation to energy availability.

Most cells respond to experimental energy limitation in the laboratory by reducing cell size and cell volume within days to weeks (Fig. A1, Supporting Information). These reductions in cell size and volume (miniaturizations) can result either from shrinking of individual cells (Novitsky and Morita 1976; Kieft *et al*. 1997) or from cell division without growth (fragmentation), which results in increased cell numbers (Amy and Morita 1983; Amy *et al*. 1993). In environmental samples, large numbers of small cells that pass through 0.2-μm filters are common and have been called 'ultramicrobacteria' (Torrella and Morita 1981) or 'minibacteria' (Watson *et al*. 1977). Many of these cells appear to be starved forms of microbes that grow to significantly larger size under energy-rich conditions (Haller *et al*. 1999; Lysak *et al*. 2010). A classic example is *Sphingopyxis alaskensis* strain RB2256, formerly regarded as a model strain of an ultramicrobacterium with an obligately oligotrophic life style (Schut, Prins and

Gottschal 1997; Cavicchioli *et al.* 2003), which was later shown to increase in cell size when grown in suitable energy-rich media (Vancanneyt *et al.* 2001). Not all ultramicrobacteria increase in size when provided with abundant energy, however, as the ubiquitous oceanic Alphaproteobacterium Candidatus *Pelagibacter ubique* and the Thaumarchaeote *Nitrosopumilus maritimus* suggest (Cho and Giovannoni 2004; Könneke *et al.* 2005).

To provide an overview of the organisms and habitats in which responses to energy limitation have been documented, we compiled published data on cell size, as indicated by cell volumes, from heterotrophic pure culture isolates and heterotrophic communities in environmental samples (Fig. 1; Table A1, Supporting Information). Despite the above-mentioned uncertainties associated with accurate determinations of cell volume, an average reduction in cell volume of approximately one order of magnitude between growing pure cultures and pure cultures that have been starved for 28 days is apparent from comparing data on the same organism in growth, stationary and starvation phase (Fig. 1A). This trend holds when comparing cell volume changes documented by the same method across 10 bacterial species from freshwater and marine environments. Here cell volumes after 28 days of starvation were reduced by a factor of 2–8 compared to their initial well-nourished state (Fig. A2A, Supporting Information; Troussellier *et al.* 1997). Moreover, though differences in cell volume between pure cultures in growth and stationary phase are not apparent when comparing all cell volume data (Fig. 1A; black versus red), 3–5-fold reductions in cell volume during stationary phase compared to growth phase are evident when comparing data from the same studies (Fig. 1A, Fig. A2B, Supporting Information; *Escherichia coli* II from Norland, Fagerbakke and Heldal 1995, *E. coli* III and *Vibrio natriegens* II from Fagerbakke, Heldal and Norland 1996). Significant reductions in cell volume can even occur within less than 1 day of energy limitation (Kjelleberg and Hermansson 1984).

When examining heterotrophic microbial communities from terrestrial soils to freshwater habitats and marine systems, it is apparent that cell volumes often vary by one order of magnitude or more within the same samples or habitats (Fig. 1B). As a result, freshwater lakes and nutrient-rich coastal waters overlap in cell volume distributions with microbial communities from oligotrophic open ocean water columns. Nonetheless, average cell volume values from open ocean water columns tend to be the lowest, as would be expected if these ocean regions had higher percentages of energy-limited cells. Thus, the existing data on microbial cell sizes in the environment, despite having been obtained by methods that differ substantially in cell volume estimates, are consistent with the overall notion of cell size reduction under energy limitation.

Cell morphology

The example of cell morphology changes illustrates the fundamental difference in how organisms respond to energy compared to nutrient limitation.

Some nutrient-limited microorganisms increase their cell volume (Kjelleberg *et al.* 1993), even compared to exponentially growing cultures (Hütz, Schubert and Overmann 2011). Others increase the cell surface area to volume (S:V) ratio without decreasing the cell volume, e.g. by adopting a more elongated or rod-shaped morphology (Kjelleberg *et al.* 1993), or by growth of specialized appendages (Poindexter 1981). The *Caulobacter* genus within the Alphaproteobacteria is a classic example of an aquatic organism that responds to nutrient limitation by increasing the S:V ratio. *Caulobacter* are found in aquatic ecosystems where P is the limiting nutrient, such as many freshwater ecosystems (Paerl 1982). Members of this genus grow a stalk (prostheca) that consists entirely of cell membranes (Poindexter and Cohen-Bazire 1964), and is able to take up organic phosphate compounds (Wagner *et al.* 2006). Under P limitation, this stalk increases to 5–40 times the length of the cell, whereas under phosphate-replete conditions stalk length typically does not exceed cell length (Poindexter 1981; Gonin *et al.* 2000).

Unlike cells experiencing nutrient limitation, energy-starved cells often decrease their cell size substantially and change from rod-shaped to coccoid morphology (Novitsky and Morita 1976; Kjelleberg *et al.* 1993; Fida *et al.* 2013). Reductions in cell size lower the total surface area, whereas shifts from rod to coccoid shape lead to lower S:V ratios. These morphological shifts are thus opposite to ones that confer improved fitness under nutrient limitation. Members of the *Arthrobacter*, a genus in the Actinobacteria that is widespread in soils and can withstand energy limitation for long time periods (Ensign 1970), are excellent examples of these morphological shifts. The average cell volume of *Arthrobacter globiformis* decreases from 0.45–0.60 μm^3 to 0.16–0.24 μm^3 in chemostats in response to 20% reductions in dilution rates (Luscombe and Gray 1974); shifts from rod-shaped to coccoid morphology have been observed in *Arthrobacter* spp. during transitions from exponential to stationary phase (Poindexter 1981). Similarly, nine out of ten bacterial isolates from marine and freshwater habitats express reductions in length: width ratios after a 28-day starvation period (Fig. 2, Table A2, Supporting Information; Troussellier *et al.* 1997). These trends from pure cultures are consistent with cell proportions in energy-depleted subseafloor sediments; here, ~43% of cells were found to be spherical and the remaining cells mostly short rods (Kallmeyer *et al.* 2012). Over 70% of cells were very small, ranging between 0.2–0.5 μm in width and 0.2–1.2 μm in length (Kallmeyer *et al.* 2012).

The extent of the cell size and shape change under starvation can vary drastically between and within species. At one side of the spectrum is what we term the 'copiotrophic starvation response' (CSR), which is characterized by reductions in cell size, often but not always involving reductive cell division, and shift from rod to spherical shape. As the name suggests, the CSR is used by microbes that can grow well under high-energy conditions, but can also endure long periods of starvation. Examples are the pathogenic *V. cholerae* (Hood *et al.* 1986) and the marine water column *Vibrio* species strain Ant-300 (Novitsky and Morita 1976). At the opposite side of the spectrum is what we term the 'oligotrophic starvation response' (OSR), which occurs in microbes that are adapted to and able to grow under oligotrophic conditions. Cells are typically small and (near-) spherical independent of energy levels, and undergo lesser, if any, reductions in size between conditions of growth and energy limitation. Examples are coryneform bacteria, including several *Arthrobacter* spp. (Boylen and Ensign 1970; Scherer and Boylen 1977), and certain chemolithoautotrophs (Johnstone and Jones 1988). Many microbes fall on a continuum between the CSR and OSR, while others only deviate in one or few traits from the endmember response. An example of the latter is the rod-shaped—not coccoid—*Candidatus P. ubique*, which apart from its rod-like shape is a textbook example of an organism with an OSR (Rappé *et al.* 2002). Differences between these two responses are not restricted to morphological features, but also apparent at the intracellular level (see section 'Cell compositional indicators of energy limitation').

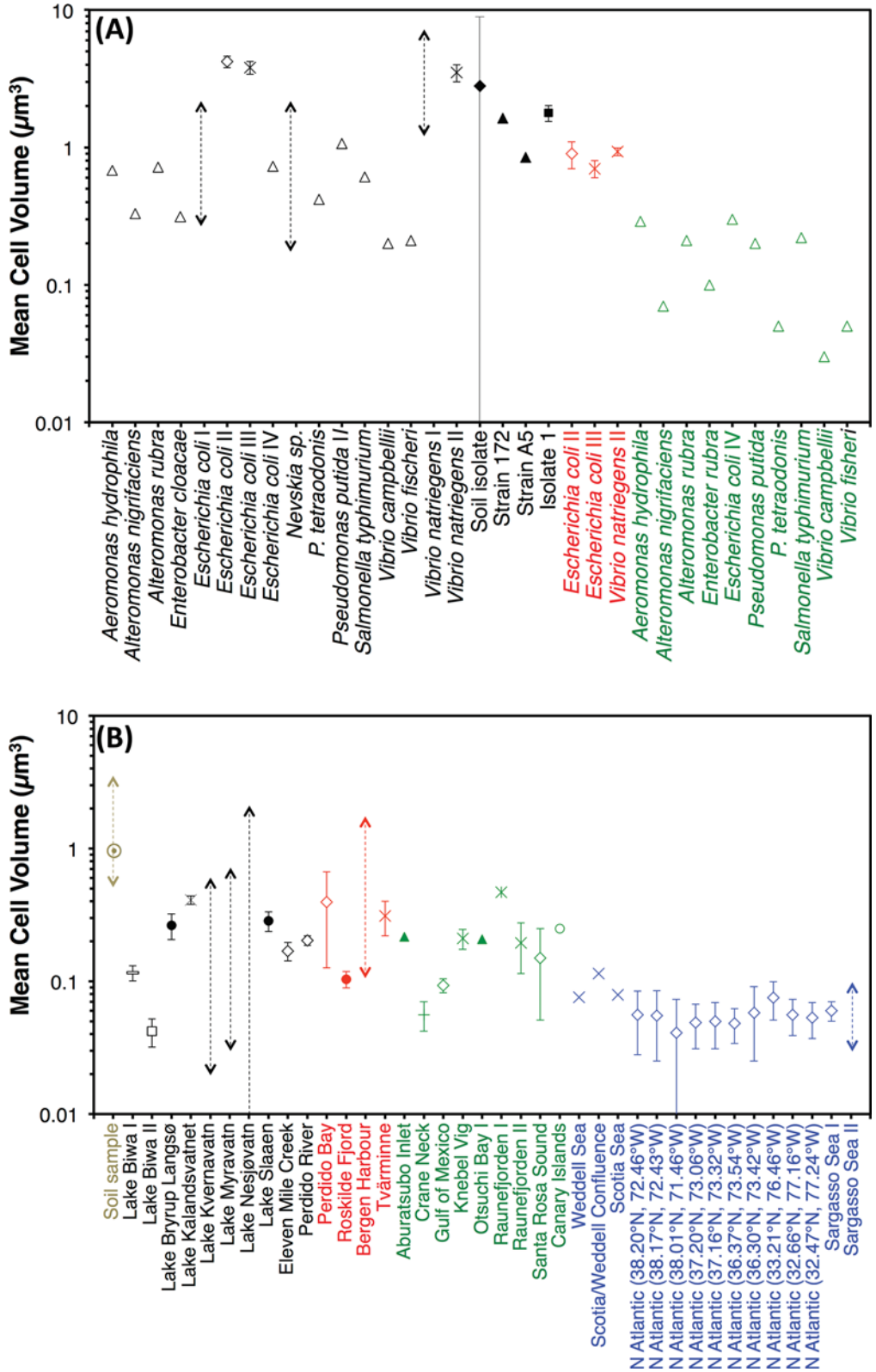

Figure 1. (A) Mean cell volumes of pure cultures during growth phase (black), stationary phase (red) and after starvation for 28 days (green). (B) Mean cell volumes in environmental samples, i.e. terrestrial soil (brown), freshwater lakes and rivers (black), estuarine and brackish waters (red), coastal and nearshore marine waters (green), and pelagic marine waters (blue). Use of the same symbols on different data points indicates that these data points are from the same publication (e.g. all empty triangles in A are from Troussellier *et al.* (1997), whereas all empty diamonds in B are from Børsheim 1990; however, note that solid symbols indicate different studies than empty symbols). Error bars indicate standard deviations. Dashed lines with arrows indicate data ranges and are shown for studies where mean cell volumes were not shown or could not be calculated based on the data provided. More detailed information on the data shown can be found in Table A1 (Supporting Information).

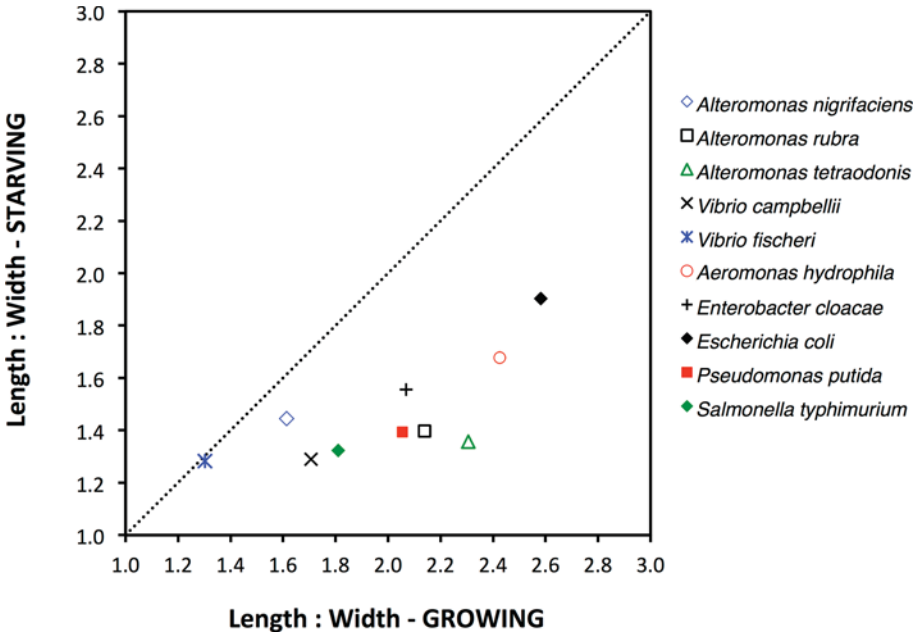

Figure 2. Comparison of length to width ratios of 10 pure culture isolates during growth phase (x-axis) and after starvation for 28 days (y-axis). The dotted line indicates a 1:1 ratio, thus indicating no difference in length to width ratio during growth phase and after starvation. Except *V. fisheri*, all pure cultures had lower length:width ratios after starvation. Along with decreases in cell volumes (Fig. A2A, Supporting Information), these reductions in cell length:width ratios are consistent with the trend toward reduced cell surface area under energy limitation (all data from Troussellier *et al.* 1997, also see Table A2, Supporting Information).

Cell motility

The ability of cells to actively move toward chemical cues provides the advantage of faster and greater access to energy sources, such as ephemeral or spatially heterogeneous patches of organic matter (Taylor and Stocker 2012). Motility also comes at a significant energetic cost, however, due to the required maintenance of motility-providing structures, such as flagella, and motility itself (Fenchel 2008; Taylor and Stocker 2012; Hoehler and Jørgensen 2013). The documented effects of energy limitation on motility are varied.

In pure culture experiments, loss of motility in response to starvation has been documented in chemotaxis assays with *Photobacterium angustum* strain S14. In these studies, the fraction of motile cells decreased from ~60% initially to <5% after 24 h of starvation (Malmcrona-Friberg, Goodman and Kjelleberg 1990). Motility could be revived in 10–50% of cells within 4 h, however, by addition of energy substrates. In tests with the Alphaproteobacterium *Sinorhizobium meliloti*, all three strains tested ceased to be motile within 8–72 h of incubation in starvation buffer (Wei and Bauer 1998). Interestingly, however, one-third to one-half of cells retained their flagella, even after 72 h. Motility in a *Pseudomonas* strain was found to decrease by >95% within 3 h of starvation. Yet, 80% of the cells had restored their motility after 27 h without further energy supply (Wrangstadh *et al.* 1990). One study even found *Vibrio* sp. strain Ant-300 to become motile only under starvation conditions (Torrella and Morita 1982). Another showed that the hyperthermophilic archaeaon *Methanocaldococcus jannaschii* expresses archaellum proteins—the archaeal version of the bacterial flagellum (Jarrell and Albers 2012)—when energy becomes limiting (Mukhopadhyay, Johnson and Wolfe 2000). Thus, both decreased and increased chemotaxis can be responses to energy limitation.

Studies in the marine environment indicate motility and motility genes to be widespread (DeLong *et al.* 2006). It was estimated that ≥20% of cells in coastal water columns and throughout the upper 35 cm in coastal sediment were motile (Fenchel 2001, 2008). Over the course of a 10-month field study of motility in coastal water off La Jolla, California, the fraction of motile prokaryotes was <5–25% during the fall and winter months, and 40–70% during spring and summer (Grossart, Riemann and Azam 2001). As revealed by parallel mesocosm studies, the percentage of motile cells followed a diel pattern, and increased during the crash of an algal bloom, implying that higher availability of algal detritus increased the energetic benefits of motility, and resulted in active colonization of (dead) algae by heterotrophic microbes (Grossart, Riemann and Azam 2001). The significant energetic benefit of colonizing these patches is consistent with high rates of hydrolytic activity on organic particles (Smith *et al.* 1992), and cell densities exceeding those in surrounding water by two to three orders of magnitude (Herndl 1988; Fenchel 2002).

In environments with ephemeral microscopic plumes, the velocity of motility is critical—even though the energetic cost of motility increases by the square of velocity (Taylor and Stocker 2012). In marine photic zones, heterotrophic bacteria often chemotactically track and cluster around phytoplankton and other heterotrophic cells to consume exudates or form other symbiotic relations of unknown nature (Mitchell, Okubo and Fuhrman 1985; Malfatti and Azam 2009; reviewed in Stocker 2012). Rapid chemotactic responses to substrate patches in slide chamber and microfluidics experiments with the marine bacterioplankton *Pseudoalteromonas haloplanktis* (Mitchell *et al.* 1995; Stocker *et al.* 2008) were shown to increase substrate exposure up to 10-fold compared to non-motile cells. The velocity of *Ps. haloplanktis* was thereby >10 times higher than in the classic model organism of chemotaxis, *E. coli*. Perhaps rapid chemotaxis is an adaptation to life in water columns, where substrate plumes vanish rapidly due to advective mixing, and rapid colonization of fresh detritus, fecal pellets and exopolymers could confer a competitive advantage.

To better understand the energetic payoffs of motility, the balance of energy gains and expenditures needs to be understood. Assuming that motility serves the chemotactic pursuit of energy, then the energy gain is the extra energy taken up by being motile, compared to being non-motile, and determined by net energy yields per substrate and the amount of substrate taken up. The energetic cost is the extra energy spent on locomotion, and the maintenance of both the motility apparatus and motility genes. Hereby the cost of locomotion varies greatly with cell size, cell shape, as well as mode and speed of locomotion. Submicron increases in cell radius may increase the energetic cost of locomotion by orders of magnitude (Mitchell 2002). Rod-shaped cells may have a superior ability to swim directionally, e.g. in chemotactic pursuit of substrate plumes, compared to spherical cells. On the other hand, spherical shape increases the impact of Brownian motion and may be advantageous to small, non-motile microbes that rely on passive dispersal (Dusenberry 1998). The energetic cost of motility is also influenced by the flagellar motor torque used by the cells (Li and Tang 2006), and the spatial extent of chemical gradients (micrometer to meter scale) (Mitchell 2002). Experiments with microbes from seawater, moreover, indicate lower average swimming speeds of starved compared to non-starved cells (Yam and Tang 2007), perhaps reflecting the higher energetic cost of fast compared to slow swimming.

Despite theoretical calculations suggesting that small motile cells might overcome the challenges of Brownian motion and spend less energy on locomotion than larger cells (Mitchell 2002), the existing literature does not indicate increased motility in small cells. A review on observed motility across 97 bacterial genera suggests the general absence of motility in cells with a mean diameter below 0.8 μm (Dusenberry 1997). In metagenomes from a deep subseafloor sediment column, where cells are small and probably severely energy limited, motility genes are rare and decrease in proportion with depth (Biddle *et al*. 2008). A decrease in the proportion of motility genes has also been reported for aphotic water columns relative to photic zones (DeLong *et al*. 2006). A higher percentage of motile cells in surface sediments and in marine photic zones suggests that the energy investment required for motility pays off especially among microbes inhabiting energy-rich or spatiotemporally patchy environments. Applying the same reasoning, motility should pay off less in energy-poor or spatiotemporally homogeneous environments.

Cell adhesion

Several studies have demonstrated phylogenetic differences between free-living and attached-living microbes in water columns (DeLong, Franks and Alldredge 1993; Crump, Armbrust and Baross 1999). These differences may change over time, for instance, some microbes that live attached most of the time may switch to living freely under high substrate concentrations or while searching for new food particles of suitable chemical composition (Riemann and Winding 2001). Nonetheless, general distinctions between attached and free-living microbes have been made. Many microbes growing on particles are characterized as copiotrophs with the ability to opportunistically exploit ephemeral and spatially heterogeneous resources by means of motility—a life history strategy, for which the term 'opportunitrophic' (Moran *et al*. 2004) was coined. By contrast, free-living microbes consist mainly of non-motile (passive) oligotrophs, that minimize energy expenditures, and are thus able to grow and survive under very low cell-specific energy fluxes (Polz *et al*. 2006)—we coin the term 'pigri-troph' ('lazy/sluggish eater') to describe the trophic strategy employed by these energetic minimalists.

In addition to providing access to energy stored in particles, increased adhesion to surfaces has been indicated as a survival strategy of copiotrophic bacteria under energy limitation, e.g. in oligotrophic environments (Dawson, Humphrey and Marshall 1981; Kjelleberg, Humphrey and Marshall 1982). A likely reason is that even non-metabolizable particles can be enriched in energy compared to surrounding fluids due to adsorption of substrates, such as fatty acids (Dawson, Humphrey and Marshall 1981; Kjelleberg, Humphrey and Marshall 1982). Consistent with this notion, numerous microbial strains increase surface adhesion under energy limitation in laboratory experiments. The Gram-negative *E*. coli and *Shewanella oneidensis* trigger adhesion by increasing hydrophobicity of their outer cell membranes (Saini, Nasholm and Wood 2011). *Vibrio* sp. strain DW1 undergoes reductive cell division and thereafter produces bridging polymers, which increase adhesion to surfaces (Kjelleberg, Humphrey and Marshall 1983). Both *Vibrio* sp. DW1 and *Pseudomonas* sp. have higher endogenous rates of metabolism when attached to surfaces under starvation conditions (Kjelleberg, Humphrey and Marshall 1983). This trend can be reversed: when sorbed energy substrates are depleted or starvation experiments are terminated by substrate addition, cells may abandon particles using flagellar locomotion (Kjelleberg, Humphrey and Marshall 1982; Kefford, Humphrey and Marshall 1986). In line with laboratory experiments showing increased adhesion of microbes to particles under energy limitation, metagenomes from deep, aphotic water columns show an increased proportion of genes related to pili synthesis relative to photic zone metagenomes (DeLong *et al*. 2006). Moreover, recent starvation studies on *Candidatus P. ubique*, which produce pili under starvation conditions (Steindler *et al*. 2011)—perhaps to attach to particles—indicate that adhesion to surfaces can even occur in genuinely oligotrophic microbes as a starvation survival mechanism.

Interestingly, the trend toward increased adhesion under energy limitation is not universal. *Klebsiella pneumoniae* inhabiting fissures in advection-controlled, subsurface terrestrial sandstone decreases exopolysaccharide (EPS) production and thereby reduces adhesion under energy starvation (Lappin-Scott, Cusack and Costerton 1988; MacLeod, Lappin-Scott and Costerton 1988). Perhaps this strategy is comparable to the behavior of some water column cells to abandon particles, after depleting them of energy substrates, to find and colonize new patches with higher energy availability, with the difference that *K. pneumoniae* relies on passive rather than active dispersal.

The published data thus indicate that whether adhesion is increased or decreased in response to starvation may depend on factors such as habitat type (e.g. water column versus rock fissure, diffusion versus advection dominated), energy substrates and broader evolutionary strategy. Future investigations will identify which environmental stimuli trigger adhesion/detachment, what the underlying physiological and intracellular mechanisms are and the extent to which differences in adhesion response are genetically hard wired and/or vary with ecological strategy, e.g. oligotrophy versus copiotrophy, opportunitrophy versus pigritrophy, etc.

CELL COMPOSITIONAL INDICATORS OF ENERGY LIMITATION

Changes in cellular composition resulting from energy limitation can be profound, often vary considerably between species, and yet follow general trends. We here discuss changes in the sections 'cell carbon (C) content', 'cell volume to C content relationships' and adjustments in cellular building blocks in the subsequent sections in response to energy limitation.

Cell C content

Assumed mean cell C contents and empirically determined cell volume to C content conversion factors are widely used to infer the total amount of C stored in living microbial biomass across ecosystems. The appeal of this approach is that by quantifying cell numbers it is possible to estimate the amount of C stored in living microbial biomass, and vice versa. Reliable factors for converting cell volume to C content of unknown cells are, however, missing (also see section 'Cell volume to cell carbon relationships'), due to inter- and intra-specific variability in cellular composition under different energy and nutrient regimes (Vrede *et al.* 2002), and different artifacts associated with various methods of cell fixation, staining and size measurement (Bölter *et al.* 2002; Romanova and Sazhin 2010).

Nonetheless, trends are apparent when examining C contents per cell across pure cultures and natural samples under different energy regimes. Estimates of mean C content per cell range over one order of magnitude under energy-rich conditions in pure cultures (16–403 fg C cell^{-1}, Fig. 3A; Table A3, Supporting Information) and eutrophic estuarine and coastal systems (5.9–258 fg C cell^{-1}, Fig. 3B; Table A3, Supporting Information). By contrast, under energy limitation in the laboratory (17–34 fg C cell^{-1}; Fig. 3A; Table A3, Supporting Information) and in oligotrophic environments of the open ocean (5.9–39.9 fg C cell^{-1}; Fig. 3B; Table A3, Supporting Information), C contents show a smaller range of variation (less than one order of magnitude). Furthermore, though C contents between energy-rich and energy-poor samples overlap, this overlap is limited to the lower end of the spectrum. Thus, the published data suggest that high average C contents per cell are only found in pure cultures under energy-replete conditions and in eutrophic natural samples, while low average C contents per cell can be found everywhere. Moreover, comparing pure cultures to natural samples indicates that mean C contents of pure cultures under energy-rich conditions are not reached in the environment, even in more energy-rich estuarine and coastal settings, and that mean cell C contents at the lower end of the spectrum in environmental samples are lower than ones after 28 days of starvation in the laboratory.

These trends are confirmed when data obtained within the same study are compared. Though typically small—and much lower compared to reductions in cell volume (Fig. A2A, Supporting Information)—slight reductions in cell C contents did occur in nine out of ten different pure culture strains over the course of a 28-day-starvation period (Fig. A3A, Supporting Information; Troussellier *et al.* 1997). Even during the switch from growth phase to stationary phase, reductions in cell C contents are measurable within less than 24 h in *E. coli* and *V. natriegens* (Fig. A3B, Supporting Information; Norland, Fagerbakke and Heldal 1995; Fagerbakke, Heldal and Norland 1996). Similarly, mean C contents per cell in natural water samples from six pelagic stations were lower than at two coastal stations in the Pacific Ocean (Fig. A3C, Supporting Information; Fukuda *et al.* 1998).

Cell volume to cell carbon relationships

If compiled data from pure culture and environmental studies in which both mean cell volumes and mean cell-specific C contents were measured are plotted against each other, two trends are immediately apparent, despite significant scatter: (1) pure culture and environmental data cluster together, suggesting they are underlain by the same general relationship, and (2) there is a statistically significant relationship between cell volume and cell-specific C content, with an average ratio of ∼100 fg cell C content per μm^{-3} cell volume if this relationship is assumed to be linear (Fig. A4, Supporting Information).

If ratios of cell C content to cell volume are compared at different energy states across pure cultures and environmental samples, they appear—on average—lower in pure cultures under energy-replete conditions than in energy-limited pure cultures or environmental samples (Fig. 4; Table A4, Supporting Information). Yet, despite this overall trend, the strong overlap between energy-replete and -limited microbes confirms once more that cellular C content to cell volume ratios are not reliable indicators of energy state. The utility of general bioconversion factors to infer C content from measured cell volumes, and vice versa, has to be called into question given that ratios vary by over one order of magnitude (mean ± SD = 226 ± 279).

General changes in cellular composition

The type and magnitude of change in the molecular composition of cells in response to energy limitation varies greatly between species and can also change within a species over the course of starvation (e.g. Galdiereo *et al.* 1994; Kieft *et al.* 1994, 1997).

Profound differences in the change of intracellular composition can be seen between organisms using the CSR and OSR. An extreme example of the CSR is *V. cholerae*, in which the lipid and carbohydrate contents decreased by 99.8 and 88.7% within 7 days of starvation, and DNA and protein contents each decreased by ∼75% over 30 days of starvation (Hood *et al.* 1986). Similar trends were reported from starvation experiments with *Nocardia corallinae* (Robertson and Batt 1973) and *Salmonella typhimurium* (Galdiereo *et al.* 1994); in the latter, carbohydrates decreased by ∼98%, lipids by 87%, proteins by 95% and DNA by 75% within 32 days (Galdiereo *et al.* 1994). Surprisingly, intracellular RNA content decreased the least, i.e. by only ∼20%, in both *V. cholerae* and *Sa. typhimurium*. Initial, rapid declines in lipids and carbohydrates may indicate use as energy sources for dormancy preparation and cell maintenance during starvation (Chapman and Gray 1981; Hood *et al.* 1986), whereas maintenance of high RNA levels during starvation could enable rapid responses to substrate pulses in environments with fluctuating energy regimes. In both species, decreases in lipid content went along with loss of cell integrity (Hood *et al.* 1986) or complete loss of the three-layered outer cell membrane (Galdiereo *et al.* 1994).

Both *V. cholerae* and *Sa. typhimurium* are pathogens and examples of microbes that have evolved to opportunistically exploit periods of extremely high energy levels, while also being capable of surviving long periods of energy limitation. The marine water column *Vibrio* sp. strain Ant-300 may live in an environment with lower energy levels, yet shows similar responses to starvation. Besides changing from rod-shaped to spherical morphology and greatly diminishing in size in the process, these microorganisms decrease protein, DNA, and RNA levels by 43, 63 and 65% within 4 days of starvation (Amy and Morita 1983), illustrating that the CSR response occurs across a range of energy conditions.

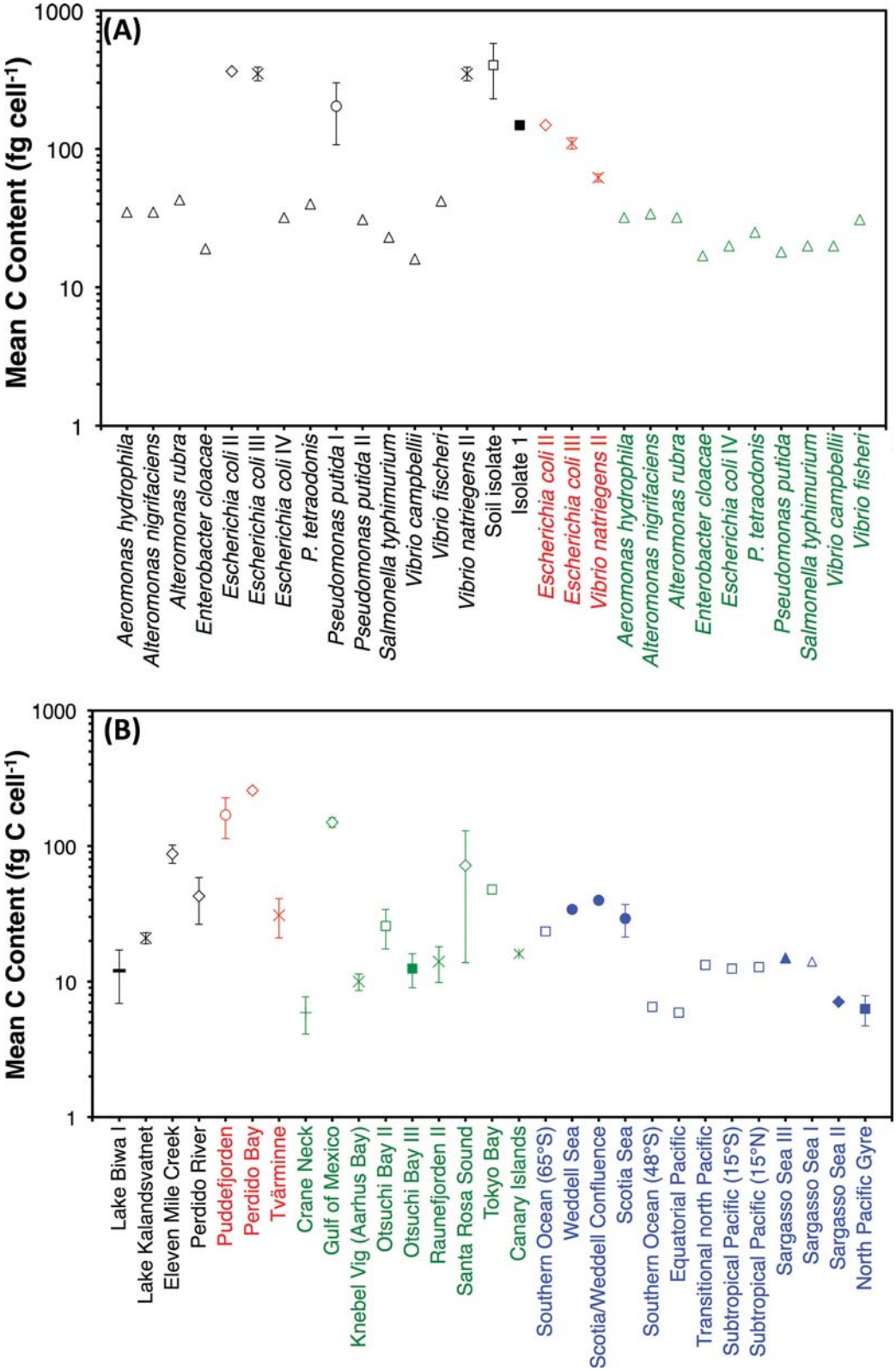

Figure 3. (A) Mean C contents per cell of pure cultures during growth phase (black), stationary phase (red) and after starvation for 28 days (green). (B) Mean C contents per cell in environmental samples, i.e. freshwater lakes and rivers (black), estuarine and brackish waters (red), coastal and nearshore marine waters (green), and pelagic marine waters (blue). Error bars indicate standard deviations. More detailed information on the data shown can be found in Table A3 (Supporting Information).

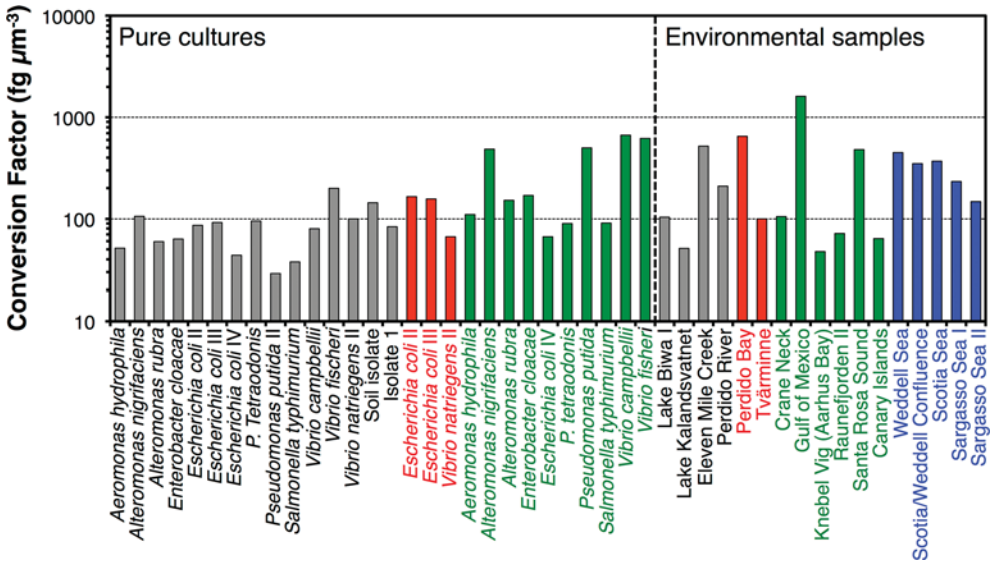

Figure 4. Cell carbon content to cell volume conversion factors from pure cultures and environmental samples, calculated from mean C content per cell (fg) divided by mean cell volume (μm^3). More information on these data can be found in Table A4 (Supporting Information).

Remarkably different changes were seen in starvation experiments with six oligotrophic types of coryneform bacteria (*A. globiformis*, *A. nicotianae*, *Brevibacterium linens*, *Corynebacterium fascians*, *Mycobacterium rhodochrous*, *N. roseum*; Boylen and Mulks 1978). Depending on the species, carbohydrate content per cell decreased by ~20–80%, protein content by ~10–50% and RNA content by ~10–65% over an 8-week starvation period. Unlike in copiotrophs, however, DNA content did not decrease, but—in five of six species—increased by 25–57% within the first few days of starvation and remained constant thereafter. Similar increases in DNA content, and decreases in RNA, protein and carbohydrates were also seen in *A. crystallopoietes* and *A. atrocyaneus* (Boylen and Ensign 1970; Scherer and Boylen 1977), raising the question whether increases in DNA content are a general strategy to survive starvation in oligotrophic microbes.

Changes in building blocks of cells using the OSR are not always as dramatic as in aforementioned coryneform bacteria. The chemolithoautotrophic ammonium-oxidizing bacterium *Nitrosomonas cryotolerans* showed essentially no changes in protein, DNA or RNA content over the course of a 10-week starvation period (Johnstone and Jones 1988). No significant changes in protein content were observed in the sphingomonad species *Zymomonas anaerobia* and *Z. mobilis* over a 6-day starvation period, though both species lost >50% of their RNA during this time (Dawes and Large 1970). The chemoheterotrophic bacterium *Alteromonas denitrificans*, after a ~10-fold decrease in protein content within the first 10 days of starvation, increased its protein content by a factor of 3–4 after 15 days of starvation (Nissen 1987).

Though changes in bulk amounts of different cellular components suffice to demonstrate dramatic shifts in intracellular composition during starvation, relating these bulk changes to individual cells may be problematic. Variability may occur at an intercellular level, due to heterogeneities in media, endogenous activity, viability/non-viability, mortality, etc. If not cleanly separated, residual components of lysed cells that vary in half-life may persist for different time periods, further confounding accurate determinations of the intracellular composition of living cells. Ultimately, measurements on the single-cell level will be necessary to capture the extent of intracellular compositional variability under starvation. Future research, e.g. involving tools such as nano-secondary ion mass spectrometry (nanoSIMS) or Raman spectroscopy (reviewed in Wagner 2009), may enable accurate determinations of how single microbial cells alter their intracellular composition in response to starvation.

Most research on cell compositional changes so far has been on cells growing under oxic conditions. The extent to which these changes apply to cells under anoxic conditions is not clear. Evidence from the methanotrophic *Methylosinus trichosporium*, in which strong reductions in cell size and protein content under oxic conditions contrast with only minor changes under anoxic conditions, suggests that cell responses to energy limitation may depend on redox conditions (Roslev and King 1994). Whether these responses are simply temporal, with the same starvation responses simply being delayed under anoxic compared to oxic conditions, or whether they differ fundamentally, with no or different cell compositional shifts occurring under anoxic conditions, is not known. Roslev and King propose that higher oxidative stress under oxic conditions could result in higher basal energy metabolism and thus faster change. It is also possible that aerobes respond differently to energy limitation induced by electron acceptor limitation than to energy limitation induced by electron donor limitation. The possibility that the starvation responses vary under different redox conditions, or in the presence/absence of required electron acceptors, needs to be kept in mind in the following sections, in which we discuss the existing literature, which is mainly on electron donor-limited aerobic microbes, and in general when considering the potential for long-term survival under energy limitation in oxic versus anoxic environments.

Proteins

Compositional changes in proteins in response to energy limitation vary considerably across closely and distantly related taxa, in part depending on whether species are adapted to growth in energy-rich or energy-poor environments (Hood *et al.* 1986; Nissen 1987).

In *Vibrio* sp. strain Ant-300, a small number of proteins are only produced under energy-replete conditions or under energy

limitation, while most are produced under both conditions (15). More detailed data are available for the marine *V. angustum* (formerly *Ph. angustum*) strain S14. This organism induces production of periplasmic, outer membrane and cytoplasmic proteins after 120 h of energy and nutrient starvation (Nyström, Albertson and Kjelleberg 1988). These intracellular proteins are synthesized *de novo* rather than from products of biomass degradation. Some proteins appeared after 3 h of starvation, while others are first detectable after 5, 10, 24 or 120 h, suggesting sequential induction of starvation-related proteins (Nyström, Albertson and Kjelleberg 1988). Parallel studies on *V. angustum* strain S14 and *Pseudomonas* sp. strain S9, moreover, have revealed that both strains increase exoprotease activity during 120 h of energy and nutrient starvation (Albertson, Nyström and Kjelleberg 1990a). In total, at least 42 new proteins are only synthesized under energy and nutrient limitation in *V. angustum*, of which 20 appear unique to C-starvation (Nyström, Olsson and Kjelleberg 1992). Certain proteins (carbon starvation protein 1 (*Csp1*); also see section 'RNA') are induced at 100-fold higher rates under energy starvation than under nutrient limitation, and are completely repressed under all other conditions (Holmquist and Kjelleberg 1993). Despite or because of these changes in proteins synthesized, *V. angustum* remain highly responsive, increasing rates of protein and RNA synthesis instantaneously after substrate addition following 9 days of starvation (Flärdh, Cohen and Kjelleberg 1992). The ability to respond rapidly to substrate pulses after starvation has been linked to 'immediate upshift proteins' (*Iup*; (Marouga and Kjelleberg 1996). Within only 3 min after substrate addition, changes in the induction rates of 18 of these upshift proteins are evident from 10-fold increases in *Iup* production rates compared to starvation conditions.

Similar protein induction patterns to those observed in *V. angustum* strain S14 under starvation have been observed in other microbial species. In *V. vulnificus*, drastic reductions in total rates of protein synthesis were accompanied by the synthesis of 34 new, starvation-induced (Sti) proteins over a 26-h starvation period. At least 23 of these Sti proteins were induced within the initial 20 min of starvation, and synthesis of all 34 Sti proteins was induced within 4 h of starvation (Morton and Oliver 1994). New proteins were also induced during starvation in the *Pseudomonas* strains *Pseudomonas fluorescens* DF57 and *P. putida* DF14, with the latter inducing outer membrane proteins in response to C-starvation (Kragelund and Nybroe 1994). *Enterococcus faecalis* responds to glucose starvation by producing 42 novel proteins within 24 h of starvation (Giard *et al.* 1997). When grown under energy and nutrient limitation, it synthesizes 51 new proteins, of which 16 are identical to those produced under glucose starvation (Hartke *et al.* 1998).

Similar trends, indicating the synthesis of specific proteins immediately after the onset as well as after days of C-starvation have been observed in *Staphylococcus aureus* (Clements and Foster 1998) and other microbial strains (Jouper Jaan, Dahllöf and Kjelleberg 1986). Given that most of the strains studied to date can be considered copiotrophs or opportunitrophs that have evolved strategies to survive fluctuating energy and nutrient regimes the extent to which these patterns in protein induction occur in microbes living under permanent energy limitation remains to be shown.

DNA

With respect to the DNA content of cells, both phenotypic adjustments under starvation conditions and evolutionary adaptations to permanent low-energy regimes have been postulated. Cells respond phenotypically to starvation conditions in the laboratory by decreasing, maintaining or increasing their intracellular DNA content. Typically, microbes using the CSR decrease their DNA content, while microbes using the OSR maintain or increase DNA content (see also section 'General changes in cellular composition'). What drives these changes, and which aspects actually change is unclear.

In the case of DNA content decreases per cell, polyploid cells, such as the radiation-resistent *Deinococcus radiodurans* (Hansen 1978), symbiotic *Buchnera* spp. and *Epulopsicium* spp. (Komaki and Ishikawa 1999; Mendell *et al.* 2008), or pathogenic *Neisseria gonorrhoeae* (Tobiason and Seifert 2010), might be able to catabolize extra genome copies for energy production, thereby not only gaining energy, but also lowering the cost of genome maintenance. Yet, many bacteria appear to tightly regulate chromosome number, limiting it to one set of copies except during replication (Nordström and Dasgupta 2006). In *Sa. typhimurium*, 75% reductions in DNA content per cell have been documented in response to starvation (Galdiereo *et al.* 1994). Yet, based on whole-genome sequencing these organisms are haploid. *Vibrio cholerae*, which have two unique chromosomes per cell (Trucksis *et al.* 1998), increase cell densities and viable cell counts by nearly one order of magnitude over a 30-day starvation period, and yet decrease the total amount of DNA by >70% (Hood *et al.* 1986). And the Gammaproteobacteria *Vibrio* sp. strain Ant-300 and *Cycloclasticus oligotrophus* reduce intracellular DNA content by one order of magnitude over 98-day starvation experiments or after 20-fold dilution of substrate concentrations, respectively (Moyer and Morita 1989b; Button and Robertson 2001). If the published data are accurate, a conceivable explanation would be that cells under growth conditions were replicating at the time of sampling and thus harboring multiple genome copies per cell. Though so far only documented for parasites, pathogens and endosymbionts, and not in the context of energy limitation, alternative explanations, such as 'genome streamlining', i.e. selective loss of gene duplicates or non-essential genes, are also possible (Maurelli *et al.* 1998; Mira, Ochman and Moran 2001), as rapid evolutionary changes can take place in microbial populations within only days to weeks even under starvation conditions (see section 'The growth advantage in stationary phase phenomenon').

Some microorganisms maintain or increase their DNA content during periods of starvation in the laboratory (Scherer and Boylen 1977; Johnstone and Jones 1988). Gene duplications and lateral gene transfer are plausible explanations. To better understand changes in DNA content within cells, it is necessary that cells of the same genotype are cultivated under starvation and energy-replete conditions and their genomes sequenced and compared.

In addition to laboratory studies, several studies have examined DNA content in natural samples. In water columns, the co-occurrence of distinct high-nucleic acid and low-nucleic acid (LNA) microbes has been invoked to suggest coexisting well-nourished and starving (Button and Roberts 2001) or active and inactive subpopulations (Lebaron *et al.* 2001). While these explanations are plausible based on pure culture data, and perhaps true for certain populations or locations, recent isolations of LNA bacteria suggest otherwise; three strains of *Polynucleobacter* from freshwater environments retained their small cell volumes and LNA characteristics even after enrichment followed by pure culture isolation (Wang *et al.* 2009).

In addition to phenotypic adjustments in DNA content, a number of genotypic traits have been linked to improved starvation survival. Lauro *et al.* (2009) compared the genomic

DNA content of the copiotrophic *Ph. angustum* strain S14 and the oligotrophic *S. alaskensis* and linked the smaller genome size in the latter to trophic strategy. *Photobacterium angustum* uses a broad spectrum of transport genes encoding for high-specificity, low-affinity uptake systems, whereas *S. alaskensis* harbors a small number of transport genes which encode for broad specificity, high-affinity uptake systems. *Photobacterium angustum* also have a higher number of extracytoplasmic proteins compared to *S. alaskensis*, consistent with high ectoenzymatic activity and growth on large particles ('marine snow'). Other relative differences include more genes linked to motility, sensory systems, defense, transcription and signal transduction. By comparison, *S. alaskensis* has relatively more genes linked to lipid transport (whereby lipids are used for energy storage), catabolism, secondary metabolite biosynthesis, degradation of aromatic/recalcitrant compounds and detoxification (Lauro *et al.* 2009). Interestingly, seven prophages were located in the genome of *Ph. angustum* compared to none in *S. alaskensis*. The higher number of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs) that may confer resistance to phage infection in *Ph. angustum* implies that this species is more sensitive to viral attack than *S. alaskensis*, perhaps due to higher growth rates, and larger burst sizes due to larger cell size.

Lauro *et al.* (2009) went on to compare genomes of other bacterioplankton that had been categorized as copiotrophic or oligotrophic based on criteria that applied to *Ph. angustum* and *S. alaskensis*—with most Gammaproteobacteria considered copiotrophs and most Alphaproteobacteria considered oligotrophs. Among the variables compared were the numbers of 16S rRNA gene copies per cell and genome size. While oligotrophic bacteria on average harbored 1.4±0.7 copies, copiotrophs on average harbored 8.4±1.8 16S rRNA gene copies per cell (Fig. 5A; Table A5, Supporting Information). The same trend toward higher copy numbers of 16S rRNA genes had been observed by Klappenbach, Dunbar and Schmidt (2000) in fast-growing, rapidly colonizing prokaryotes compared to slow-growing, slowly colonizing prokaryotes in soil. Since the ability to grow fast and colonize rapidly is also a typical trait of copiotrophs, it is likely that the two studies by Lauro *et al.* and by Klappenbach *et al.* were comparing the same fundamental ecological strategies, albeit in different environments.

Lauro *et al.* also compared average genome size across copiotrophs and oligotrophs. Although mean genome size was higher in copiotrophs (4.8±0.7 vs 4.0±1.9 Mb), the strong overlaps in error bars indicate that this difference is not consistent across all groups (Fig. 5B; Table A5, Supporting Information). In fact, the largest genome (7.78 Mb) among all bacterioplankton species compared in this study belongs to *Planctomyces maris* (Table A5, Supporting Information), a species that is considered oligotrophic, and exceeds that of the copiotroph with the smallest genome, *Flavobacteria bacterium* BBFL7 (3.08 Mb), by a factor of 2.5. This absence of a clear relationship between genome size and trophic strategy is confirmed by meta-analyses on relationships between gene content and genome size across free-living species of prokaryotes from a wide range of habitats; here species with larger genomes were linked to environments in which resources are scarce but diverse and in which there is little disadvantage to slow growth, such as soil (Konstantinidis and Tiedje 2004).

RNA

As mentioned earlier (section 'General changes in cellular composition'), changes in intracellular RNA pools in response to starvation vary, ranging from maintenance to drastic reductions in RNA content. These changes are in part attributable to adjustments in RNA composition and intracellular controls on RNA stability. We will briefly discuss the limited data on starvation responses concerning the ribosomal RNA (rRNA) content and then focus on the larger body of literature concerning mRNA.

Changes in rRNA content have been indicated in the starvation- and stress-resistant copiotroph *Ph. angustum* S14, which reduced its number of ribosomes over the course of a 9-day starvation experiment (half-life: 79 h). Despite these cutbacks, the ribosome content remained in 'oversupply' with respect to what was required for observed rates of protein synthesis (Flärdh, Cohen and Kjelleberg 1992). This finding is consistent with previous studies on *V. cholera* and *Sa. typhimurium*, both of which showed lower relative decreases in RNA compared to protein, DNA, carbohydrate or lipid contents under starvation (Hood *et al.* 1986; Galdiereo *et al.* 1994). Maintenance of a relatively high rRNA content throughout periods of starvation may thus be an evolutionary adaptation of opportunitrophs, which enables rapid response to increases in energy availability via rapid surges in mRNA translation followed by catabolic enzyme synthesis.

In addition, the synthesis and maintenance of long-lived mRNA may explain the high RNA content throughout periods of starvation, and starvation-induced dormancy in certain cells. A study on fruiting bodies of *Myxococcus xanthus* was among the first to show the existence of long-lived mRNA; this led to the hypothesis that under extended starvation conditions long-lived mRNA replaces short-lived mRNA, in some cases compromising the cells' ability to respond quickly to changing energy regimes, but—more importantly under these conditions—reducing the amount of energy spent on mRNA synthesis (Nelson and Zusman 1983). Since then, many studies have confirmed that microbes increase mRNA half-lives under starvation

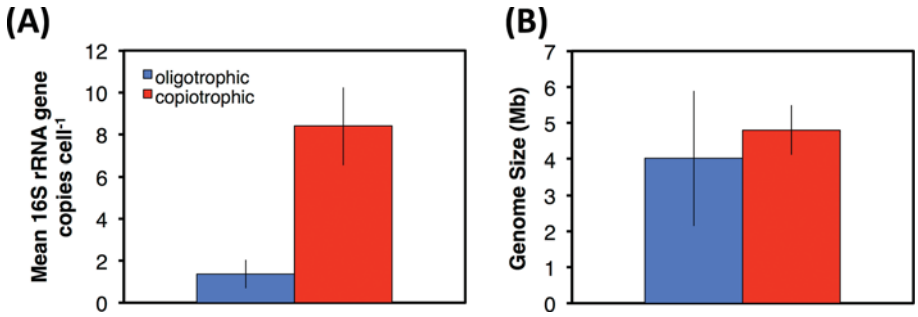

Figure 5. Comparison of (**A**) mean 16S rRNA gene copies per cell and (**B**) mean genome size per cell. Means calculated on microbial strains categorized as 'oligotrophic' and 'copiotrophic' by Lauro *et al.* (2009). More information on the data can be found in Table A5 (Supporting Information).

conditions (Redon, Loubiere and Cocaign-Bousquet 2005; Dressaire *et al.* 2013). *Escherichia coli* and the halophilic archaea *Haloferax volcanii* increase the stability of mRNA encoding for certain proteins at low growth rates and during stationary phase compared to exponential growth phase (Nilsson *et al.* 1984; Evguenieva-Hackenberg and Klug 2009). The mean mRNA half-life of *Ph. angustum* S14 increased 6-fold, from 1.7 to 10.3 min, over the course of a 24-h starvation period, with certain starvation-specific proteins encoded by mRNA with half-lives up to 70 min (Csp1; Albertson, Nyström and Kjelleberg 1990b). These shifts in mRNA pools are rapidly reversed upon substrate addition. For instance, as discussed in section 'Proteins', *Ph. angustum* S14, when provided with energy substrates after a 48-h starvation period, was found to synthesize 18 Iup proteins within 3 min after substrate addition (Marouga and Kjelleberg 1996). The ability to respond with such rapidity was ascribed to 'silent' and stable mRNA transcripts, which were present throughout the starvation period, the translation of which into Iup was induced by substrate addition. As with rRNA content, opportunitrophic cells may particularly benefit from these silent transcripts, which enable rapid metabolic responses to oligotrophic environments with ephemeral boosts in energy supply.

In addition to changes in mRNA composition, many studies have documented the post-transcriptional regulation of mRNA stability, translation and turnover. RNA is degraded by internal cleavage via endoribonucleases, or nucleotide by nucleotide from the end by exoribonucleases (Evguenieva-Hackenberg and Klug 2011). First insights into intracellular regulation of mRNA half-life came from observed associations of ribonuclease E (RNAse E) and polynucleotide phosphorylase (PNPase) with mRNA in *E. coli*, in which the presence of these proteins appeared to control the half-life of the mRNA (Carpousis *et al.* 1994; Py *et al.* 1994). Since then, it has become clear that all three domains share similarities in the mechanisms of controlling mRNA degradation, e.g. in using poly(A) polymerase, or attaching adenine (A)- or adenine-uridyl (AU)-rich sequences to 3′ ends of mRNA (Evguenieva-Hackenberg and Klug 2009; Belasco 2010). Yet, the function of these sequences differs. In eukaryotes, A- and AU-rich sequences facilitate binding of poly(A)- and AU-binding proteins and shield mRNA from exoribonuclease attack (Wilusz, Wormington and Peltz 2001; Bevilacqua *et al.* 2003). By contrast, in Bacteria and Archaea these A- and AU-rich tails destabilize mRNA (Evguenieva-Hackenberg and Klug 2011). Bacteria furthermore possess a multiprotein complex, termed degradosome, that controls mRNA stability, which differs from vesicles, termed exosomes, used by Eukarya and many Archaea (Romeo, Vakulskas and Babitzke 2013). Though the protein composition of this degradosome varies across different groups of Bacteria (Carpousis 2007), and even within the same strain under different environmental conditions (Jäger *et al.* 2004), similarities between distantly related species have been described. For instance, the degradosome of *E. coli* typically consists of RNase E, PNPase, RNA helicase B (RhlB) and enolase—proteins that, with the exception of RhlB, also occur in degradosomes of *Ph. angustum* (Carpousis 2007; Erce, Low and Wilkins 2010). While prokaryotes shield 5′ ends of mRNA from exoribonuclease attack by attaching triphosphate groups (Bacteria) or translational initiation factors (Archaea), Eukarya shield 5′ ends with a methylguanosine cap at the 5′ end (Wilusz, Wormington and Peltz 2001). Furthermore, Bacteria use pronounced mRNA secondary structure (e.g. stem loops) to protect internal cleavage via endoribonucleases (Evguenieva-Hackenberg and Klug 2011), and recent studies have indicated a key role of small regulatory RNAs in up- or downregulating mRNA turnover (Lalaouna *et al.* 2013).

Despite the vast advances in describing various general mechanisms of post-transcriptional regulation in microbes, little is known about the interplay of these mechanisms that leads to increased mRNA half-life under energy limitation.

Lipids

Lipid compositional changes in cells under starvation stress have been observed across a range of pure cultures, with most studies so far on Gram-negative Gammaproteobacteria. Compositional changes within the Gammaproteobacteria are often similar, showing shifts from unsaturated to saturated fatty acids, but differ from observed compositional changes within the Actinobacteria.

In *Vibrio* sp. strain Ant-300, phospholipids decrease by 65% over a 21-day starvation period and co-occur with a 59% increase in neutral lipids (Oliver and Stringer 1984). Similarly, phospholipids, which make up ∼99.9% of total lipids in the non-starving, Gram-negative Gammaproteobacterium *V. cholera* only account for 83.6% of lipids after 30 days of starvation; this decline is, again, concomitant with a relative increase in the proportion of neutral lipids (Hood *et al.* 1986). Changes in lipid composition in *V. cholerae* have been linked to a high turnover of *cis*-monoenoic fatty acids of membrane phospholipids coupled to the production of higher saturated fatty acids, cyclopropyl derivatives of *cis*-monoenoic fatty acids and *trans*-monoenoic fatty acids (Guckert, Hood and White 1986). Similar, strong increases (37.5%) in saturated fatty acids along with reductions in unsaturated fatty acids have been documented for the barophilic, marine, Gram-negative Gammaproteobacterium *Psychromonas* sp. strain CNPT-3 (Rice and Oliver 1992).

Similar to the above examples of Gammaproteobacteria, the soil Alphaproteobacterium *Sphingomonas* sp. strain LH128 was found to increase the proportion of saturated fatty acids (Fida *et al.* 2013). Yet, the effects of 16-day starvation experiments on lipid composition with the Gammaproteobacterium *Pse. aureofaciens* and the Actinobacterium *A. protophormiae* showed divergent outcomes (Kieft, Ringelberg and White 1994). While *Pse. aureofaciens*, similar to other Gammaproteobacteria, increased ratios of saturated to unsaturated fatty acids and cyclopropyl fatty acids to their monoenoic precursors, *A. protophormiae* changed little in lipid composition. Only minor changes were also observed in 64-week starvation experiments involving terrestrial subsurface isolates of *Pse. fluorescens* and another *Arthrobacter* sp., suggesting that lipid compositional responses of microbes to starvation are not universal (Kieft *et al.* 1997).

Carbohydrates

Compositional changes in response to starvation have also been observed in carbohydrates. During periods of starvation, cells can maintain low endogenous metabolic rates by catabolizing energy storage compounds, such as poly-β-hydroxybutyrate (Jones and Rhodes-Roberts 1981; Poli *et al.* 2011), trehalose (Schimz and Overhoff 1987) or glycogen (Zevenhuizen 1992). In *V. cholera*, relative losses of the 3- and 5-carbon sugars ribose and *N*-acetylglucosamine have been reported to co-occur with an increase in 6-carbon sugars, such as glucose. These changes are likely due to a selective loss of hydrophilic O-side chains associated with the outer membrane, and consistent with increases in hydrophobicity reported from other microbes under starvation conditions (Kjelleberg and Hermansson 1984; Hood *et al.* 1986; also see section 'Cell adhesion'). Similarly, starving *Pseudomonas* sp. have been shown to increase EPS production

under energy limitation (Wrangstadh, Conway and Kjelleberg 1986; Wrangstadh *et al.* 1990). Yet, the fact that energy-limited *Klebsiella* strains reduce EPS production (MacLeod, Lappin-Scott and Costerton 1988) illustrates that starvation responses can include both increases and decreases in EPS production. Thus, changes in carbohydrates depend on the specific starvation response of the organism studied, e.g. whether adhesion resulting from increased hydrophobicity or EPS production confers a survival advantage, or not.

THE MINIMUM ENERGY REQUIREMENTS OF LIFE

Microbial cells differ not only in their morphological and cell compositional responses to starvation. Pronounced differences occur also in the minimum cell-specific energy flux that is required for survival over time—both between different species and within the same strain—depending on the physicochemical conditions in the surrounding environment.

Physiological states of microbial life

The minimum energy required to sustain microbial populations has been examined directly in laboratory incubations (Tijhuis, van Loosdrecht and Heijnen 1993; Lin, Westerhoff and Röling 2009) and model environments (Penning and Conrad 2006) and inferred from carbon turnover and respiration rates in the environment (Biddle *et al.* 2006; Lomstein *et al.* 2012; Røy *et al.* 2012). First studies on this energy requirement were performed on yeast in the late 19th century (Duclaux 1898). Building on this and other pioneering work (e.g. Monod 1942; Herbert, Elsworth and Telling 1956), the term 'maintenance energy' was introduced to describe the energy required by microorganisms to maintain essential physiological functions over time (Pirt 1965). Attempts to quantify this cell-specific energy requirement have relied mainly on cell cultures grown under steady-state conditions in continuous-flow reactors, i.e. chemostats (Pirt 1965, 1982; Stouthamer and Bettenheimer 1973), and resulted in highly divergent estimates (Tijhuis, van Loosdrecht and Heijnen 1993). Explanations for these divergent estimates include interspecific differences in maintenance energy requirements (Stouthamer and Bettenheimer 1973), even between closely related species (Lin, Westerhoff and Röling 2009). Maintenance energy requirements can even vary within the same species, depending on incubation temperature (Tijhuis, van Loosdrecht and Heijnen 1993), carbon substrate (Hempfling and Mainzer 1975), redox conditions (Stouthamer and Bettenheimer 1973) and cultivation technique (Grünberger *et al.* 2012, 2013). While maintenance energy requirements were determined at high growth rates in some studies (Tännler, Decasper and Sauer 2008), they were mathematically extrapolated from conditions of slow growth to conditions of zero growth in others (Tijhuis, van Loosdrecht and Heijnen 1993).

Due to the decrease in maintenance energy at decreasing growth rate (Pirt 1965; Stouthamer and Bettenheimer 1973), extrapolations from slow growth have been considered most relevant to the environment, where energy limitation is widespread and slow or zero growth likely to be the norm (Morita 1997). Nonetheless, maintenance energy estimates based on chemostat cultures appear to exceed the minimum energetic cost of survival in the environment by orders of magnitude (Morita 1997; Price and Sowers 2004). A likely reason is that, even at minimum flow settings, microbial growth is necessary to balance the continuous loss of biomass due to outflow; thus, conditions of very slow or zero growth cannot be studied directly in chemostats. Generalizations to the environment are also flawed because energy consumption in the environment may fall along a continuum of three physiological states with highly discrepant energy requirements (Morita 1997; Hoehler 2004). (1) A 'growth' state, where the full energy demand of a cell is met, and cells have excess energy available for growth and division (Fig. 6A). (2) A 'maintenance state', where the full energy demand of a cell is met, but excess energy required for growth is limiting, thus only allowing for low growth rates (Fig. 6B); maintenance energy extrapolations to zero growth from chemostats may describe this state. (3) A 'survival state', where cells grow extremely slowly—if at all. In the 'survival state', CSR cells express visual signs of energy shortage, such as reductions in size and changes in morphology, e.g. from rod- to coccoid-shaped, and/or filamentous or aggregated to single-cell life style (Amy and Morita 1983; Finkel 2006; Fig. 6C). Both CSR and OSR cells may direct their anabolic activity mostly to the replacement and repair of essential biomolecules, and must continue to synthesize housekeeping proteins which repair DNA, stabilize RNA transcripts and maintain membrane potential (Morita 1997; Keene 2007). The required energy comes from metabolic reactions which—albeit occurring at much lower rates than in the growth or maintenance state—still produce geochemical gradients that attest to this activity. This residual metabolic activity, which enables the maintenance and repair of essential biomolecules, distinguishes the survival state from full dormancy, as in endospores, and thus enables survival under extreme energy limitation over geologic time scales (Johnson *et al.* 2007).

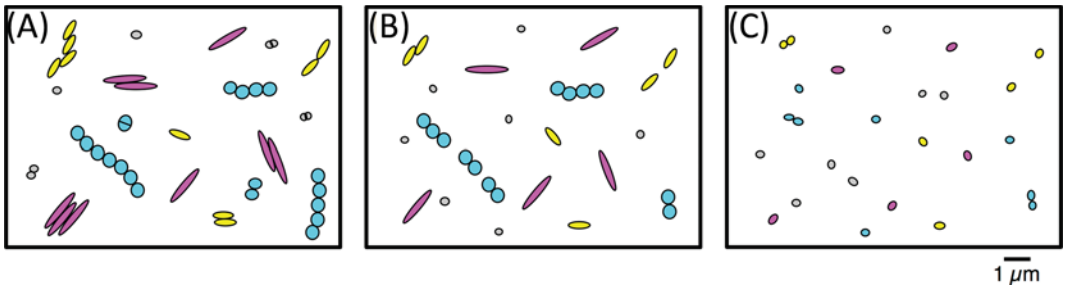

Figure 6. Concept sketch of microbial populations in different physiological states. (**A**) Growing populations under energy-replete conditions, as indicated by large numbers of dividing cells, and the presence of typical copiotrophic large, rod-shaped cells (pink, yellow) and filaments (turquoise). (**B**) Population in maintenance state large cells but fewer dividing cells than under growth conditions. (**C**) Microbes in survival state, as evidenced by uniformly small cell sizes, absence of rod-shaped cells, and fewer filaments. Note that cells with the OSR (gray) are impossible to differentiate in terms of size or morphology independent of physiological state. At best the presence/abundance of dividing cells might offer clues to the physiological state of these microorganisms.

Threshold energy requirements

The amount of energy required for repairing essential biomolecules over time is likely to vary across organisms and with physicochemical conditions. Constraining this 'basal power requirement' (BPR; Hoehler and Jørgensen 2013), a term we will use from hereon to avoid confusion with the term 'maintenance energy' from the traditional chemostat literature, is important to understand the cell-specific minimum energy requirement for survival, and to predict the 'biotic fringe' (Shock 2009), i.e. the habitable boundaries of life on Earth.

The total energy that is available per cell is the sum of the Gibbs energy yields of each substrate utilized multiplied by its cell-specific turnover rate. The sum of energies obtained from each substrate over time thus determines whether a cell meets its BPR. Certain energy substrates are drawn down to threshold concentrations below which energy yields appear too low to be harvested. These threshold free energy values are believed to reflect the 'biological energy quantum' (BEQ), i.e. the lowest amount of energy that can be conserved by an organism (Thauer and Morris 1984; see the section 'Glossary' for detailed definition of BEQ). Empirical support for the relevance of the BEQ concept to microbial communities is limited so far to certain types of anaerobic metabolism characterized by low-energy yields, such as sulfate reduction, methanogenesis and acetogenesis from H_2, in some cases methanogenesis from acetate, and certain secondary fermentation reactions (Hoehler 2004; Schink and Stams 2013). In the presence of O_2, or other anaerobic electron acceptors of high redox potential, such as nitrate, Mn(IV) or Fe(III), and in the metabolism of the vast majority of organic compounds, Gibbs free energy yields are well above the BEQ, even at very low H_2 or C substrate concentrations (Lovley and Goodwin 1988; Hoehler *et al.* 1998; Lever 2012; also see section 'Specialist versus generalist strategies'). Here other factors, e.g. relating to substrate production and cell-specific uptake rates, limit the power available to single microbial cells rather than thermodynamic factors.

The energetic cost of biomass synthesis

The energetic cost of biomass synthesis has frequently been ignored in studies of life under severe energy limitation. Yet, this energetic cost, which varies greatly under different physicochemical conditions, could be a key determinant of long-term survival and population size of microbes under different energy regimes. Here we will first discuss the energetic cost of cellular building block synthesis and polymerization, and then discuss the uncertainties that limit our ability to accurately assess how much energy cells spend on biosynthesis reactions.

Monomer synthesis. Due to the fact that the synthesis of cellular building blocks typically involves redox reactions, the energetic cost of their synthesis depends on the oxidation state of the environment. Assuming chemoautotrophic synthesis of all cellular building blocks, McCollom and Amend (2005) calculated that the energetic cost of building block synthesis per cell is at least one order of magnitude higher under microoxic conditions with 10% O_2 saturation compared to anoxic conditions typical of methanogenic environments (Table 1). This finding may help explain why (a) cell-specific maintenance energies are higher in aerobic compared to anaerobic bioreactors (Tijhuis, van Loosdrecht and Heijnen 1993), (b) the expression of starvation-related symptoms is delayed under anaerobic compared to aerobic conditions in starvation experiments (Roslev and King 1994) and (c) calculated BPRs in oxic subsurface sediments are likely 30-fold higher than in sulfate-reducing, anoxic subsurface sediments (this study, section 'Field-based studies of the BPR').

Among the main cellular components, proteins account for the largest portion of total biomass and C content, and also require the biggest energetic expenditures, requiring 56.6% of the total energy required for monomer synthesis under microoxic conditions, and 44.1% under methanogenic conditions (McCollom and Amend 2005, Table 1B). While lipid monomer synthesis is the next largest energy sink in aerobes (19.8%), RNA nucleotide synthesis is the second biggest energy sink in anaerobes (32.8%). This shows that the energetic cost of cellular component synthesis is affected differently by the redox state of the environment. This is also reflected in the ratios of energetic costs of cellular components. While all cellular components require higher energy inputs for monomer synthesis under oxic compared to anoxic conditions, the ratio of energetic cost varies between oxic and anoxic settings. For instance, based on our calculations, lipid synthesis is energetically 34 times more costly under oxic compared to anoxic conditions, while RNA synthesis is only 5.2 times more costly (Table 1B).

Macromolecule synthesis. Since the main cellular components are macromolecules, e.g. produced by polymerization of monomers, and most reactions involved in macromolecule assembly consume energy, calculations of the energetic cost of cell synthesis are incomplete without including these energetic costs, which have only been quantified in a few studies (Stouthamer 1973; Neidhardt, Ingraham and Schaechter 1990).

As polymer synthesis typically does not involve redox reactions, the energetic cost of polymerization should be comparable under oxic and anoxic conditions. Moreover, not all polymerization reactions are believed to be endergonic; for instance, the formation of saccharide chains, such as lipopolysaccharides and glycogen, may proceed without energy investment in *E. coli* (Neidhardt, Ingraham and Schaechter 1990; Table 2). This is not true of proteins, nucleic acids, lipids and murein, all of which consume energy in the form of energy-rich phosphate (Table 2). Among these groups of cellular macromolecules, the polymerization of protein consumes by far the most energy (~90%), followed by RNA (~7%) and DNA (~1.5%), whereas lipid and murein synthesis appear to each consume <1% of the total energy spent on macromolecule formation (Table 2).

In protein and nucleic acid polymerizations, a major energy investment is the initial activation of amino acids, ribonucleotides and deoxyribonucleotides (Neidhardt, Ingraham and Schaechter 1990). This activation consists of the conversion of amino acids to aminoacyl-tRNAs, nucleotide monophosphates to nucleotide triphosphates and deoxynucleotide monophosphate to deoxynucleotide triphosphates, respectively. In the synthesis of proteins, the incorporation of aminoacyl-tRNAs to peptides is equally energy consuming as the initial activation, whereas proofreading, assembly and modification require comparatively minor energy inputs. For RNA formation, removal of segments or primary transcripts, as well as post-transcriptional modification also require energy input, whereas in DNA synthesis unwinding of the DNA helix, discontinuous synthesis, proofreading and postreplication repair, negative supercoiling and methylation add to the energetic cost and have been quantified to a greater or lesser extent. Synthesis of lipids and murein appears less complex and energetically less costly than synthesis of proteins and nucleic acids.

Comparing the energetic costs of monomer synthesis and polymerization at a per cell level, it appears that—under oxic conditions—the cost of monomer synthesis vastly exceeds that

Table 1. (**A**) Cellular contents of various cellular building blocks, and the energetic costs of their biosynthesis under oxic and anoxic conditions. (**B**) Overview of absolute and % energetic cost of synthesis of main cellular components under oxic and anoxic conditions, as well as ratios of absolute energetic costs.

(A)

			Monomers cell-1		C contents per cell		Oxic		Anoxic	
Cellular components	Building blocks (monomers)	mg (g cells)$^{-1}$ [a]	mol cell^{-1} [c]	mono-mers cell^{-1}	C atoms mono-mer^{-1}	C atoms cell^{-1}	ΔGr' (kJ mol^{-1})	ΔGr' (kJ cell^{-1})	ΔGr' (kJ mol^{-1})	ΔGr' (kJ cell^{-1})
Protein	*Amino Acids*									
	Alanine	42.2	1.4E-17	8.3E+06	3	2.5E+07	1329	1.8E-14	67	9.2E-16
	Arginine^{+}	42.7	7.1E-18	4.3E+06	6	2.6E+07	2671	1.9E-14	362	2.6E-15
	Asparagine	26.4	5.8E-18	3.5E+06	4	1.4E+07	1423	8.2E-15	163	9.4E-16
	Aspartate	26.6	5.8E-18	3.5E+06	4	1.4E+07	1360	7.9E-15	101	5.8E-16
	Cysteine	9.2	2.2E-18	1.3E+06	3	4.0E+06	1181	2.6E-15	131	2.9E-16
	Glutamate	35.6	7.0E-18	4.2E+06	5	2.1E+07	2001	1.4E-14	111	7.8E-16
	Glutamine	31.8	6.3E-18	3.8E+06	5	1.9E+07	2060	1.3E-14	170	1.1E-15
	Glydne	38.1	1.5E-17	8.9E+06	2	1.8E+07	678	1.0E-14	48	7.1E-16
	Histidine	12.2	2.3E-18	1.4E+06	6	8.2E+06	2398	5.5E-15	299	6.8E-16
	Isoleucine	31.6	7.0E-18	4.2E+06	6	2.5E+07	3277	2.3E-14	128	8.9E-16
	Leucine	48.9	1.1E-17	6.5E+06	6	3.9E+07	3268	3.5E-14	118	1.3E-15
	Lysine^{+}	41.6	8.2E-18	5.0E+06	6	3.0E+07	3119	2.6E-14	179	1.5E-15
	Methionine	19.0	3.7E-18	2.2E+06	5	1.1E+07	2295	8.5E-15	–14	–5.2E-17
	Phenylalanine	25.4	4.4E-18	2.7E+06	9	2.4E+07	4383	1.9E-14	184	8.2E-16
	Proline	21.1	5.3E-18	3.2E+06	5	1.6E+07	2434	1.3E-14	124	6.6E-16
	Serine	18.7	5.2E-18	3.1E+06	3	9.4E+06	1180	6.1E-15	130	6.7E-16
	Threonine	25.0	6.1E-18	3.7E+06	4	1.5E+07	1837	1.1E-14	157	9.6E-16
	Tryptophan	9.6	1.4E-18	8.2E+05	11	9.0E+06	5130	7.0E-15	301	4.1E-16
	Tyrosine	20.7	3.3E-18	2.0E+06	9	1.8E+07	4205	1.4E-14	216	7.2E-16
	Valine	23.6	1.0E-17	6.1E+06	5	3.1E+07	2623	2.7E-14	103	1.0E-15
	Total amino acids	550	1.3E-16	7.9E+07	N/A	3.8E+08		2.9E-13		1.7E-14
Nucleic Acids	*Nucleotides*									
RNA	AMP^{2-}	54.4	4.5E-18	2.7E+06	10	2.7E+07	4031	1.8E-14	882	4.0E-15
	GMP^{2-}	38.6	3.5E-18	2.1E+06	10	2.1E+07	3780	1.3E-14	841	2.9E-15
	CMP^{2-}	69.9	5.6E-18	3.4E+06	9	3.0E+07	3824	2.1E-14	674	3.8E-15
	UMP^{2-}	41.9	3.7E-18	2.3E+06	9	2.0E+07	3761	1.4E-14	612	2.3E-15
	Total RNA	205	1.7E-17	1.0E+07	N/A	9.9E+07		6.7E-14		1.3E-14
DNA	$dAMP^{2-}$	7.7	6.8E-19	4.1E+05	10	4.1E+06	4188	2.9E-15	830	5.7E-16
	$dGMP^{2-}$	7.3	6.8E-19	4.1E+05	10	4.1E+06	3936	2.7E-15	787	5.4E-16
	$dCMP^{2-}$	8.3	6.8E-19	4.1E+05	9	3.7E+06	3980	2.7E-15	620	4.2E-16
	$dTMP^{2-}$	7.4	6.5E-19	3.9E+05	10	3.9E+06	4551	3.0E-15	560	3.7E-16
	Total DNA	31	2.7E-18	1.6E+06	N/A	1.6E+07		1.1E-14		1.9E-15
Lipid	*Fatty acid anions*									
	Palmitate	32.4	3.7E-18	2.2E+06	16	3.5E+07	9912	3.6E-14	254	9.3E-16
	Oleate	19.9	2.0E-18	1.2E+06	18	2.2E+07	11058	2.3E-14	350	7.2E-16
	Palmitoleate	24.7	2.8E-18	1.7E+06	16	2.7E+07	9758	2.7E-14	310	8.7E-16
	Myristate	4.3	5.6E-19	3.4E+05	14	4.7E+06	8612	4.8E-15	214	1.2E-16
	β-Hydroxymyristate	9.3	1.1E-18	6.8E+05	14	9.5E+06	8468	9.5E-15	280	3.1E-16
	Total fatty acids	91	1.0E-17	6.1E+06	N/A	9.9E+07		1.0E-13		2.9E-15
Saccharides[b]	*Monosaccharides*									
Lipopoly-saccharide (LPS)	Glycerol	22.1	1.0E-17	6.2E+06	3	1.9E+07	1600	1.7E-14	130	1.3E-15
	Heptose	7.7	1.6E-18	9.7E+05	7	6.8E+06	3334	5.4E-15	395	6.4E-16
	Galactose	2.2	5.4E-19	3.3E+05	6	2.0E+06	2861	1.5E-15	342	1.9E-16
	Rhamnose	2.0	5.4E-19	3.3E+05	6	2.0E+06	2906	1.6E-15	290	1.6E-16
	Total LPS	34.0	1.3E-17	7.9E+06	N/A	2.9E+07		2.5E-14		2.3E-15
Murein	Glucoseamine	3.9	6.5E-19	3.9E+05	6	2.3E+06	3020	1.9E-15	386	2.5E-16
	N-Acetylglucosamine	10.5	1.4E-18	8.2E+05	8	6.6E+06	3803	5.2E-15	443	6.1E-16
	N-Acetylmuramic acid	10.6	1.1E-18	6.4E+05	11	7.0E+06	5201	5.5E-15	582	6.2E-16
	Total murein	25.0	3.1E-18	1.9E+06	N/A	1.6E+07		1.3E-14		1.5E-15
Glycogen	Glucose	25.0	1.4E-18	8.6E+05	6	5.2E+06	2855	4.1E-15	335	4.8E-16
	Total glycogen	25.0	1.4E-18	8.6E+05	N/A	5.2E+06		4.1E-15		4.8E-16
	Total saccharides	84.0	1.8E-17	1.1E+07	N/A	5.1E+07		4.2E-14		4.3E-15

Table 1 (*continued*.)

Cellular components	Building blocks (monomers)	mg (g cells)$^{-1}$ [a]	Monomers cell-1: mol cell^{-1} [c]	Monomers cell-1: monomers cell^{-1}	C contents per cell: C atoms monomer^{-1}	C contents per cell: C atoms cell^{-1}	Oxic: ΔGr' (kJ mol^{-1})	Oxic: ΔGr' (kJ cell^{-1})	Anoxic: ΔGr' (kJ mol^{-1})	Anoxic: ΔGr' (kJ cell^{-1})
Other										
	Ethanolamine	N/A	N/A		2		N/A	N/A	N/A	N/A
	Diaminopimelic acid	N/A	N/A		7		N/A	N/A	N/A	N/A
	Putrescine	N/A	N/A		4		N/A	N/A	N/A	N/A
	Spermidine	N/A	N/A		7		N/A	N/A	N/A	N/A
	Total amines	N/A	N/A		N/A		N/A	N/A	N/A	N/A
TOTAL		960						5.1E-13		4.0E-14

(B)

	Oxic: ΣΔG' (kJ cell^{-1})	Oxic: %	Anoxic: ΣG' (kJ cell^{-1})	Anoxic: %	Ratio cost: Oxic:Anoxic
Protein	2.9E-13	56.6	1.7E-14	44.1	16.5
RNA	6.7E-14	13.1	1.3E-14	32.8	5.2
DNA	1.1E-14	2.2	1.9E-15	4.8	5.9
Lipid	1.0E-13	19.8	2.9E-15	7.5	34.1
Lipopoly- saccharide	2.5E-14	4.9	2.3E-15	5.9	10.8
Murein	1.3E-14	2.5	1.5E-15	3.7	8.6
Glycogen	4.1E-15	0.8	4.8E-16	1.2	8.5
Sum	5.1E-13	100	4.0E-14	100	Mean: 12.9

[a]Assumes the same proportions of cellular building blocks as in McCollom and Amend (2005), but the weight percentages from Neidhardt, Ingraham and Schaechter (1990). Thus, for amino acids, individual amino acid contents were adjusted proportionally to reflect a cellular protein content of 55.0% instead of 63.1% by multiplying the value of each building block in mg (g cells^{-1}) by 55.0%/63.1%. These conversions to the values from Neidhardt *et al.* were done to make calculations of the energetic cost of polymerization, which were based on the Neidhardt *et al.* values, comparable.
[b]Saccharides were subdivided as in Neidhardt, Ingraham and Schaechter (1990) into lipopolysaccharides, murein, and glycogen. All compounds under 'Other' were ignored in further calculations.
[c]Assumes estimated median cell-specific dry weight of subseafloor microbes of 29 fg, from Kallmeyer *et al.* (2012).

of polymerization. Under the assumed conditions, ~95% of the energy spent on cell synthesis is spent on the synthesis of building blocks, and only ~5% on polymerization (Fig. 7A). Hereby especially the energetic cost of lipid and murein monomer synthesis greatly exceeds that of polymerization, by factors of ~470 and ~98, respectively, whereas the energetic cost of protein, RNA and DNA building block synthesis is only ~13, ~38 and ~30 times higher than the cost of building block polymerization. By comparison, under anoxic conditions, the total energetic cost of building block synthesis appears to be only 1.6-fold higher than the energetic cost of polymerization, with amino acid polymerization even requiring more energy than amino acid synthesis (Fig. 7B).

Uncertainties in our calculations of the energetic cost of biosynthesis

Several factors result in uncertainties with respect to the calculated cost of biomolecule synthesis. For instance, the calculated cost of building block synthesis is based on reduced nitrogen (NH_4^+) and sulfur (H_2S) sources for N and S requirements (as in McCollom and Amend 2005) under both aerobic and anaerobic conditions. However, microbes inhabiting oxic environments may utilize more oxidized N and S sources, such as NO_3^- and SO_4^{2-}, which would further drive up the cost of building block synthesis. Furthermore, the calculated cost of building block synthesis assumes autotrophic synthesis from solely inorganic building blocks. Yet, many/most microbes in the environment may utilize low-molecular weight organic precursors, e.g. acetate, to synthesize these building blocks, or even take up these building blocks directly from their surrounding environment (Takano *et al.* 2010). Synthesis from organic precursors could considerably lower the energetic cost of building block synthesis, whereas direct uptake would bring it to zero (apart from the energetic cost of import into the cell). Similarly, cells might be able to considerably lower the cost of polymerization by importing and directly utilizing oligomers, as long as these are sufficiently small to be imported into the cell (≤600 Da; Weiss *et al.* 1991). Thus, there is considerable uncertainty with respect to how much, or even if, the energetic cost of building block synthesis is indeed higher than that of polymerization under all or most conditions in the environment.

While, despite uncertainties with respect to precursor molecules, the energetic cost of monomer synthesis can be constrained based on thermodynamic constants, and measurements and minimum estimates of reactant and product concentrations in natural samples, the energetic cost of polymerization is more difficult to quantify. The range of polymers synthesized within cells is vast, and proceeds via many different anabolic pathways, many or even most of which are likely to be unknown. The energetic cost of post-synthesis modifications, e.g. methylations of certain amino acids (Paik, Paik and Kim 2007), folding of proteins into their tertiary and quaternary configurations

Table 2. Energetic cost of polymerization calculated for a cell with a monomeric content as in Table 2.

Macromolecules	Monomers mol cell^{-1}	Mol ~P (mol reaction)$^{-1}$ [a]	Energetic cost of polymerization (g cells)$^{-1}$		Energetic cost of polymerization cell^{-1}		
			mol ~P	kJb	mol ~P	kJb	%
Protein	1.3E-16		1.9E-02	7.8E-01	5.4E-16	2.3E-14	90.1
Activation (amino acids to aminoacyl-tRNAs)	1.3E-16	2	9.0E-03	3.8E-01	2.6E-16	1.1E-14	43.7
Incorporation (aminoacyl-tRNAs to peptides)	1.3E-16	2	9.0E-03	3.8E-01	2.6E-16	1.1E-14	43.7
Proofreading	1.3E-16	0.1	4.5E-04	1.9E-02	1.3E-17	5.5E-16	2.2
Assembly and modificationv	1.3E-16	0.024	1.1E-04	4.5E-03	3.1E-18	1.3E-16	0.5
RNA	1.7E-17		1.4E-03	6.0E-02	4.2E-17	1.7E-15	7.0
Activation (NMPs to NTPs)	1.7E-17	2	1.2E-03	5.0E-02	3.5E-17	1.5E-15	5.8
Discarded segments of primary transcripts	3.3E-18	2	2.3E-04	9.6E-03	6.7E-18	2.8E-16	1.1
Modification	3.8E-19	1	1.3E-05	5.5E-04	3.8E-19	1.6E-17	0.1
DNA	2.7E-18		3.1E-04	1.3E-02	9.1E-18	3.8E-16	1.5
Activation (dNMPs to dNTPs)	2.7E-18	2	1.9E-04	7.8E-03	5.4E-18	2.3E-16	0.9
Unwinding of helix	1.3E-18	2	9.3E-05	3.9E-03	2.7E-18	1.1E-16	0.5
Discontinuous synthesis	1.3E-21	12	5.6E-07	2.3E-05	1.6E-20	6.8E-19	0.0
Proofreading & postreplication repair	4.7E-19	2	3.2E-05	1.4E-03	9.4E-19	3.9E-17	0.2
Negative supercoiling	N/A	ND	4.7E-07	1.9E-05	1.3E-20	5.7E-19	0.0
Methylation	N/A	ND	9.3E-08	3.9E-06	2.7E-21	1.1E-19	0.0
Lipid	1.0E-17	0.5	1.8E-04	7.4E-03	5.1E-18	2.1E-16	0.9
Lipopolysaccharide	1.3E-17	0	0	0	0	0	0
Murein[a]	3.1E-18	1	1.1E-04	4.4E-03	3.1E-18	1.3E-16	0.5
Glycogen[e]	1.4E-18	0	0	0	0	0	0
TOTAL			2.1E-02	8.6E-01	6.0E-16	2.5E-14	100.0

[a]All values taken from Neidhardt, Ingraham and Schaechter (1990).

[b]Assumes the energetic cost of one energy-rich phosphate (~P) to equal the energetic cost of ADP phosphorylation, which was previously determined to be 41.9 kJ mol^{-1} (Tran and Unden 1998); thus, we assume in all calculations that $\Delta G\sim P = \Delta GADP\text{->}ATP = \Delta GAMP\text{->}ADP = \Delta GGDP\text{->}GTP = 41.9$ kJ mol^{-1}.

(Onuchic *et al.* 1997), or post-transcriptional regulation of RNA (see section 'RNA'), remain guesswork, and were not included in previous estimates (e.g. Neidhardt, Ingraham and Schaechter 1990).

Further unknowns are the impacts of environmental variables, such as temperature and monomer concentrations, on the pathways and energetic costs of various polymerization reactions. Could polymerization of RNA and proteins occur without energetic cost, albeit controlled via the known mechanisms of transcription and translation, within cells in ways analogous to spontaneous and abiotic mechanisms of RNA and amino acid polymerization outside the cell (Leman, Orgel and Ghadiri 2004; Powner, Gerland and Sutherland 2009; Pino *et al.* 2011)? Energy would presumably still be necessary for the intracellular machinery to select and determine the monomer sequence that is essential to RNA or protein function. Or does intracellular polymerization instead always occur through building blocks that have been activated in the same energetically costly way? In the latter case, does this activation always cost the same amount of ATP, and do published data (e.g. Tran and Unden 1998; LaRowe and Helgeson 2007) accurately represent the range of energies spent by cells per ATP? Even if polymerization is energetically favorable and occurs at zero energetic cost within the cell, energetic costs will be associated with the hydrolysis of polymers (depolymerization), which is required for the breakdown and repair of defect enzymes, nucleic acids, lipids and other essential biomolecules. The potential energetic cost of these repair mechanisms is discussed next.

Cell damage by abiotic reactions

A substantial fraction of the energy spent by cells under extreme energy limitation is likely to go into maintenance operations, such as repair and replacement of damaged biomolecules, cross-membrane transport of ions, nutrients and/or energy substrates, or compensation for substrate or membrane potential leakage. The accurate quantification of energy cells spend on these maintenance operations is paramount to determining the BPR.

We will here (this section, and sections 'Energetic cost of racemization' and 'Energetic cost of depurination') focus on amino acid racemization and DNA depurination (for definitions see the section 'Glossary'). Proteins can be damaged by reactions besides racemization, e.g. deamidation, isomerization (Ishikawa and Clarke 1998; Clarke 2003), and DNA can be damaged by reactions other than depurination, e.g. hydrolysis, methylation, cross-linking, oxidation (Lindahl 1993; Morita *et al.* 2010; Vågbø *et al.* 2013). Repair of biomolecules other than protein or DNA is also likely to cost significant amounts of energy. For instance, RNA is also damaged by depurination reactions—albeit at lower rates than DNA (Lindahl 1993)—in addition to cleavage, methylation, RNA–RNA and RNA–protein cross-linking, and at least some of this damaged RNA is repaired (Chan, Zhou and Huang 2009, Wurtmann and Wolin 2009, Vågbø *et al.* 2013). Racemization is also not limited to amino acids, but also occurs in other chiral molecules, including DNA and RNA nucleotides and sugars (Bada and Miller 1987). Damage and damage repair also occur in lipids (Foreman-Wykert, Weiss and Elsbach 2000; Imlay 2013).

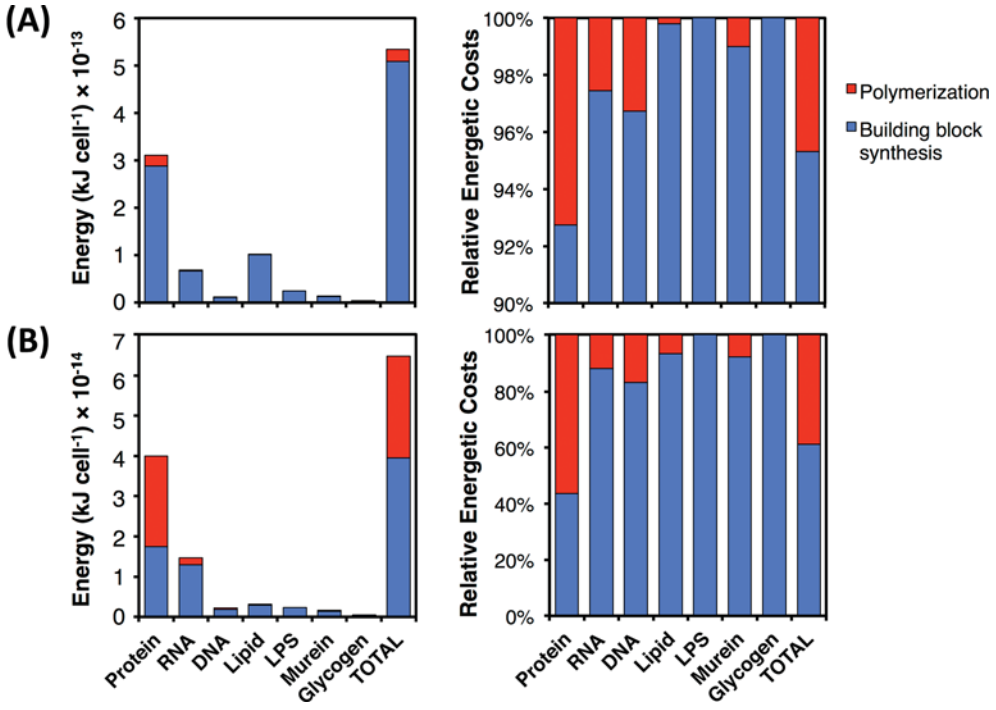

Figure 7. Quantitative comparison of the energetic costs of monomer synthesis and polymerization required for the synthesis of a cell using data from Tables 1 and 2. (**A**) Oxic environments and (**B**) anoxic environments. We assume that the energetic cost of polymerization is the same under oxic and anoxic conditions, but that the energetic cost of monomer synthesis is highly different (data as calculated and shown in Tables 1 and 2). Note the differences in data ranges between the left panels of A and B and the right panels of A and B.

Due to limited data on membrane leakage, energetic cost of cross-membrane transport, and rates of biomolecule-damaging reactions other than amino acid racemization and DNA depurination, we do not discuss these data further. Despite these limitations, our calculations based on published rate constants of racemization and depurination provide insights into the factors that may ultimately control the energetic cost of survival and consequently microbial population size in severely energy-limited systems.

Amino acid racemization rates

Of the abiotic processes known to cause biomolecule damage, amino acid racemization has garnered the most attention to date, perhaps because it is considered to be energetically most costly. Racemization rate constants have been determined for free amino acids (Bada 1982), and for amino acids that were likely to be bound in peptides and proteins (Engel, Zumberge and Nagy 1977; Steen, Jørgensen and Lomstein 2013). Racemization rate constants were higher in proteins and natural samples than in single amino acids (Kriausakul and Mitterer 1978, Steen, Jørgensen and Lomstein 2013). Since the bulk of amino acids within microbial cells is bound in proteins, racemization rate constants based on protein-bound amino acids may better reflect racemization rates within cells.

Examining racemization rate constants across the known temperature limits of life (−40 to +120°C) (Price and Sowers 2004), it is clear that racemization rates are driven by temperature (Fig. A5A, Supporting Information). Racemization rates of the four amino acids examined deviate from one another by one order of magnitude or less when cross compared at any given temperature. Yet, when examined individually and extrapolated across the entire temperature range, rate constants span six to eight orders of magnitude, suggesting 1–100 million times higher rates of racemization damage at 120°C than at −40°C (Fig. A5A, Supporting Information). Based on this relationship with temperature, it can be calculated that on average every amino acid per cell is racemized once per year at temperatures of 80–103°C (Fig. A5B, Supporting Information). By contrast, at temperatures of 15–31°C, on average every amino acid per cell is racemized only once every 1000 years. Of the amino acids compared, 'aspartic acid + asparagine' consistently has the highest racemization rates, whereas 'glutamic acid + glutamine' and alanine have the lowest racemization rates, with 'glutamic acid + glutamine' having the lowest rates below ∼60°C, and alanine having the lowest rates above ∼60°C.

DNA depurination rates

Examining depurination rate constants across the known temperature limits of life (−40 to +120°C) indicates that depurination rates increase exponentially with temperature, showing an order of magnitude increase with approximately every 16°C increase (Fig. A6A, Supporting Information). As a result, at a temperature of 84°C nucleotides are on average depurinated once per year. By contrast, at 37°C nucleotides are on average depurinated only once per millennium, and at –9.2°C nucleotides are on average depurinated only once per million years (Fig. A6B, Supporting Information).

In addition to being controlled by temperature, the cell-specific energetic costs of amino acid racemization and DNA depurination depend on redox conditions, as a result of the higher energetic cost of amino acid and nucleotide synthesis under oxic compared to anoxic conditions (also see sections 'Energetic cost of racemization' and 'Energetic cost of depurination'). Moreover, proteome and genome size play a role, with the number of amino acid racemization and DNA depurination reactions per cell under a given set of temperature and redox conditions

being directly correlated with proteome and genome size, respectively.

Life at energy turnover rates approaching the BPR

With respect to the existing literature on life under energetic conditions approaching the BPR, there is a wide discrepancy between laboratory- and field-based investigations. As mentioned earlier (section 'Physiological states of microbial life'), microbial 'maintenance energy' requirements inferred from chemostat experiments are orders of magnitude higher than BPR estimates from long-term energy-limited systems, suggesting that laboratory studies have in most cases overestimated the energy required by single cells under energy limitation. Nonetheless, the potential for laboratory-based investigations to eventually provide accurate quantitative measures of cell-specific energy turnover requirements of single, energy-limited cells is greater than for field-based investigations. In the following section, we consequently first discuss suitable techniques for studies of the BPR in the laboratory, and then—based on published data—present estimates of the BPR in long-term energy-limited subseafloor sediments. We conclude with a compilation of laboratory- and field-based estimates of BPRs and compare these to modeled energetic burdens imposed by amino acid racemization and DNA depurination reactions.

Laboratory-based techniques to study long-term energy limitation

Laboratory-based experiments are critical to understanding microbial energy requirements, as they have the advantage of lower complexity and better control over growth and energy conditions compared to environmental studies. Even simple incubation setups, such as batch reactors, can provide important insights to life under energy limitation. Small-volume biological samples in microbially impassable but chemically permeable vessels, e.g. dialysis bags (Blom *et al.* 2010), suspended in large reservoirs of low-energy media, can be used to study energy-limited microbes under relatively constant energy availability for extended time periods. The use of batch reactors two decades ago, moreover, resulted in the discovery of a new phase in the bacterial life cycle, the so-called long-term stationary phase (Finkel and Kolter 1999). In incubations where no energy substrates are added except initially, this phase is fifth after the well-documented lag, exponential, stationary and death phases (Finkel 2006). During the long-term stationary phase, stable population sizes persist over the course of months to years in the absence of substrate addition (Finkel 2006), and may provide insights into the survival of microbial populations that are cut off from fresh energy supplies over long time periods. Yet, quantitative estimates of energy consumption by microbes in the long-term stationary phase are still rare (Riedel *et al.* 2013). A disadvantage of batch reactors compared to continuous-flow reactors is that *in vitro* energy inputs, energy yields and chemical changes to media, such as build-up of (toxic) metabolic products over time, cannot be regulated by adjusting flow rates and media composition.

Recycling fermenters ('retentostats'), i.e. continuous-flow reactors where cells are retained by a filter membrane (Tappe *et al.* 1996, 1999; Colwell *et al.* 2008; Davidson *et al.* 2009), have an advantage over chemostats or batch reactors in that they enable both biomass retention and control of substrate concentrations. As with batch reactors and chemostats, a limitation of conventional retentostat models is that, due to the incubation volumes of milliliters (Tappe *et al.* 1996) to liters (Boender *et al.* 2009), only average energy consumption per cell, not energy consumption by individual cells, can be determined. It is simply not possible to ensure even energy availability per cell in large incubation volumes because of heterogeneities in fluid flow, e.g. due to edge effects, and cell distributions, e.g. due to wall growth, biofilm or particle formation (Jannasch 1967). As a result, energy availability per cell is potentially highly variable, with cells in certain locations dying of starvation, while others are growing and dividing at high rates. To complicate things further, genetically identical cells express individual differences in phenotype, known as phenotypic heterogeneity or phenotypic noise—even when grown in a homogeneous environment (Elowitz *et al.* 2002; Umehara *et al.* 2007). Thus, the average energy turnover per cell does not provide reliable insights into the BPR or maintenance energy turnover of individual cells within a population.

More promising than conventional large-volume batch or flow-through reactors in terms of studying cell-specific energy turnover are microfluidics-based chemostats and retentostats (Yeo *et al.* 2011; Yin and Marshall 2012). Using microfluidics chambers and chip devices—also known as lab-on-a-chip (LOC) devices—(so far) ranging from microliters down to picoliters in chamber volumes, single microbial cells can be distributed to individual chambers and growth conditions controlled on a micrometer scale, where diffusion and thermal conductivity are fast enough to eliminate microgradient formation (Fig. 8; Lee *et al.* 2011; Grünberger *et al.* 2012). Thereby the response of individual microbial cells to variables, such as substrate and electron acceptor concentration, or temperature, can be studied across replicates under stringently controlled conditions. Moreover, by isolating single cells prior to cultivation, competition is eliminated. Consequently, enrichment of slow-growing, pigritrophic microbes might be facilitated in laboratory media in which these organisms would otherwise be outcompeted by faster growing, opportunitrophic strains (Fig. 8A and B, right bottom panels).

Currently, a common challenge with microfluidics chips is that, due to volume reduction, long flow times are necessary to obtain outflow volumes large enough for 'off-chip' chemical analyses. This imposes limitations on the sampling frequency and ability to monitor activity within flow chambers. Moreover, sensitive techniques to study the identity and molecular composition of single cells, such as polymerase chain reaction (PCR) and DNA sequencing, Fluorescence In Situ Hybridization (FISH), Fluorescence-Activated Cell Sorting or nanoSIMS are destructive and thus not suitable for growth monitoring over the course of incubations (Grünberger *et al.* 2012, 2013; Yin and Marshall 2012). These challenges are likely to be overcome with the development of analytical tools, such as optical probes and microsensors, such as atomic force mass sensors, with sufficient sensitivity to perform real-time concentration or molecular composition measurements 'on-chip'. New microfluidics chips with integrated temperature, pH, O_2 and CO_2 analytical probes demonstrate the feasibility of these non-destructive tools, which have the added advantage over conventional assays that sample preparation is not necessary (Acha *et al.* 2000; Greener, Abbasi and Kumacheva 2012). Combining flow cytometry with microfluidics, the non-destructive sorting and isolation of single cells has become possible via 'microfabricated fluorescence-activated cell sorters (Takahashi *et al.* 2004; Mao *et al.* 2012). First applications of attenuated total reflection Fourier transform infrared probes suggest that on-chip monitoring of low concentrations of reactants and products will be possible in

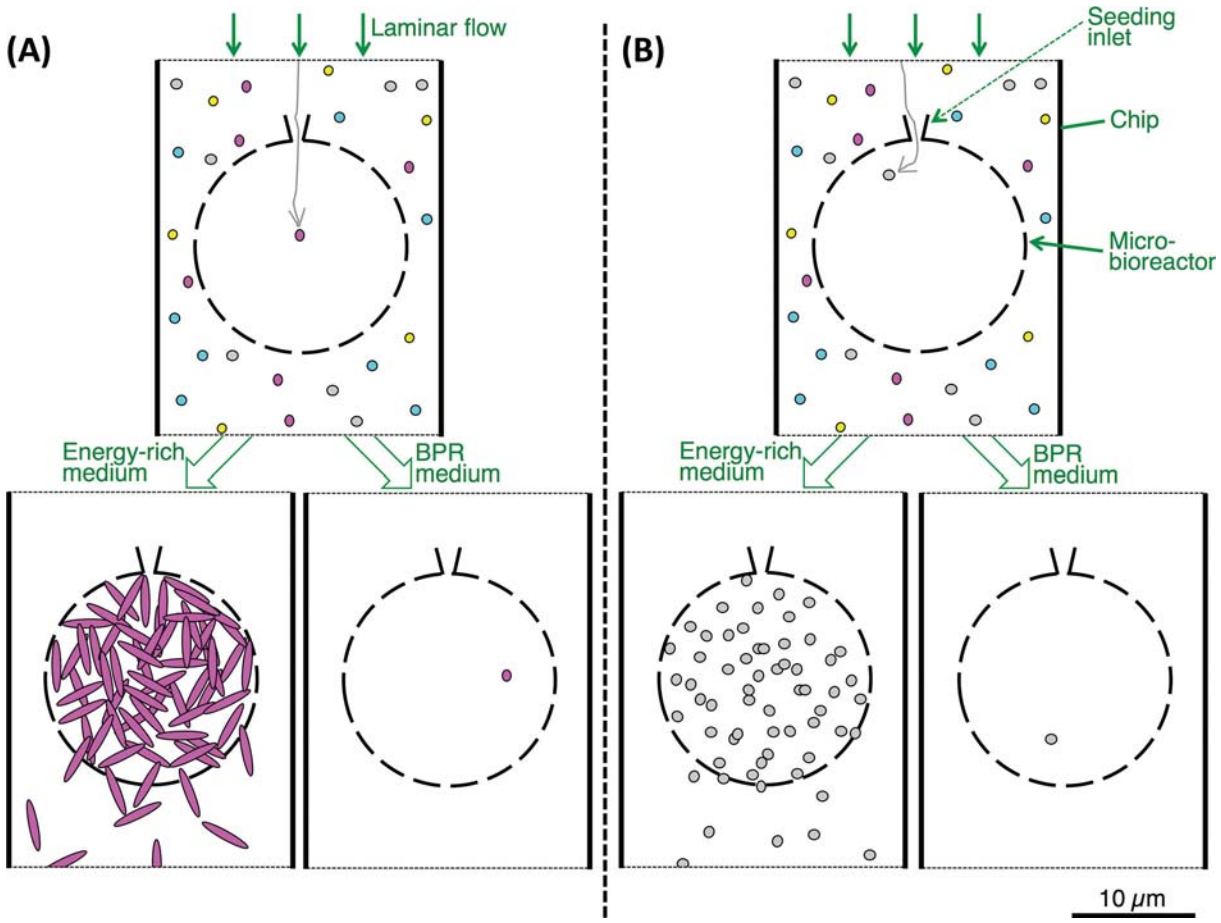

Figure 8. Cultivation of single cells in microbioreactor (based on Grünberger *et al.* 2012). A cell suspension, e.g. consisting of a cell extract from an energy-limited environmental sample, is pumped through microfluidics channels into LOC devices. A seeding inlet facing the incoming flow traps a cell from the cell suspension into a microbioreactor. Overflow channels result in outflow of cells, if populations reach a threshold size (bottom left panels in A and B; for more info, see Grünberger *et al.* 2012). Under scenario (**A**), the trapped cell is of the CSR type, i.e. if it is incubated with energy-rich medium, the cell increases dramatically in size, switches from coccoid to rod-shaped morphology, and divides (bottom left panel). If this CSR type cell is incubated in low-energy medium that supports the BPR, but no growth or cell division, the cell retains its original coccoid morphology (bottom right panel). Under scenario (**B**), the trapped cell is of the OSR type. Thus, even after incubation in energy-rich medium, the cell retains its small size and coccoid morphology (left panel). Nonetheless, growth and cell division both occur. If this OSR type cell is incubated in low-energy medium that supports the BPR, but no growth or cell division, the outcome is as in scenario (A): no visible change occurs in cell morphology, and the cell is visually indistinguishable from an CSR type cell.

the near future (Zaera 2012). Other useful tools include Raman microspectroscopy and Raman microscopy, by which element/isotope incorporation into biomolecules of living cells (Huang *et al.* 2004, 2007), molecular constituents of cells (Ivleva *et al.* 2009), and chemical changes in media and substrata can be monitored (Fletcher, Haswell and Zhang 2003; Zaera 2012)—all non-destructively.

Tools for real-time monitoring of microbial populations that can be incorporated into these LOC applications include fluorescent stains and antibodies, and have often been applied in combination with flow cytometry. Fluorescent stains can be added to distinguish live from dead cells (Boulos *et al.* 1999; Ferrari and Gillings 2009), identify viable and physiologically active cells (Berney *et al.* 2007), detect activities of specific enzymes (Kalyuzhnaya, Lidstrom and Chistoserdova 2008; Ashida *et al.* 2010), test membrane integrity (Hoefel *et al.* 2003) or identify Gram-positive cells (Holm and Jespersen 2003). Membrane potentials can also be measured and manipulated to study uptake mechanisms and uptake rates (Farinas, Chow and Wada 2001), or to identify dormant and germinated spores (Laflamme *et al.* 2005; Magge *et al.* 2009). Antibodies tagged with fluorophores can be used to identify microbes with cell wall, capsular or flagellar antigens (Brehm-Stecher and Johnson 2004), or detect specific microbes or viruses (e.g. Ferrari *et al.* 1999; Li, He and Jiang 2010; reviewed in Ishii, Tago and Senoo 2010). Even techniques without any form of labeling, such as the CASY (Cell Counter + Analysis) technology, with high-throughput differentiation of live from dead cells across microbial populations based on electric current exclusion, may in the future be miniaturized for LOC applications at the single-cell level.

As tools of real-time chemical and biochemical monitoring in microbioreactors continue to evolve, it will eventually become possible to monitor catabolic and anabolic activity of energy-limited single cells over the course of incubations. Combined with end-point analyses, such as DNA sequencing, FISH or SIMS (nanoSIMS, time-of-flight-SIMS), with its ability to analyze the atomic and molecular composition of cells in three dimensions at so far unrivaled resolution (Wagner 2009; Musat *et al.* 2012), microbial identity as well as intra- and extracellular constituents of starving cells will be determined. Novel insights to physiological adaptations and phylogenetic differences in the ability to cope with energy limitation will emerge, as will an

understanding of the genetic traits and physicochemical variables that determine the BPR. So-called micro total analysis systems , which have already been widely applied in other fields (Garcia *et al*. 2003; Dittrich and Manz 2005), will create new possibilities for the study of single cells under severe energy limitation, and thereby vastly expand our understanding of the minimum energy requirements of microbial life.

Field-based studies of the BPR

Direct monitoring of energy turnover per cell in the field is currently not possible, and time will show whether it will ever become possible. So far, field studies have relied on calculations of average energy consumption per cell (reviewed in Hoehler and Jørgensen 2013). To reliably determine the average energy consumption per cell in the environment, accurate quantifications of live (active) cell numbers, metabolic rates and *in situ* free energy yields of energy-producing reactions are essential. Based on recent studies of the deep biosphere involving two different quantification methods, total cell numbers in this energy-depleted environment are well constrained (Morono *et al*. 2009); yet, cell numbers of specific phylogenetic groups estimated by FISH, quantitative polymerase chain reaction (qPCR), slot-blot hybridization and intact polar lipids are not always consistent with each other or with total cell counts (Lipp *et al*. 2008; Lloyd *et al*. 2013; Xie *et al*. 2013), and the extent to which inactive or dead cells are included in the various quantification methods is not clear (Riedel *et al*. 2013). Similarly, estimates of activity based on modeling of measured concentration gradients can differ greatly from estimates based on incubations with isotopic tracers (Parkes *et al*. 2005; Wang *et al*. 2008; Jørgensen and Parkes 2010), due to methodological shortcomings and cryptic processes, which cannot be quantified based on measured concentrations (Holmkvist, Ferdelman and Jørgensen 2011). To complicate issues further, energy yields of the vast majority of metabolic reactions in the environment are unknown, due to the absence of sensitive methods to quantify porewater concentrations of most energy substrates and metabolic products. The case of H_2 illustrates that even measured porewater concentrations of well-studied energy substrates can vary significantly depending on the measurement method (Lin *et al*. 2012b). These inconsistencies illustrate that existing methods need to be refined and the range of measurements expanded to enable the average energy turnover of cells inhabiting energy-limited environments to be accurately constrained.

Despite the long list of shortcomings and challenges, fundamental constraints can be made on energy turnover per cell based on measured or modeled electron acceptor gradients and total cell numbers. Given that many subseafloor sediments have been cut off from fresh energy supplies for thousands to millions of years, it seems plausible that microbial communities inhabiting these energy-poor environments are living at cell-specific energy turnover rates close to the BPR. Under this assumption, published data of cell counts and electron acceptor turnover in oxic and anoxic subsurface sediments allow estimates of the BPR of both aerobic and anaerobic microorganisms.

Cell-specific energy turnover in oxic sediments

Cell-specific energy turnover by aerobic metabolism has been calculated by dividing total cell numbers—thus assuming all cells to be performing aerobic metabolism—by measured and modeled O_2 consumption rates (Fig. 9). Based on these data, cell-specific energy turnover decreases by three orders of magnitude within the upper 10 m of marine sediment, and approaches an asymptote below. The cell-specific energy flux at this asymptote, which might reflect the BPR of aerobic microbes, depends on the average Gibbs free energy yield of aerobic metabolism, which is not known, and thus has to be approximated. If cells gain 100 kJ per every mole of O_2 consumed, this BPR lies near 3×10^{-14} kJ cell^{-1} yr^{-1} ($\sim 1 \times 10^{-18}$ W cell^{-1}). On the other hand, if cells gain 1000 kJ mol^{-1} per every O_2 consumed, then this asymptote falls near 3×10^{-13} kJ cell^{-1} yr^{-1} ($\sim 1 \times 10^{-17}$ W cell^{-1}). Though neither the Gibbs free energies of aerobic catabolic reactions nor the main energy substrates of aerobes in subseafloor environments have been determined for low-energy environments, the assumed range appears well inclusive of that of four amino acids (aspartate, glutamic acid, serine, alanine), one monosaccharide (glucose), one lipid building block (palmitate), two nucleotides (AMP^{2-}, $dAMP^{2-}$), acetate and H_2 based on conservative calculations (Fig. A7, Supporting Information). Given that microbial necromass, consisting of these and similar C compounds, and H_2, the product of radiolysis, are plausible main energy sources of aerobic subseafloor microbes, it is likely that our calculated range of cell-specific energy fluxes reflects that of microbes inhabiting oxic subsurface environments.

Cell-specific energy turnover in anoxic sediments

Cell-specific energy turnover by anaerobic metabolism has been calculated by dividing numbers of sulfate reducers, determined by slot blot, FISH, qPCR or assuming that 10% of total microbial communities are sulfate reducers, by measured and modeled electron acceptor, i.e. sulfate, consumption rates (Fig. 10). Due to the lower Gibbs free energies obtained with electron acceptors of anaerobic metabolism, and the fact that anaerobic terminal remineralizing microbes such as sulfate reducers typically metabolize small, organic molecules that are the products of monomer fermentation (rather than remineralizing monomers all the way to CO_2 as is typical of aerobes), we assume Gibbs free energies of –10 and –100 kJ per mol of sulfate. These values are inclusive of Gibbs free energy yields per sulfate determined for sulfate reducers under natural conditions and in chemostats (Hoehler 2004).

As with aerobes, cell-specific energy turnover in sulfate reducers appears to decrease by three orders of magnitude within the upper 10 m of sediment, and approaches an asymptote below (Fig. 10). Assuming Gibbs free energy yields of –10 kJ mol^{-1} of sulfate, this asymptote, which may reflect the BPR of sulfate reducers in cold subsurface sediments, lies around 10^{-15} kJ cell^{-1} yr^{-1} ($\sim 3 \times 10^{-20}$ W cell^{-1}). If the Gibbs free energy yield per sulfate is −100 kJ mol^{-1}, then this asymptote is one order of magnitude higher, around 10^{-14} kJ cell^{-1} yr^{-1} ($\sim 3 \times 10^{-19}$ W cell^{-1}). The agreement of data from different, cold subseafloor settings suggests that the observed trends in BPR might be universal to cold, sulfate-reducing sediment.

Our estimates of the BPR of aerobes and sulfate reducers indicate that sulfate reducers have 30-fold lower BPRs than aerobes. This difference is consistent with the energetic cost of biosynthesis, which is considerably higher under oxic compared to anoxic conditions (as discussed in section 'The energetic cost of biomass synthesis'). Moreover, it is of fundamental importance to point out that the data we use to calculate cell-specific energy turnover are exclusively from environments with *in situ* temperatures of 2–8°C. While these calculated values may apply to the upper (tens of) meters of most marine sediments globally, many environments—including coastal marine sediments—have considerably higher seasonal or year-round temperatures. In these locations, the BPR will almost certainly be seasonally or constantly higher, due to higher rates of biomolecule damage (also

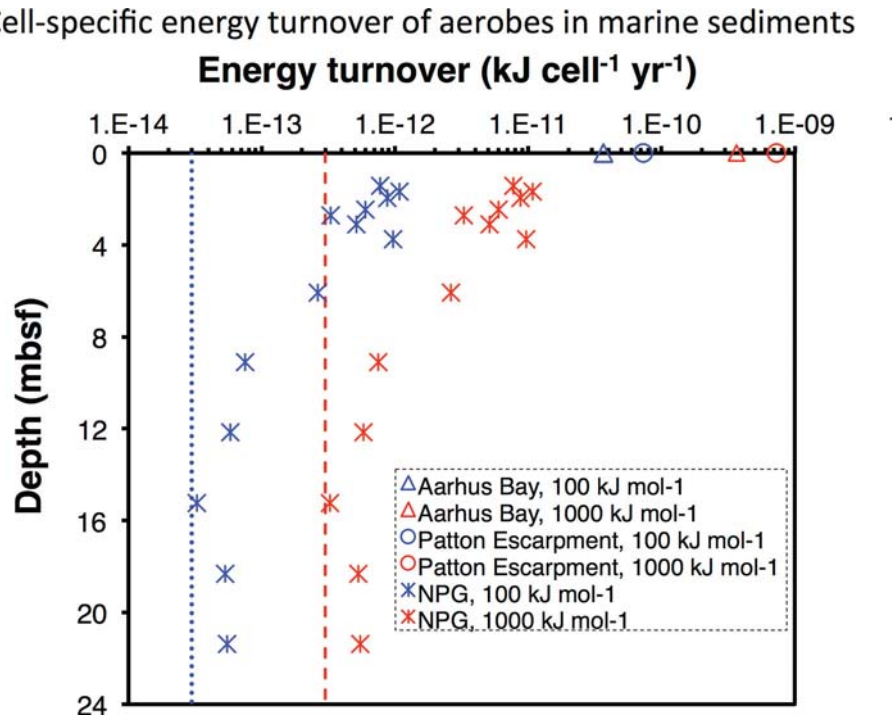

Figure 9. Mean annual energy turnover per cell in oxic marine sediments, calculated by multiplying cell-specific O_2 consumption rates (in mol) by Gibbs free energy yields (per mol) of O_2 consumed. Due to the lack of data on organic substrate concentrations in oxic marine sediments, and resulting absence of *in situ* energy yields, we assume Gibbs free energy yields of –100 (blue symbols, dotted blue line) or –1000 (red symbols, dashed red line) kJ per mole of O_2 consumed. These values are based on calculated Gibbs free energy yields of aerobic remineralization of four amino acids (aspartate, glutamate, serine, alanine), one monosaccharide (glucose), one lipid building block (palmitate), two nucleotides (AMP^{2-}, $dAMP^{2-}$), acetate, and H_2 based on conservative calculations under the reactant and product concentrations assumed by McCollom and Amend (2005) for microaerobic environments (more info in text, section 'Field-based studies of the BPR', and Fig. A7, Supporting Information). Data sources for cell-specific O_2 respiration rates: Aarhus Bay (Rasmussen and Jørgensen 1992), Patton Escarpment (Reimers 1987) and North Pacific Gyre (NPG) (Røy *et al.* 2012).

see sections 'Energetic cost of racemization' and 'Energetic cost of depurination').

Independent of electron acceptors, our calculations, moreover, indicate decreases in cell-specific energy flux by three orders of magnitude within the upper meters of sediment. This trend was calculated under the assumption that all aerobic or sulfate-reducing microbes present are indeed active. A recent study has, however, shown that the number of active/alive cells may be one to two orders of magnitude below the total number of cells after a 200-day starvation experiment (Riedel *et al.* 2013). If this trend holds true for subseafloor sediments, and there is a depth-related increase in the fraction of inactive cells, then cell-specific energy turnover may decrease by considerably less than three orders of magnitude. In this case, the BPR would be considerably higher than we have calculated. To determine whether the calculated BPRs are accurate, it will be essential to determine whether cells included in these calculations are indeed alive and active, and, ideally, measure metabolic activity of single cells.

Energetic cost of racemization

The influence of temperature on racemization rates (section 'Energetic cost of racemization' may have profound consequences for microbial BPRs at different temperatures. We have calculated the energetic cost of racemization repair under four different repair scenarios (Fig. 11) using the mean racemization rate of the four amino acids (Fig. A8, Supporting Information). We assume the same energetic costs of amino acid monomer synthesis and polymerization as in section 'The energetic cost of biomass synthesis'. We compare these energetic costs to cell-specific energy turnover rates from natural sediments (Figs 12 and 13) and from starved pure cultures in the laboratory (Davidson *et al.* 2009; Riedel *et al.* 2013), taking into account temperature effects on racemization rates.

Oxic sediments

For aerobes, the lowest cell-specific energy turnover rates measured in lower depth horizons of North Pacific Gyre (NPG) sediment are roughly one order of magnitude above the energetic cost under the energetically most costly scenario 4 (Fig. 11A). The same is true of average cell-specific energy turnover rates at station 1 in the South Pacific Gyre (SPG), the least active station within the SPG in D'Hondt *et al.* (2009). By contrast, mean cell-specific energy turnover in the more active SPG station 10, NPG surface sediment, coastal surface sediment from Aarhus Bay and in energy-starved cultures expressing the 'Growth Advantage in Stationary Phase' phenotype (GASP; more info in section 'The growth advantage in stationary phase phenomenon' and 'Glossary') is —two to five orders of magnitude higher than under the energetically most costly scenario 4. The lower rates observed in subsurface sediments of the NPG and SPG compared to the GASP study could have a methodological explanation. While in the GASP study (Riedel *et al.* 2013) only the minority of actively respiring cells was taken into consideration, energy turnover rates in environmental samples were calculated based on total cell counts, assuming that all cells are equally

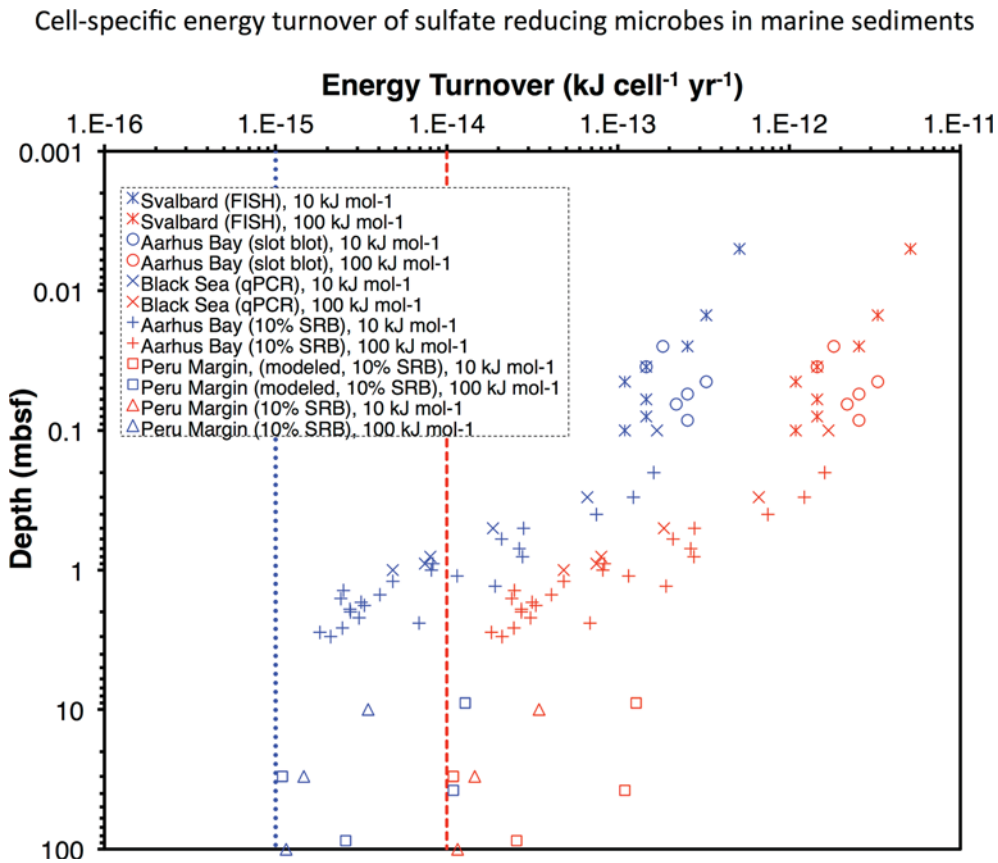

Figure 10. Mean annual energy turnover per cell of sulfate-reducing bacteria (SRB) in anoxic marine sediments, calculated by muliplying cell-specific SO_4^{2-} reduction rates (in mol $cell^{-1}$ yr^{-1}) by Gibbs free energy yields (per mol) of SO_4^{2-} consumed. We assume Gibbs free energy yields of –10 (blue symbols, dotted blue line) or –100 (red symbols, dashed red line) kJ per mole of SO_4^{2-} consumed (more info in the text, section 'Field-based studies of the BPR'). Data sources for cell-specific sulfate respiration rates: Svalbard (Ravenschlag *et al.* 2000), Aarhus Bay (slot blot) (Sahm *et al.* 1999), Black Sea (Leloup *et al.* 2007), Aarhus Bay (Leloup *et al.* 2009, Holmkvist, Ferdelman and Jørgensen 2011), Peru Margin (modeled) and Peru Margin (both J. Kallmeyer, unpublished). SRB were assumed to represent 10% of the total microbial community in calculations involving the data by Leloup et al. (2009) and J. Kallmeyer, unpublished.

active—an assumption that has yet to be validated. In fact, it cannot be ruled out with certitude that—as in the GASP study (Riedel *et al.* 2013)—the vast majority of cells counted in energy-depleted environmental samples are dormant (Lomstein *et al.* 2012). In this case, racemization repair might also only account for a small fraction of energy turnover at SPG-1 and in the NPG, even under the energetically most costly scenario 4.

Anoxic sediments

For sulfate-reducing microbes, the cell-specific energy turnover at the BPR in subsurface sediment, as well as in GASP experiments, is close to what would be expected under scenario 4 (Fig. 11B). By contrast, the only surface sediment examined has two to three orders of magnitude higher cell-specific energy turnover rates. Intriguingly, across the studied temperature range of ~65°C, the predicted temperature-driven increase in energetic cost of racemization is reflected in the cell-specific energy turnover rates of both subsurface samples and GASP incubations. This could indicate that for sulfate reducers, or anaerobes in general, the energetic cost of racemization damage repair accounts for a major fraction of the BPR in strongly energy-limited environments. Moreover, it could mean that starvation experiments in the laboratory, that are only few weeks in duration, reproduce energy limitation and induce microbial energy efficiency comparable to energy-depleted subsurface environments. The fact that cell-specific energy turnover is one to two orders higher in GASP incubations of laboratory isolates compared to the cold subsurface environments in Fig. 11B would then be mainly due to temperature-dependent increases in racemization rates–not lower energy efficiency of *in vitro*-grown GASP cells compared to cells living in the cold subseafloor.

Our observed temperature-related trends in racemization rates and cell-specific energy turnover rates are consistent with interpretations by Röling, Head and Larter (2003) based on oil reservoirs, and recently by Onstott *et al.* (2013) based on aspartic acid racemization rates at different temperature regimes in the South African Witwatersrand Basin goldmines. Accordingly, if racemization repair indeed consumes a large fraction of the energy available to energy-starved anaerobic microbial communities, then the increase in energy spent on racemization repair at higher temperatures is likely to present an increasing energetic burden. Consequently—if one assumes the same energy availability across habitats—the carrying capacity of microbes would decrease the higher the temperature is in a given habitat. The fact that racemization rates in cold subsurface sediments with temperatures of 1–5°C are likely to be three to four orders of magnitude lower than at 80°C may explain why microbes are found at considerable abundance in cold, anoxic subsurface sediments (e.g. Parkes *et al.* 1994; Kallmeyer *et al.* 2012), but are often below detection in subsurface oil reservoirs with temperatures of ≥80°C (Head, Jones and Larter 2003; Röling, Head and Larter 2003).

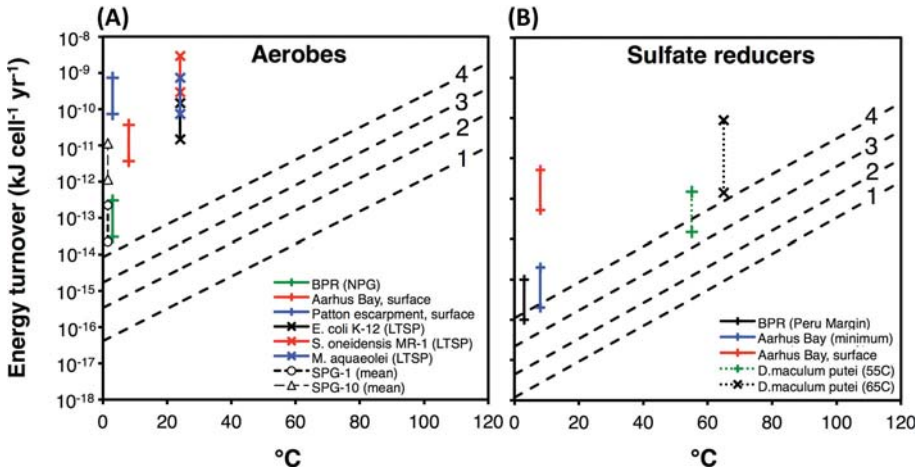

Figure 11. Cell-specific energetic cost of racemization repair in relation to temperature compared to cell-specific energy turnover, determined in environmental samples and laboratory-based starvation experiments with pure culture isolates. The left panel shows data for aerobes, and the right panel data for sulfate reducing microbes. The cell-specific energetic cost of racemization was modeled based on the calculated mean racemization rate of the four amino acid groups examined by Steen *et al.* (Fig. A2, Supporting Information). Due to uncertainties regarding the mode of intracellular repair of racemization damage, we assume four different repair scenarios, shown as dashed lines and numbered 1–4: scenario 1 (black): racemized amino acids are excised from their respective amino acid polymers and replaced individually (only factors in cost of monomer synthesis and polymerization); scenario 2 (blue): the entire protein is replaced when 10% of the amino acids within a protein have been racemized; scenario 3 (green): proteins are replaced when 2% of the amino acids within that protein have been racemized; and scenario 4 (red): the entire protein is replaced as soon as one amino acid within has been racemized. We further assume (a) that all amino acids are protein bound; (b) 250 amino acids per protein (based on median values of 267 and 247 amino acids per bacterial and archaeal proteins, respectively ; Brocchieri and Karlin 2005); (c) the energetic cost of amino acid polymerization does not change with temperature and is as shown in Table 2 for aerobic and anaerobic conditions, respectively; (d) cells have the amino acid content shown in Table 1; and (e) the energetic cost of amino acid synthesis under aerobic and anaerobic conditions is as shown Table 1 and changes in this energetic cost related to temperature are negligible within this context (consistent with Fig. A2, Supporting Information). The sources of cell-specific energy turnover data and temperatures of aerobes are as follows: BPR (NPG) (this study, Fig. 9, 3°C), Aarhus Bay, surface (BB Jørgensen, pers. comm., 8°C), Patton escarpment, surface (as in Riedel *et al.* 2013, based on Reimers 1987, 3°C), *E. coli* K-12, *Sh. oneidensis* MR-1 and *Marinobacter aquaeolei* VT8 (Riedel *et al.* 2013, all 24°C), and SPG-1 and SPG-10 means (D Hondt *et al.* 2009, both 1.5°C). The sources of cell-specific energy turnover data and temperatures of sulfate reducers are as follows: BPR (Peru Margin) (this study, Fig. 10, 3°C), Aarhus Bay (minimum) (this study, based on data compiled from Leloup *et al.* 2009 and Holmkvist, Ferdelman and Jørgensen 2011, 8°C) and *D. putei* (Davidson *et al.* 2009, 55°C and 65°C, as indicated).

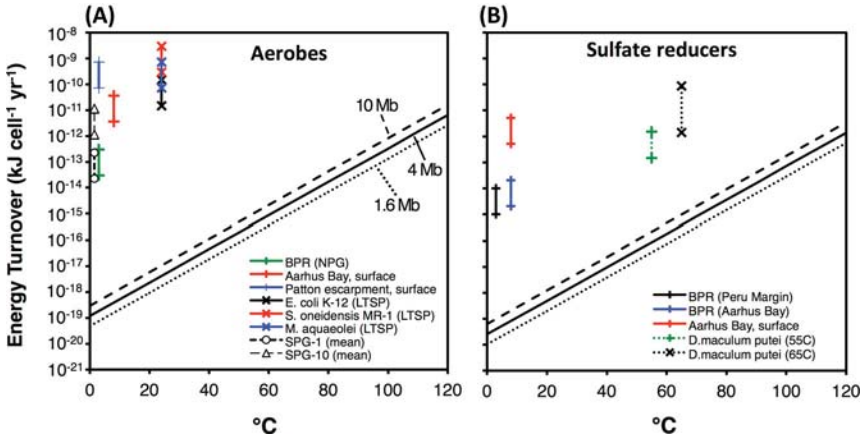

Figure 12. Cell-specific energetic cost of DNA depurination repair in relation to temperature compared to cell-specific energy turnover, determined in environmental samples and laboratory-based starvation experiments with pure culture isolates. We assume that all depurinated nucleotides are excised and replaced via the single-nucleotide base excision repair mechanism (BER). Accordingly, we assume the energetic cost of repairing a single nucleotide to consist of the sum of the energetic costs of (a) synthetizing the replacement nucleotide (Table 1), and (b) polymerizing that nucleotide on both sides, so it becomes a part of an intact nucleotide chain, and thus DNA molecule (two polymerization reactions per BER, each of which with the energetic cost of polymerization as shown in Table 2). The left panel shows data for aerobes, and the right panel data for sulfate reducing microbes. The cell-specific energetic cost of depurination was modeled based on the calculated mean depurination rate constant in Lindahl and Nyberg (1972) and modeled in relation to temperature (dotted, solid and dashed black lines). Due to uncertainties and natural variability in the genome size of energy-starved microbes, we assume three different genome sizes: 1.6 Mb (dotted), which is the genome size we assume in our calculations of cell-specific energetic cost of monomer synthesis and polymerization (Tables 1 and 2) and is near the size of the smallest genomes identified for free-living organisms; 4 Mb (solid), which is an average genome size for oligotrophic and copiotropic microbes alike (Fig. 5); and 10Mb (dashed) as an upper end member, which is well above average microbial genome size. The sources of cell-specific energy turnover data and temperatures of aerobes are as follows: BPR (NPG) (this study, Fig. 9, 3°C), Aarhus Bay, surface (BB Jørgensen, pers. comm., 8°C), Patton escarpment, surface (as in Riedel *et al.* 2013, based on Reimers 1987, 3°C), *E. coli* K-12, *Sh. oneidensis* MR-1 and *Marinobacter aquaeolei* VT8 (Riedel *et al.* 2013, all 24°C), and SPG-1 and SPG-10 means (D'Hondt *et al.* 2009, both 1.5°C). The sources of cell-specific energy turnover data and temperatures of sulfate reducers are as follows: BPR (Peru Margin) (this study, Fig. 10, 3°C), Aarhus Bay (minimum) (this study, based on data compiled from Leloup *et al.* 2009 and Holmkvist, Ferdelman and Jørgensen 2011, 8°C) and *D. putei* (Davidson *et al.* 2009, 55 and 65°C, as indicated).

Even if the energetic cost of racemization is considerably lower, and better described by scenarios 1, 2 or 3, the observed increase in minimum cell-specific energy turnover with temperature provides a hint to a general phenomenon: a strong temperature-dependence of the BPR due to an exponential increase in cell-damaging abiotic reactions with increasing temperature. Once this relationship—which in addition to racemization could be driven by temperature-dependent increases in other biomolecule-damaging reactions, mistakes in transcription and translation, and increased cytoplasmic membrane leakage—has been more clearly established, normalization of cell-specific energy efficiency for ambient temperature could be achieved due to the linear relationship between the natural log of energy turnover per cell and ambient temperature.

Energetic cost of depurination

As with amino acid racemization, temperature-driven changes in depurination rates (section 'Cell damage by abiotic reactions') could have significant consequences for microbial BPRs. When compared to the cell-specific energy turnover across the same sedimentary environments and GASP studies shown in the context of racemization, it is apparent that the energetic cost of DNA depurination is vastly lower than the energetic cost of racemization and thus likely to account for only a small fraction of total energy turnover (Fig. 12). The reasons are threefold: (a) the number of DNA nucleotides is 50 times lower than the number of amino acids per cell (McCollom and Amend 2005); (b) racemization rate constants are higher than depurination rates at the *in situ* and *in vitro* temperatures included here; according to our calculated relationship between mean amino acid racemization rate and temperature (Figs A5 and A6, Supporting Information), depurination rate constants exceed racemization rate constants only at temperatures >74°C; and (c) unless racemization damage is routinely repaired by single-monomer excision and substitution (scenario 1), as is DNA depurination, protein damage repair bears a significantly higher energetic cost, due to the replacement of entire proteins rather than single amino acids (scenarios 2–4; Fig. 11).

Our calculations indicate that under oxic conditions, the energetic cost of depurination is four to nine orders of magnitude lower than the cell-specific energy turnover, whereas under sulfate-reducing conditions the energetic cost of depurination is two to seven orders of magnitude lower than the cell-specific energy turnover. Hereby the difference in energetic cost of repairing small genomes (1.6 Mb), medium-sized genomes (4 Mb) or large genomes (10 Mb) from depurination damage is minor compared to the total energy that is being turned over per cell over time, suggesting that maintenance of large genomes per se only adds minor energy expenditures to the BPR. Based on the energetic cost of depurination repair—and assuming that (1) total energetic costs associated with DNA repair from other damaging reactions or proofreading are in the same order of magnitude, and (2) cells are able to regulate gene expression and concomitant protein synthesis in relation to demand—it would appear that small genome size confers at most a small advantage under extreme energy limitation.

ADAPTATIONS TO LOW-ENERGY SUPPLY

The energy that is theoretically available for biomolecule synthesis in a given setting is set by external factors, such as temperature, redox and fluid chemistry, and pressure. Knowledge of these external factors per se is insufficient to predict how large microbial populations are, or what the BPRs of various groups of microbes present are. This is because microbes are not optimally adapted to energy limitation. Evolutionary constraints, due to genetically determined biochemical pathways, physiologies, behaviors and physicochemical tolerance regimes, will impose energetic inefficiencies that preclude populations from being at their theoretical carrying capacity. Nonetheless, the genetic and physiological traits that improve the ability of microbes to cope with low-energy conditions are astonishing and vast. We discuss some of these next.

Cell-specific traits that control the energetic cost of anabolism

At an intracellular level, the efficiency of energy utilization and the mode of biosynthesis are likely to be important variables that influence the BPR. For instance, whether an organism synthesizes essential biomolecules autotrophically, *de novo* from available building blocks, or by recycling larger fragments is likely to have major implications for cell-specific energy consumption (Takano *et al.* 2010). Due to the considerable energetic cost of amino acid polymerization (section 'The energetic cost of biomass synthesis'), one might expect the ability to recycle oligomers or even large peptide fragments to provide a survival advantage in many environments. The specific biochemical pathway used for amino acid synthesis will also influence the energetic cost, as is evident from the very different energetic investments required for known pathways of C fixation (Russell and Martin 2004; Berg *et al.* 2010). Biomolecule half-life will further determine the rate at which energy needs to be invested into repair or replacement. Besides racemization rates, key determinants of protein half-life include the amino acid composition (Zhou *et al.* 2008), the molecular structure of the protein (Dice 1987), intracellular regulation of protein stability (Bougdour *et al.* 2008), ability to repair protein damage (Ishikawa and Clarke 1998) and external stressors, such as temperature, pressure, ionizing radiation and desiccation (Holden and Baross 1995; Krisko and Radman 2010). An example that illustrates the importance of temperature and pressure in controlling biomolecule stability within cells is the delay in heat shock response in thermophilic microbes under increasing pressure; here, increasing pressure can mitigate the disordering effects of increasing temperature by stabilizing biomolecules and ultimately allowing for higher temperature tolerance (Holden and Baross 1995). Given the wide range of stressors and thermodynamic conditions in nature, the minimum energy consumption required per microbial cell for synthesis and replacement of essential biomolecules will vary considerably across different environments. Yet, within any energy-limited environment, microbes capable of maximizing the net difference of total energy gain from catabolic reactions and total energy expenditure on essential biosynthesis reactions are likely to have an advantage.

GASP phenomenon

So far, the study of mutants expressing the GASP phenotype in the laboratory may have produced the most direct insights into physiological adaptation to severe energy limitation in nature. These experiments have shown mutant strains—with new alleles that confer a competitive advantage under energy

limitation—to evolve and take over batch incubations with pure or mixed cultures after only 10 days without substrate replenishment (Zambrano *et al.* 1993; Helmus *et al.* 2011). Currently, the extent to which multi-week to multi-year laboratory experiments in long-term stationary phase, as with GASP mutants, provide insights to life under energy limitation over thousands to millions of years, as in the subseafloor biosphere, still remains unclear. Cultures expressing GASP are dynamic, with growth and succession of mutant strains occurring continuously despite near-constant total population size (Finkel and Kolter 1999; Finkel 2006; Riedel *et al.* 2013). By contrast, microbial communities that have starved for millennia in the environment may be static, with near-zero mortality and cell division, and virtually all energy used for cell maintenance rather than growth (Lomstein *et al.* 2012). Nonetheless, the discovery of GASP mutants with improved ability to withstand energy limitation due to single genetic mutations (Zinser and Kolter 1999) raises the question whether similar genetic changes might enable natural communities to survive energy limitation over much longer time scales.

Phenotypic plasticity as a mechanism to cope with low-energy conditions

Microbes react to energy levels via a range of phenotypic responses. In addition to adjusting their cell morphology and cellular composition (sections 'Morphological and behavioral changes during energy limitation' and 'Cell compositional indicators of energy limitation'), these phenotypic responses include changes in substrate transport systems and pathways of energy conservation. Given the ubiquity of energy limitation, these phenotypic responses may be considered integral parts of the ecological niche and life cycle of many microbes. Next, we discuss several examples of such phenotypic responses to low-energy conditions.

With respect to the uptake of carbon substrates, many microbes harbor multiple membrane transport systems that are differentially expressed depending on energy conditions. For instance, *Vibrio* sp. strain Ant-300 imports arginine into the cell using a high-affinity active transport system under oligotrophic conditions. In arginine-enriched seawater, however, this organism switches to chemotaxis coupled with a low-affinity system (Geesey and Morita 1979). Similarly, a fermentative marine *Vibrio* isolate was shown to switch from a constitutive system to a different, high-affinitiy uptake system to take up mannitol after 5 weeks of energy starvation (Davis and Robb 1985). *Photobacterium angustum* strain S14 was found to use two different uptake systems for leucine import. The different affinities for leucine in this organism were reflected in half-saturation constant (K_m) values differing by more than one order of magnitude (0.76 μM vs 20 μM; Mårdén, Nyström and Kjelleberg 1987). In addition to carbon substrates, the use of two uptake systems has been documented for the electron acceptor sulfate; depending on ambient sulfate concentrations, the marine sulfate reducer *Desulfobacterium autotrophicum* differentially expresses a low-affinity uptake system with a K_m of 150 μM, and a high-affinity uptake system with a K_m of 8 μM (Tarpgaard 2013). Based on these data, it appears likely that the presence of both high- and low-affinity uptake systems within the same organism is widespread in microbes, and enables growth and survival across a wide range of energy conditions.

In addition to high substrate affinity, the ability to lower endogenous metabolic rates enhances fitness under low-energy conditions. In comparisons across different strains of enteric bacteria, Gram-positive cocci, pseudomonads and *Arthrobacter*, the *Arthrobacter* strains survived starvation 10–100 times longer due to their ability to drastically lower their endogenous metabolism (Ensign 1970; Poindexter 1981). Experiments with *Vibrio* sp. strain Ant-300 showed that cells that had been conditioned to slower growth in dilute media had higher survival rates under starvation conditions than cells conditioned to fast growth in energy-replete media (Moyer and Morita 1989a). Similarly, in comparisons of *Arthrobacter* sp. to *Pse. fluorescens* strains isolated from surface and subsurface habitats, the *Arthrobacter* sp. showed higher survival rates under starvation for 64 weeks (Kieft *et al.* 1997). However, contrasting with *Vibrio* sp. strain Ant-300, there was no difference in starvation survival between *Arthrobacter* sp. and *Pse. fluorescens* strains from surface or subsurface environments (Kieft *et al.* 1997). This underscores yet again that the physiological capacity to survive sustained energy shortage is a life history trait of many organisms, rather than an adaptation that is unique to severely and long-term energy-limited habitats.

In addition to changes in enzyme and protein kinetics, certain microbes are known to shift their energy metabolism under energy limitation. Many strains of sulfate reducing and acetogenic microbes are able to switch from one respiration pathway to another or even to fermentative metabolism (Drake, Küsel and Matthies 2006; Rabus, Hansen and Widdel 2006). *Methanosarcina acetivorans*—when grown on CO as energy source—switches from formate and acetate synthesis at high CO concentrations to methanogenesis at low CO concentrations (Rother and Metcalf 2004). A diverse range of chemoorganotrophic microbes inhabiting marine photic zones have photoproteins, called proteorhodopsins, which enable survival without growth via light-driven proton pumping under low-energy conditions (Stingl *et al.* 2007; González *et al.* 2008; Gómez-Consarnau *et al.* 2010). Research on *Vibrio* sp. strain AND4 has shown that this organism only performs phototrophy during stationary phase, but not during active growth (Akram *et al.* 2013). Even the oligotrophic marine strain SAR11 is known to switch from chemoorganotrophy to this form of light-mediated ATP production under very low organic carbon substrate concentrations (Steindler *et al.* 2011). The ability of planktonic chemoheterotrophs to switch to phototrophic life style—not only using photoproteins, but also via aerobic anoxygenic photosynthesis involving bacteriochlorophyll, e.g. *Roseobacter* spp.—is widespread (Kirchman and Hansen 2013; Karl 2014). Conceivably, the ability to use both solar energy and ambient chemical compounds as energy sources critically enhances short- and long-term survival in water columns, where (1) light availability varies with water depth and season, (2) organic carbon concentrations fluctuate in response to seasonal fluctuations in phytoplankton populations and riverine discharge, and (3) nutrient availability limits photosynthesis seasonally or year-round.

Fundamental differences in starvation states

As discussed earlier, microbes respond to energy limitation by a spectrum of phenotypic changes, of which we have identified the CSR and OSR as the two end member responses (section 'Cell morphology'). To complicate things further, however, microbes characterized by the CSR react by at least two distinct phenotypic responses, both of which are characterized by classic CSR-traits such as cell size reduction, shift to more coccoid morphology, low metabolic activity and changes in protein expression patterns. One of these phenotypic responses, the 'starvation-survival state' (Morita 1990) is characterized by reductive cell division resulting in formation of ultramicrobacteria, decreasing

viability over time, and increasing time to restore viability the longer the starvation period continues; cells remain culturable (Amy and Morita 1983; Thorne and Williams 1997). To more accurately describe this starvation response, we suggest renaming it to 'survival through cell division' (STCD) strategy. By contrast, in the 'viable but non-culturable' (VBNC) state, cells reduce cell size without cell division, and typically to a lesser degree than in the STCD, express different proteins, retain viability, but lose culturability (Heim *et al.* 2002; Oliver 2005).

Unlike the STCD, the VBNC is not specific to energy limitation, but can also be induced by other environmental stressors, e.g. shifts in temperature, salinity or redox conditions (Oliver 2005). Which of the two states occurs is often neither stated nor clear based on the variables measured in the existing starvation literature, and cannot be determined based on morphology alone (Fida *et al.* 2013). Moreover, experiments with the pathogens *V. vulnificus* and *E. faecalis* have shown that both states are—at least in some cases—inducible in the same organism (Oliver, Nilsson and Kjelleberg 1991; Heim *et al.* 2002). In both organisms, the STCD was induced by maintaining cells at the original growth temperature (room temperature) after the onset of starvation, whereas the VBNC state was induced when the incubation temperature was lowered to 4–5°C. Yet, the example of *Sphingomonas* sp. strain LH128, in which the VBNC state was induced at the same temperature at which initial growth had occurred (Fida *et al.* 2013), suggests the variables triggering the STCD and VBNC are not entirely understood and may vary.

Interestingly, protein expression patterns of *E. faecalis* in the VBNC state, despite having unique characteristics, more closely resemble those of growing cells than cells in the STCD (Heim *et al.* 2002). Nonetheless, profound changes in physiology occur in the VBNC state. The hydrocarbon-degrading Alphaproteobacterium *Sphingomonas* sp. strain LH128, after being kept in starvation media for 6 months, had drastically reduced the expression of genes involved in transcription and protein synthesis, but increased the expression of genes regulating gene expression, gene translocation, and catabolism of fatty acids and rRNA (Fida *et al.* 2013). As is characteristic of the VBNC, these cells showed no lag time in response to substrate addition afterwards and immediately resumed hydrocarbon catabolism.

The fact that the same microorganisms can enter at least two different states in response to energy limitation corroborates the fundamental importance of energy limitation as a driving force in microbial evolution. Future research will provide a better understanding of the environmental cues that determine which state is induced, and reveal possible reasons why certain phenotypic states might provide survival advantages over others under a given set of environmental conditions.

Low-energy conditions as an evolutionary driving force?

As discussed in the previous sections, many microorganisms reveal high phenotypic plasticity in their response to energy limitation, e.g. by adjusting their metabolic strategy, metabolic activity, cell division rates and cell morphology. In addition, many traits that represent adaptations to energy limitation are likely to be genetically 'hard-wired'. Distinctions between these forms of adaptation are not always clear, especially when examining environmental samples. For instance, the frequent shift toward smaller cell morphology that occurs within days in starving cultures is due to phenotypic plasticity rather than genetic shift (Amy and Morita 1983; Thorne and Williams 1997; Finkel 2006). Yet, the fact that genetically distinct GASP mutants evolve in starvation experiments after days to weeks and enable cells with these fitness-enhancing mutations to outcompete all other cells indicates a central role for genetic adaptation in surviving energy limitation, even over relatively short time spans (Zambrano *et al.* 1993; Finkel and Kolter 1999; Helmus *et al.* 2011). It is thus conceivable that microbes inhabiting environments that have been deprived of fresh energy inputs over geologic time scales have genetically evolved in response to these conditions, perhaps even via GASP-like mutations.

Several well-characterized microbial species illustrate the possibility of genome evolution in response to low-energy conditions, and suggest that certain existing microbial isolates might be suitable models for the study of life under long-term energy limitation. Strains of photosynthetic *Prosthecochloris* sp. subsist under lower light conditions, and hence lower available light energy than other low-light adapted green sulfur bacteria, by producing larger light-harvesting antennae (chlorosomes), which increase the gain of excitation energy by the reaction center, and by other, so far unknown, cellular mechanisms (Fuhrmann *et al.* 1993; Marschall *et al.* 2010). Obligate acetate-fermenting methanogenic *Methanosaeta* sp. have evolved a different, higher affinity enzymatic pathway for acetate metabolism than the facultatively acetate-fermenting *Methanosarcina* sp. (Penning and Conrad 2006; Smith and Ingram-Smith 2007). While the prolific, r-selected *Methanosarcina* sp. quickly outgrow *Methanosaeta* at high acetate concentrations, *Methanosaeta* sp. are classic K-strategists, that are slow-growing but highly efficient, and outcompete *Methanosarcina* sp. in energy-limited environments by drawing acetate concentrations below the threshold concentration required by *Methanosarcina* (Jetten, Stams and Zehnder 1992; for definition of r- and K-strategists, see the section 'Glossary', for review, see Andrews and Harris 1986). *Desulfotomaculum putei*, a sulfate reducer originally isolated from the terrestrial subsurface (Liu *et al.* 1997; Davidson *et al.* 2009)—after a few days in starvation media and after correction for temperature-dependent increases in racemization damage—have comparable cell-specific energy turnover rates to sulfate reducers from long-term energy-depleted subsurface sediments (section 'Energetic cost of racemization').

Genetic adaptations that result in high efficiency of energy utilization are likely to confer a survival advantage in environments that are energy depleted over geologic time scales. These adaptations may not have evolved as adaptations to long-term energy limitation, however, since energy limitation is ubiquitous, and adaptations to energy limitation may just as well have evolved under shorter term energy limitation, as the example of GASP mutations illustrates. In fact, the extent to which microbes continue to evolve in habitats that are cut off from free energy inputs over geologic time periods is not clear. For favorable adaptations to occur and spread through populations, cell growth and replication are considered necessary. In sediments, one might then expect to see changes in genomes, e.g. increased numbers of duplications in metabolic genes, or increased evidence of GASP-like mutations throughout microbial populations, from more energy-rich recently deposited layers to highly energy-depleted deeper layers. Similarly, one might expect lower BEQs in deeply buried layers. Yet, if average generation times of 300–1000 yr in cold subsurface sediments (Shipboard Scientific Party 2003; Jørgensen 2011; Lomstein *et al.* 2012) reflect those of individual cells, then the rate of evolution should be extremely low assuming that mutation rates of *E. coli* apply (~0.001 mutations genome^{-1} generation^{-1}; Lee *et al.* 2012). This low rate of evolution, combined with the limited potential for dispersal in compacted, diffusion-controlled sediments, would argue against the

spreading of favorable GASP-like mutations throughout subseafloor sediments—even over geologic time. Consequently, selective survival of already low-energy adapted microbes that were present in the initial 'inoculum', i.e. sediment at the time of its deposition to the sediment surface, would be more likely than evolutionary adaptation to energy limitation within deeply buried sediments.

Specialist versus generalist strategies

Evidence so far suggests that the concept of threshold energy yields may mainly be relevant for low-energy intermediates, such as H_2 and acetate, which are microbially turned over at high rates. Highly efficient substrate specialists may outcompete other microorganisms for these substrates, by drawing their concentrations down to levels at which Gibbs free energies of metabolic reactions are too low to be harvested by less efficient organisms. Even in energy-limited environments, however, the energy yields per mol of most substrates are much higher than the threshold energy yields of 0 to -20 kJ mol^{-1} determined for H_2 or acetate in pure culture and sediment incubations (Lever 2012). For these substrates, the payoff of specialization through extreme energy efficiency—i.e. the ability to thermodynamically exclude less-efficient competitors by drawing substrate concentrations down to threshold energy yields—is not given. Instead, metabolically versatile microbes that utilize wide substrate spectra may have a survival advantage over substrate specialists due to their ability to access larger total energy pools (Gottschal, de Vries and Kuenen 1979; Egli 1995; Lever 2012). A potential trade-off of this more generalistic metabolic strategy is that the metabolism of additional substrates may require further genes and enzymes to maintain. Yet, this added energetic cost might be minor compared to other energetic challenges these cells face (see section 'Energetic cost of depurination'), and the energy gained by access to additional energy sources is potentially high. Interestingly, evidence suggests that—instead of genome reductions—duplications of large chromosomal regions and thus genome expansions are common among microbes in starvation mode (Gevers *et al.* 2004; Porwollik *et al.* 2004).

The benefit of multiple gene copies under energy limitation may arise from (1) the increased probability of fitness-enhancing mutations, e.g. resulting in wider substrate spectra, and (2) the presence of an intact 'spare' gene copy—in case a mutation disrupts an essential gene function on one of the gene copies (Finkel 2006). Published data are in support of at least the first explanation. In *Arthrobacter*, a genus that is widespread in the terrestrial subsurface, gene duplications, followed by divergent evolution of individual gene copies, have been linked to the ability to degrade a wider range of polymeric substrates (Mongodin *et al.* 2006). In *Burkholderia xenovorans* strain LB400, the up to ~30% larger genome size compared to closely related strains has been interpreted to explain the greater niche breadth of strain LB400 compared to other strains (Chain *et al.* 2006). Wide substrate spectra may, however, not always require increased genome size. For instance, the O-demethylase of the thermophilic acetogen *Moorella thermoacetica* is used for the demethylation of at least 20 natural methoxylated monomeric lignin derivatives, in addition to plant hormones, and man-made pesticides (Daniel *et al.* 1991; El Kasmi, Rajasekharan and Ragsdale 1994). Studies with GASP mutants indicate that small, localized mutations, including point mutations, can result in enzymes that enable utilization of a wider range of amino acids and thereby create a survival advantage in energy-starved cultures (Zinser and Kolter 1999; Finkel 2006). Reductions in genome size in low-energy adapted pelagic heterotrophs, by minimization of intergenic spacers to on average three bases (Giovannoni *et al.* 2005), moreover, indicate that even genome reductions do not necessarily go hand in hand with gene deletions.

Biomass turnover and mortality

A remarkable finding of laboratory-based starvation experiments is that while total microbial populations remain nearly constant in size during long-term stationary phase, the microbial community composition continues to be dynamic after years of incubation (Fig. 13). Every few weeks, new mutants evolve which outcompete less competitive older mutants and become dominant. And in spite of the dominance by certain strains, an astonishing genetic diversification produces many new morphotypes (Finkel 2006). Similar to long-term incubations in the laboratory, microbial populations surviving energy limitation on geologic time scales in the environment remain nearly constant in size over time. It is, however, unknown whether these populations, like GASP mutants, are in a state of dynamic equilibrium, where growth via cell division balances mortality—thus the long-term stationary phase would persist over geologic time scales, possibly with microbial generation times spanning hundreds to thousands of years (Fig. 13)—or whether individual microbial cells enter a new phase (phase 6) characterized by non-growth and near zero mortality over geologic time (Fig. 13, inset). If it indeed exists, this phase could be defined as the 'long-term non-growth phase'.

Current estimates of average biomass turnover times of energy-limited microbes in subseafloor sediments are 300–1000 yrs (Shipboard Scientific Party 2003; Jørgensen 2011; Lomstein *et al.* 2012). Yet, very little is known about the variability across individuals within these communities. Low-energy-adapted microbes may continue to metabolize, and even grow, at a low rate (Lennon and Jones 2011; Lomstein *et al.* 2012). Moreover, long periods of low activity or dormancy may be punctuated by bouts of activity, e.g. if cell death, or desorption of previously unavailable organic molecules from mineral matrices, produce local patches of elevated energy availability. If, under these circumstances, cell growth and division are indeed occurring, then perhaps the spatial distributions of cells offer clues. Due to the energetic cost of locomotion (Mitchell 2002; Berg 2003; section 'Cell motility') and/or physical obstructions by lack of pore space, microbial motility is likely to be increasingly limited—not only by available energy, but also due to sediment compaction. This means that cells that are growing and multiplying will be forced to remain in close proximity with one another—even though this enforces greater partitioning of energy resources and may lower the available energy per cell. Thus, an increase in associations of genetically identical cells with depth may provide a hint that cell division is ongoing. Clearly, alternative explanations for such observations are possible. A potential strategy to tackle the conundrum of whether cell growth is taking place might include the study of sediment columns that are vertically heterogeneous with respect to grain size. Here, cell distributions could be compared across (A) high-porosity, fine-grained shallow subsurface sediments that would allow for motility; (B) highly-compacted low-porosity, fine-grained sediment/sedimentary rock layers from the deep subseafloor with small pore sizes that restrict motility; and (C) unconsolidated, coarse-grained deep subseafloor layers, in which movement through active locomotion or Brownian

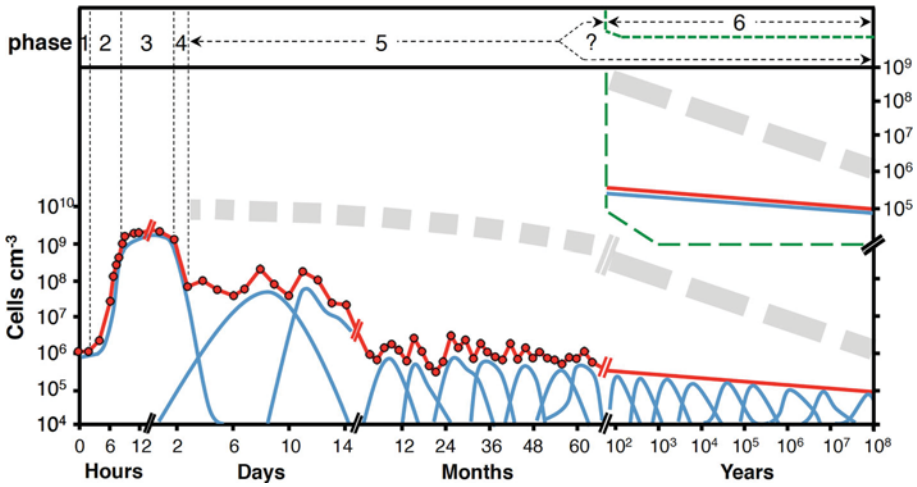

Figure 13. Concept sketch expanded from Finkel (2006) to show potential changes to microbial populations cut off from new energy inputs over geologic time scales. The five known phases of the microbial life cycle (1: lag, 2: exponential, 3: stationary, 4: death, 5: long-term stationary) are shown in the top panel. Red dots in graph indicate cell concentrations of individual strains at time points sampled. Blue lines suggest cell concentrations of individual mutants (GASP mutants) within that strain. The shaded, dashed gray line reflects the expected population trend over time if the same trends observed for individual strains and GASP mutants in pure cultures are reflected in the size of the entire microbial community in a complex environmental sample over time. Whether populations remain in the long-term stationary phase over geologic time scales of hundreds to hundreds of millions of years is unclear. If yes, one would expect complete population turnover within a species after each fitness-enhancing mutation, though perhaps on a much longer time scale than in GASP experiments (hence, time on *x*-axis changes to log-scale after 60 months). Alternatively, microbial species and populations might enter a final phase of the microbial life cycle, the non-growth phase (phase 6; see inset in uppermost panel and in main graph, outlined by green dashed lines). In phase 6, cells would cease to replicate and spend all their available energy on survival functions, such as essential biomolecule repair and synthesis. Of the three basic physiological states described in the text, the growth state corresponds to phase 2, the maintenance state to phase 3 and the survival state to phase 5 and/or phase 6.

motion would be expected to lead to cell dispersal or cell scattering over time (Fig. 14).

Another unresolved question concerns the role of mortality due to predation and viral lysis under long-term energy limitation in the environment. Can, for instance, the very low biomass production rates in most subsurface sediments (Hedges and Keil 1995; D'Hondt *et al.* 2004, 2009) support populations of bacterial or protozoan predators? Viruses that are outside of hosts over long time periods are likely to lose their ability to infect hosts due to their incapacity to repair depurination damage. If microbes are merely repairing biomolecules rather than growing and dividing, then lysogeny may enable survival rather than growth of viruses, and in fact this notion is supported by studies on subseafloor sediments (Engelhardt *et al.* 2013). If at all, viruses would then only minutely increase cell mortality by increasing the energetic cost of genome maintenance due to larger genome size. Since viruses leave an immunological fingerprint in the form of CRISPR repeats (Horvath and Barrangou 2010), further genetic studies, e.g. of subseafloor genomes, may elucidate viral survival (growth) strategies in low-energy communities. Similarly, the potential importance of protozoan predation on microbes could be examined by phylogenetic studies on eukaryotic 18S rRNA, 18S rRNA genes and functional genes linked to predation, such as genes and gene transcripts involved in phagocytosis (King *et al.* 2012). Evidence in support of living microbial predator populations comes from the detection of 18S rRNA of potentially predatory *Ciliophora* in deeply buried, anoxic sediments of the Peru Margin and Peru Trench (Edgcomb *et al.* 2010).

CRYPTIC AND UNDETECTED ENERGY SOURCES AND METABOLISMS

While the (average) energy that is available to cultured chemoorganotrophic organisms in laboratory-based starvation experiments can at least be constrained by analyses on media and cellular composition, cell numbers and rates of biomass turnover, similar assessments with natural samples are difficult. The vast majority of microbes are uncultured and their energy metabolism is unknown. Methods for the comprehensive analysis of organic matter pools in soils and sediments have yet to be developed; thus, well-constrained estimates of the energy stored in organic matter, let alone the fraction that is biologically available, are missing. And even habitats that have been cut off from fresh energy inputs for thousands to millions of years often host remarkably high microbial biomass (Parkes *et al.* 1994; D'Hondt *et al.* 2004), raising the question whether important *in situ* energy sources have been overlooked. Besides organic matter, weathering reactions, seismic processes and radioactive decay may all release energy and supply a significant fraction of energy consumed by energy-limited microbial communities over geologic time scales. Past studies, especially in marine sediments, have mainly focused on electron acceptor distributions (e.g. D'Hondt *et al.* 2004, 2009). These can provide clues to the distribution of activity, but not necessarily to the energy available to microbes.

To better constrain the energy available from organic matter, it will be essential to characterize and quantify the composition of its dissolved and solid-phase fractions. A vast diversity of dissolved organic compounds is likely to be present in any water or sediment sample from the environment. Information on the concentrations and isotopic compositions of individual organic compounds will help reveal their biotic and abiotic sources, as well as which are used by microbes. Fourier transform-ion cyclotron resonance-mass spectrometry offers a powerful tool to this end (D'Andrilli *et al.* 2010), although its analysis requires initial structural assumptions about the molecules making it best used in combination with other methods of chemical characterization. Similarly, little is known about the composition of detrital solid-phase organic matter, which undergoes chemical transformations, e.g by thermal maturation, geopolymerization

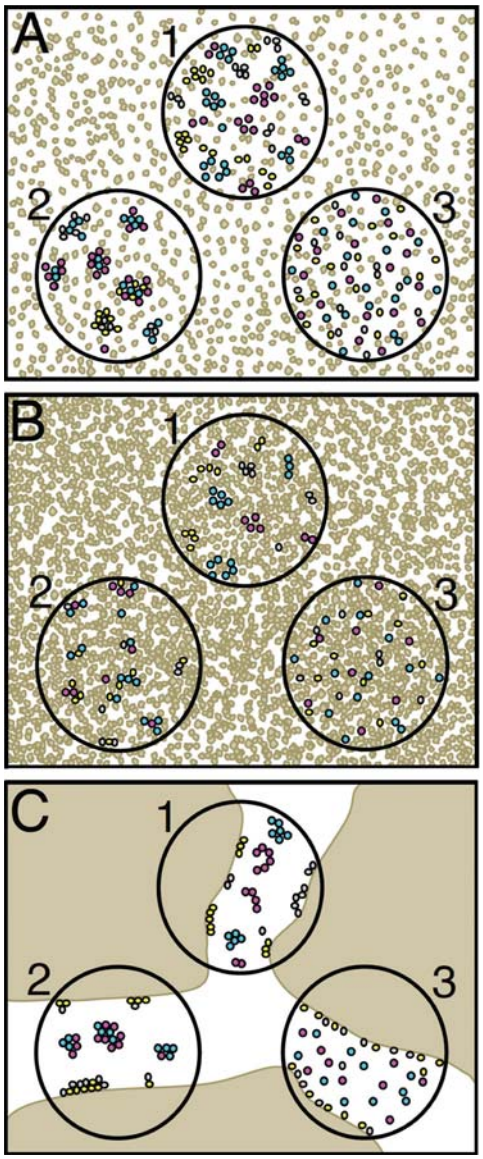

Figure 14. Concept sketch of microbial cell distributions in (**A**) fine-grained shallow subsurface sediment with high porosity and large pore space; (**B**) fine-grained deep subseafloor sediment/sedimentary rock with low porosity and small pore space; and (**C**) unconsolidated, coarse deep subseafloor sediment with low porosity and large pore space, assuming a low-diversity community of four microbial species shown in pink, gray, turquoiseand yellow. Circles within each graph indicate different microbial distribution patterns. Within each graph, top circles (1) illustrate microbial communities in which cells remain together after division, due to lack of motility or benefits of associating with kin. Bottom left circles (2) illustrate communities in which cells form associations (consortia) with cells of different species, e.g. due to mutualistic benefits. Bottom right circles (3) illustrate communities in which cells are scattered apart and maintain distance to their neighbors. Changes in distribution patterns among the same microbial species may provide clues to environmental conditions. For example, species that are spread apart in fine-grained surface sediments (A) but remain in contact with other cells, including their own species, in compacted, fine-grained subseafloor sediments (B), could indicate both cell division and absence of active or passive movement in the latter (B). Cells that cluster with other cells in A, but not in B or C, may indicate mortality-induced absence of cell clusters. Cells that remain in clusters in A–C, on the other hand, would suggest benefits to forming tight associations irrespective of sedimentary properties. Though they are not conclusive evidence, cell distribution patterns in surface compared to deep subsurface sediments indicate differences in ecological drivers, and may thus be useful in evaluating hypotheses regarding cell division, motility and effects of pore space on microbial communities.

or sulfurization processes that render it uncharacterizable by conventional methods (Burdige 2007). A large fraction of the organic matter pool may, moreover, be shielded from microbial degradation due to sorption to mineral surfaces and refractory macromolecules (Hedges and Keil 1995; Nielsen, Calamai and Pietramellara 2006). The extent to which this sorbed fraction is released and becomes bioavailable again over time is not understood. In the deep biosphere, refractory, solid-phase organic matter may account for most of the organic matter pool and, in addition to microbial necromass, support the majority of organisms (Lomstein *et al.* 2012). Recent developments in nuclear magnetic resonance techniques, by which formerly uncharacterizable fractions can be analyzed, offer great promise that the fractions of the sorbed and refractory organic matter pools that are slowly consumed by microbes can soon be classified (Abdulla, Minor and Hatcher 2010; Yu *et al.* 2011) and their thermodynamic properties and catabolic potential calculated (LaRowe and Van Cappellen 2011).

In addition to organic matter, abiotic and geologic processes may play a more vital role as energy sources in long-term energy-starved habitats than previously thought. Certain minerals, e.g. iron-bearing compounds, are considered inert over $\leq$10 kyr, but undergo significant chemical weathering over longer time periods (Canfield, Raiswell and Bottrell 1992). Peaks in Fe(II) and Mn(II) concentrations in oxygen- and nitrate-depleted subseafloor sediments (Wang *et al.* 2008; Riedinger *et al.* 2014) indicate that microbial metal reduction extends far beyond surface sediments, where reduction of reactive species takes place (Canfield *et al.* 1993). Reactions of seawater with minerals in the Earth's crust support life at the seafloor and far below, e.g. by O_2- or nitrate-dependent iron oxidation and abiotic production of H_2 from olivine (Bach and Edwards 2003), or Fischer-Tropsch-type synthesis, by which organic substrates are abiotically produced (McCollom and Seewald 2007; Neubeck *et al.* 2011). Radiolytic production of H_2 from water, driven by natural radioactive decay, could be a significant but widely ignored microbial energy source on Earth and beyond (Lin *et al.* 2005; Blair *et al.* 2007; D'Hondt *et al.* 2009). In addition, geologic processes may free substantial amounts of energy to communities in the deep biosphere. Geothermal heating results in thermal degradation of recalcitrant organic matter and H_2 production (Horsfield *et al.* 2006; Parkes *et al.* 2011). Mechanical energy released during plate tectonics, earthquakes and crustal flexing may release electron donors which are subsequently taken up by microbes (Kita, Matsuo and Wakita 1982; Sleep and Zobeck 2007). In addition, redox reactions with a wide variety of transition metals provide a typically ignored, potential source of energy to life in these systems (Berg *et al.* 2010).

Much remains to be learned about the energy sources used by microbes in subseafloor sediments. Indeed, future studies may reveal that energy sources other than organic matter are equally important or more important in deep subsurface environments, such as deeply buried sediments (Fig. 15). With the identification of novel organic, inorganic and geologic energy sources to microbes inhabiting energy-limited environments, experiments can be designed to study the microorganisms that use them. While the majority of these microorganisms will most likely belong to uncultivated taxonomic groups, new metabolisms may also be found in 'well-characterized' groups. The detection of large populations of aerobic heterotrophic *Rhizobium* spp. and aerobic bioluminescent *Photobacterium* spp. in subsurface sediments suggests that even well-studied microorganisms may have unknown metabolic capabilities when faced with severe energy limitation (Süss *et al.* 2008).

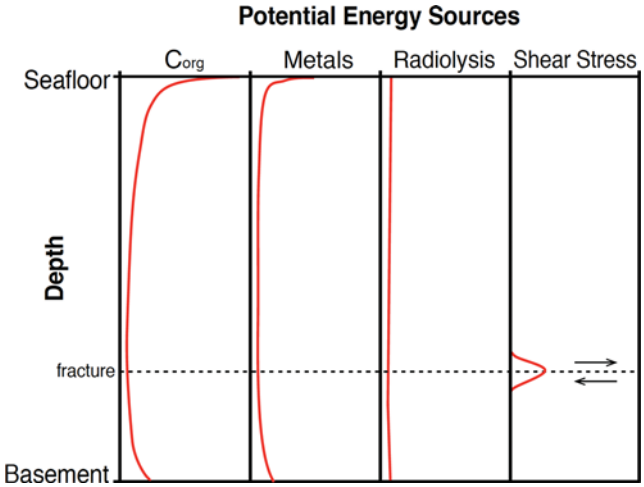

Figure 15. Concept sketch illustrating potential quantitative changes in microbial energy sources with depth across an ancient sediment column, from the sediment–water interface (seafloor) to the sediment–basement interface (basement), in a seismically active location of the world's oceans. 'C_{org}' stands for energy available from organic matter breakdown and remineralization. The C_{org} deposited to the sediment surface is mainly produced by photosynthesis and derives from the overlying water column or terrestrial sources. C_{org} entering bottom sediments from underlying basalt can either derive from circulating seawater, which enters the basaltic basement at rocky outcrops (Fisher and Wheat 2010), or be produced *in situ*, by chemosynthetic microbial communities within the basaltic basement (McCarthy *et al.* 2010; Lin *et al.* 2012a; Lever *et al.* 2013). 'Metals' refers to energy available from metal cycling, including slow weathering reactions of iron compounds. 'Radiolysis' indicates energy available from molecular hydrogen (H_2) produced by splitting of water molecules by radioactive elements (e.g. uranium, thorium, potassium). Increases in energy availability from metals and radiolysis in bottom sediment can be attributed to inputs from the underlying oceanic crust. 'Shear stress' stands for energy produced by friction between the upper and lower part of the sediment column as seismic activity induces fracturing; the existing evidence of seismically induced 'shear stress' as an important microbial energy source in the subseafloor remains circumstantial.

OUTLOOK

Energy limitation is likely to be the predominant physiological condition among a large part of all microorganisms on Earth. Traditional microbiological and biochemical approaches have typically focused on life under conditions of high-energy availability, where rapid growth takes place, and populations can be characterized over short time scales. As a result, much less is known about microbial life under low-energy conditions, even though the ability to maintain essential cell functions throughout periods of energy limitation and non-growth may have a similarly profound influence on the evolution of microbes as the ability to grow and replicate under energy-replete conditions. While the fast-growing microbes in Earth's surface environments strongly affect elemental cycles on short time scales, the energy-limited or non-growing majority that is cut off from fresh energy inputs in subsurface environments greatly influences elemental cycles over geologic periods. To understand microbial survival under energy limitation, knowledge gained from state-of-the-art technologies on environmental samples, e.g. for the characterization and tracing of microbial energy sources, can be combined with newest tools of microbial cultivation, e.g. retentostats or chip-based microbioreactors. Laboratory-based experiments can only to a limited extent recreate conditions in microbially diverse natural environments that have been starved over geologic time scales, such as deeply buried sediments. Therefore, an important component of research on life in maintenance or survival mode must be the continued study of energy-limited microbes in nature. Yet, because energy availability and energy turnover in the environment are difficult to constrain on a single-cell level, further advances to existing methods of single-cell cultivation and single-cell activity monitoring in the laboratory will be necessary to place accurate constraints on the BPR. By testing theory-based predictions of microbial population sizes, energy sinks and energy sources through the direct study of energy-limited populations in both the laboratory and the natural environment, a better understanding of microbial life in its predominant physiological state will be possible.

SUPPLEMENTARY DATA

Supplementary data is available at FEMSRE online.

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Glossary

Basal Power Requirement (BPR): Energy required per cell for survival over time. A significant fraction of this power is probably used to repair damage incurred to essential biomolecules, such as DNA and certain proteins.

Biological Energy Quantum (BEQ): The lowest amount of energy that can be conserved by living organisms. Originally believed to be the minimum amount of free energy necessary to phosphorylate 1 mol ADP to 1 mol ATP, it has since been shown that some microorganisms can live and grow under conditions where Gibbs free energy yields per 1 mol of substrate are considerably lower than required per synthesis of 1 mol of ATP. In these microorganisms, the BEQ appears to instead be set by the energetic cost of ion (H^+, Na^+) translocation by ATP synthase (Thauer and Morris 1984; Mayer and Müller 2014). Evidence suggests that the average number of H^+ translocations required by H^+-driven ATP synthases to phosphorylate ADP to ATP varies from 2.7–5 (Fritz *et al.* 2008; Pogoryelov *et al.* 2012; Wikström and Hummer 2012; Mayer and Müller 2014). Consequently, the BEQ of organisms relying on H^+-driven ATP synthases to convert ADP to ATP equals the energetic cost to produce 1 mol of ATP divided by the number of H^+ translocations required per phosphorylation of ADP to ATP. The same principles presumably apply to the less studied Na^+-driven ATP synthases (Thauer *et al.* 2008; McMillan *et al.* 2011). Ion translocations by ATP synthases are powered by a membrane potential, which is produced by translocating H^+ and/or Na^+ against the concentration gradient, and thus in the direction opposite to the translocation by the ATP synthases. The energy that is required for these membrane potential-producing translocations comes from catabolic reactions. Interestingly, the lower end of measured Gibbs energy yields from microbial catabolic reactions approaches the theoretical minimum energy required for ion translocation by ATP synthase (+20 to +10 kJ mol or even less; Schink 1997; Hoehler *et al.* 2001; Jackson and McInerney 2002). So far, calculations assuming an energetic cost of ADP phosphorylation of 41.9 kJ mol^{-1} (Tran and Unden 1998), 3–5 ion translocations per molecule of ATP synthesized and an energy efficiency of close to 100% are consistent with BEQ values as low as ~10 kJ mol^{-1} (Hoehler *et al.* 2001). Lower values are in theory possible with ATP synthases operating at >5 ion translocations per ATP phosphorylation. Similarly, factors affecting the energetic cost of ADP phosphorylation, such as intracellular ATP:ADP ratios below the value of 10:1 of growing cell populations (Schäfer, Engelhard and Müller 1999), and external variables, e.g. temperature, could result in BEQ values significantly below 10 kJ mol^{-1}.

Copiotroph; Organism requiring high energy or nutrient conditions for growth.

Copiotrophic Starvation Response (CSR): Typical response of copiotrophic microorganisms to severe energy limitation, characterized by reductions in cell size, shifts in composition of cellular building blocks, gene and protein expression, and commonly a shift from rod-shaped to coccoid morphology.

Death phase: Fourth phase of microbial life cycle (after lag, growth, and stationary), characterized by a net decrease in population as large numbers of cells lyse due to starvation.

Depurination: Chemical reaction by which a deoxyribonucleoside or ribonucleoside with a purine base (adenine or guanine)

loses this purine base by cleavage of the β-N-glycosidic bond, which binds this base to a deoxyribose or ribose sugar.

Energy limitation: Growth of an organism is limited by the total amount of energy that is available to the organism.

Exponential phase: Second phase of microbial life cycle (after lag phase), characterized by exponential growth of population, as each generation of cells doubles. Growth is not limited by energy availability.

Growth Advantage in Stationary Phase (GASP): Mutants, evolving through favorable mutations during the long-term stationary phase, which have higher fitness than other cells, and thus take over microbial populations during the long-term stationary phase. As favorable mutations occur repeatedly over time, dominant mutants are outcompeted by following generations with even higher fitness.

Growth state: The full energy demand is met. Cells have unlimited energy available for growth and division.

K_m: Half-saturation constant, i.e. concentration at which an enzyme reaches half its reaction rate for a given solute. We use this concept also to refer to the relationship between solute concentration outside the cell and its rate of transport into cells via uptake proteins.

K-strategist: Organisms with low maximum growth rates, but high energy efficiency and thus growth efficiency; typically dominate stable environments, where they occur close to the carrying capacity of that environment.

Lag phase: Initial phase after a culture of microorganisms has been inoculated. Cells prepare for growth by acclimation, damage repair, energy and nutrient uptake, and synthesis of metabolic machinery required for growth in the next phase (exponential phase).

Long-term non-growth phase (LNGP): A possible sixth phase in the microbial life cycle, which—if it exists—follows after the long-term stationary phase (Fig. 13, inset). Unlike in the long-term stationary phase, energy availability is so limited that cell growth and division are no longer supported. All energy available to cells is likely to be spent on essential biomolecule replacements and repair.

Long-term stationary phase: Fifth phase of the microbial life cycle. During this phase, which follows population reductions during the death phase, microbial population size stabilizes and persists over months to years (Finkel and Kolter 1999; Finkel 2006). Despite the absence of substrate addition, populations are dynamic, with favorable mutations resulting in a succession of dominant mutants expressing the GASP phenotype. Whether this phase persists over geologic time (Fig. 13), e.g. in subsurface environments that are cut off from new energy inputs, is not known.

Maintenance energy: Energy required by microorganisms to maintain essential physiological functions over time; has mainly been quantified in cell cultures grown under steady-state ('maintenance') conditions in continuous-flow reactors. The maintenance energies established from these experiments are likely to be considerably higher than the BPR (also see section 'Physiological states of microbial life').

Maintenance state: The full energy demand of a cell is met, but excess energy required for significant growth is lacking. Maintenance energy extrapolations to zero growth from chemostats may describe this state.

Membrane leakage: The loss of anions, cations, and molecules across the cell membrane. The resulting reductions in membrane potential, energy substrates, nutrients, and biomolecules can pose a threat to microbial cells, especially in energy-limited environments, due to energetic costs associated with the transport of many anions, cations and molecules into the cell, as well as the synthesis of new biomolecules.

Nutrient limitation: Growth of an organism is restricted by availability of a scarce element or compound.

Oligotroph: Organism that is capable of growing at low energy or nutrient conditions (Weber 1907).

Oligotrophic starvation response (OSR): Cells undergo minor, if any, reductions in cell size under energy limitation. Found in cells, that are well adapted to life and growth in low-energy environments, and typically maintain small sizes even when energy is non-limiting.

Opportunitroph: Copiotrophs are specifically adapted to life under fluctuating energy regimes and are able to exploit and grow rapidly during bouts of high energy or nutrient availability in otherwise low-energy or low-nutrient environments.

Pigritroph: Microbes that survive and grow at low cell-specific energy fluxes due to a passive ('lazy', 'sluggish') life style. Cells are typically small, single, immotile, and unable to increase their metabolism much beyond its low ordinary rate, even under energy-replete conditions.

Racemization: Interconversion of stereoisomers that are mirror images of each other (enantiomers). In organisms, the change in conformation resulting from racemization can alter the function of biomolecules and thus harm the organism, if not repaired.

r-strategist: Opportunitrophic organisms with high-potential growth rates that often dominate in unstable or unpredictable environments, e.g. where energy availability fluctuates strongly.

Stationary phase: Third phase of microbial life cycle (after lag and exponential phase). Zero population growth as cell division rates are balanced by cell mortality due to energy or nutrient depletion or buildup of toxic metabolites.

Survival state: Cells are strongly energy limited, and—besides not growing, or only growing very slowly—express visual signs of energy shortage. Cellular activity is restricted to repairing damaged essential biomolecules, such as DNA and certain proteins, and maintaining membrane potential.

Survival through cell division (STCD): Starvation response found in copiotrophic microorganisms, where cells respond to severe energy limitation by reductive cell division, forming ultramicrobacteria. Typically viability decreases over time, but cells remain culturable.

Viable but nonculturable (VBNC): Starvation response found in copiotrophic microorganisms, where cells respond to severe energy limitation by size reduction without cell division, loss of culturability, but maintenance of viability.