

Crystal ball – 2009

In this feature, leading researchers in the field of environmental microbiology speculate on the technical and conceptual developments that will drive innovative research and open new vistas over the next few years.

How to use a crystal ball in environmental microbiology: developing new ways to explore complex datasets

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A crystal ball is an instrument which – when used properly – helps to gain information on the past, present or future by other means than common human senses and the standard technologies supporting them, with the purpose to use this information to generate knowledge and to aid decision making. Hence, it could be a very useful tool in environmental microbiology, which deals with an enormous number and diversity of unknown populations, processes and habitats, many of which escape human scales, and almost all of which are not traceable in the past – if we would only know how to use the crystal ball. Esoteric handbooks say that: (i) one must have a specific question to ask, (ii) one must be able to completely concentrate on this question and be very patient until the fog goes away and some information-containing images develop, then (iii) one must accept what one sees and not be blocked by preconceived opinions, finally (iv) the crystal ball is a sensitive tool that must be well maintained, cultivated and surrounded by positive energies. So, doesn't this sound like the good advices we get from our statistics experts and data base managers in how to deal with large and complex data sets?

Let us face it, the big challenges in environmental microbiology – such as estimating total microbial diversity and its temporal and spatial patterns in a given habitat, studying evolution and succession of populations, recording and predicting the effects of global change, deciphering biological interactions, understanding the links between genomes and metabolomes in organisms – can only be solved by supernatural clairvoyance, or by a painstaking effort to improve observation, data recording, data accessibility and availability. In other words, the urgent questions in environmental microbiology are known, the methods and technologies are largely available, but the culture and art of retrieving and dealing with

large multidisciplinary data sets have remained underdeveloped in our field, and are still largely missing from the education of new generations of scientists.

The coming years will be associated with dramatic changes in the way we hitherto have approached microbial diversity and functions in natural environments. The drivers of these profound changes are already noticeable. First, major environmental issues (think global warming, ocean acidification, species extinction, altered land use) create a number of real-life, large-scale natural experiments with uncertain outcome, and increase the pressure to provide more predictive knowledge. Second, neighbouring disciplines such as oceanography and the geosciences have already prepared for new scales of global earth observation to which environmental microbiology could be hooked in many advantageous ways. Third, powerful sequencing tools are now offering a more detailed snapshot of the extensive diversity in natural environments. Fourth, the current revolution in single-cell imaging is just starting to open new dimensions in our understanding of what microbial life is about. Not only can we now look at who is there, but also at what and when they do it on an individual basis. Microbial ecology is showing the signs of a profound mutation of concepts, approaches and methods because microbial diversity and functions can now be questioned routinely over a huge range of spatial, temporal scales and environmental gradients, just as macrobial studies.

From those simultaneously occurring scientific revolutions that describe the infinitely small and immensely big patterns of microbial life, exciting progress will logically come from the merging of those fields, so that butterfly effects at the single-cell level can be detected in their complex and ecosystem-sustaining context. Although successful results have been obtained by the description of specific cells to ecosystems, it will be also needed to place the pieces of the big puzzle back together: the understanding of the ever-increasing -omics databases will not be fully comprehended if we impose our compartmentalized vision of the world to the objects of our research: Terrestrial and marine realms, sediment, water column and atmosphere are all interconnected. Moreover, macro- and microorganisms are also connected in ways we have not understood. More studies are needed that investigate biological interactions and compare diversity patterns across taxa, not only to determine whether the same scaling rules apply, but also to identify common structuring factors, and the taxonomic levels at which

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comparisons are meaningful. Furthermore, the under-explored topics of intra-community dynamics, community turnover and functional complementarities within habitats and communities require further effort. Intra-community dynamics could indeed be the unsuspected keys to the many bizarreries of microbial communities that make them appear so structurally and functionally unpredictable.

So, at first sight, our crystal ball shows us, looking into the crystal ball. The closer we get to the real world with all its complexity, the larger our databases of sequences and contextual parameters become, the more we need to focus on specific questions, to develop tools for data exploration and visualization, and to be able to retrieve images representing knowledge from the fog – just as the successful fortune teller who knows how to handle the crystal ball. And here is a deeper look into our funky crystal ball for future developments in environmental microbiology: analytical methods will be borrowed from fields as distant as astrophysics or climate research, as they are the closest to deal with the same types of computer-intensive challenges we are facing. Both positive and negative results of analyses will be reported so that redundant work will be avoided, especially in the context of the limited computer power and time available for each analysis. As computing power becomes more available, Bayesian statistics, for instance, will be favoured by environmental microbial ecologists who wish to assess the likelihood of all competing hypotheses within the space of all possible models that would apply to a given dataset. Analytical pipelines will automatically data mine and produce new information, available for the participating scientific community, and this reward is high enough to make the effort worthwhile. The growing knowledge of the whole field will be represented in a unique, constantly updated and well-curated, publicly available instrument. The current lab-centred approaches will be replaced by something more structured, bigger and transparent – a giant crystal ball maintained by a global community effort.

Living organisms are information traps: making Synthetic Biology innocuous

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Synthetic Biology aims at reconstructing life *de novo*. This asks for construction of two chemically distinct components: the cell factory and the program running it. An experiment of whole genome transplantation has been produced by one laboratory, driving a recipient host to give birth to a cell of a different species, that of the transplanted genome (Lartigue *et al.*, 2007). In the cell,

the program is separated from the machine, exactly as it is in computers. We expect that this feat will be reproduced elsewhere, and that variations on this theme will clarify the idiosyncrasies of the operating systems that drive organisms into life (we already recognize three of them, in *Archaea*, *Bacteria* and *Eukarya*). Analysis of genome channelling will help the model of the cell-as-a-computer to develop further (Mingorance *et al.*, 2004). Thus, *information* is placed at the heart of all our scientific exploration. We can now conjecture that Information will be considered as an authentic category of Nature, on a par with Matter, Energy, Space and Time. Indeed, with the present reader, I share some exchange of information: this uses some of the four standard categories, but not much! Information *per se* is therefore an essential category. It will soon be accepted generally that information cannot be derived from the classical ones. An entirely novel mathematical formalism will be invented to represent information, as well as experiments aiming at decyphering the articulation between Information and the four standard categories: we need the equivalent of Einstein's $E = mc^2$, with information at its core. Leaving the abstract world of mathematics, we will soon push this conjecture much further, and understand that living organisms are information traps. This will have enormous consequences on our understanding of several major processes associated to life. Ageing for one, and of course initiation of cancer. But we will also begin to see evolution, and learning or memory, as directly associated to the capacity of living organisms to trap information, whatever its source. This knowledge will reshape our view of Synthetic Biology.

Babies are born very young

Not convinced? Here is the short argument. Aged organisms make young ones: to perpetuate life requires *creation of information*. Making a progeny is ubiquitous, and there must be genes involved in the process. Creation of information does not require energy (Landauer, 1961). Yet accumulation of valuable information needs to make room, and energy is needed there to prevent destruction of already accumulated information (Landauer, 1961; Bennett, 1988). We will identify biochemical degradative processes, and energy sources, that implement the process *in vivo*. We will also understand how orthogonal processes, using different enzymes and energy sources, permit locally autonomous accumulation of information (learning should not be too much affected by general metabolism, for example). This ability to trap information has considerable consequences for Synthetic Biology: while we wish to make factories which produce fine chemicals, depollute our environment or synthesize bio-fuels, we will face a terrible dilemma. Either we will make

the equivalent of our classical factories, and omit in our cell constructs the genes that permit accumulation of information – and this will result in cells that will multiply only for some time, then die. Or, aiming at perennial factories, we may wish to include genes that are used to catch and hold information. We will then end up with cells that, contrary to our desire, will begin to act according to whatever information has been created, rapidly forgetting the goal for which the cell factories have been constructed. In this context ‘scaling up’ will be a nightmare. But it will be a rosy dream for those who are afraid by genetically modified organisms and the like: the only dangerous organisms will be those that already exist, having evolved so as to harness information capture in a very efficient way. This will put us back to reason: the really dangerous bugs to come are not domestic, they are those transplanted from foreign countries to virgin ones (Xie *et al.*, 2000; Global invasive species database: <http://www.issg.org/database/species/search.asp?st=100ss>; National invasive species information centre: <http://www.invasivespeciesinfo.gov/>).

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C-MORE/Agouon Institute young investigators perspective on the future of microbial oceanography

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During the 2007 summer training course ‘Microbial Oceanography: from Genomes to Biomes’, hosted by the Center for Microbial Oceanography: Research and Education (C-MORE) and sponsored by the Agouon Institute, 16 international students (graduate as well as post-doctoral) were given the opportunity to improve their knowledge in microbial oceanography. Some of the leading microbial ecologists and oceanographers in the world were recruited to participate as faculty for this course and offered an exceptionally interactive environment. Three major topics were addressed during the course: (i) Microbial Control of Biogeochemistry, (ii) Microbial Growth and Metabolism and (iii) Biodiversity and Evolution of Microbial Physiology.

Dr Alexandra Worden (Monterey Bay Aquarium Research Institute) led a colloquium focusing on the *Environmental Microbiology* 2005 ‘crystal ball’ article in which leading researchers describe their vision of what practical and theoretical developments will drive the field of environmental microbiology in the coming years. Students were encouraged to think about and articulate what intellectual and technological advances they believed would shape innovative research in microbial oceanography in the coming years. The following contributions are the opinions and ideas developed by some of the students during the research training course that highlight what we believe will take centre stage in microbial oceanography in the near future.

Ecosystem metabolism: realizing prokaryotic physiology and growth

While it would seem microbial production and respiration might simply be a game of addition and subtraction, the end result of determining metabolic balance in the sea remains one of the more elusive topics in microbial oceanography. The reasons for this are complex, with the focal point of the issue being our inability to accurately determine ecosystem balance: Are aquatic ecosystems net heterotrophic or net autotrophic? How flexible are microbial physiologies and how does this flexibility influence biochemical stoichiometry? How applicable is Redfield stoichiometry over large temporal and spatial scales, and how will the changing atmospheric CO₂ regime influence

cellular stoichiometries? Are there spatial and temporal decouplings in the net system metabolism and what processes underlie such dynamics? One of the first steps to addressing these questions would be to identify and characterize the full breadth of extant prokaryotic metabolisms.

Knowledge of environmental prokaryotic diversity and functioning has grown exponentially in the last 30 years. Historically, the central dogma has been that organic carbon production was separated over some time and space scales from organic carbon decomposition, which reflected the conceptual framework of autotrophy versus heterotrophy. In the early 2000s, views of carbon fluxes and energy budgets were changed by the realization that a significant number of the previously defined heterotrophic marine bacterioplankton may actually grow photoheterotrophically, obtaining energy from sunlight and reducing power and nutritional substrates from organic matter. Two almost contemporaneous discoveries contributed to changing views of marine bacterioplankton physiology and its contributions to oceanic carbon flux and energy budget. One was the discovery of the global presence of bacteria containing bacteriochlorophyll (e.g. Kolber *et al.*, 2001); the other was the discovery of bacterial rhodopsins, proteorhodopsins (Béjà *et al.*, 2000). The phototrophy conferred by such mechanisms has the potential to supplement energy derived from respiration in fueling essential functions such as cellular maintenance and active growth (Béjà *et al.*, 2000; Gómez-Consarnau *et al.*, 2007). On the other hand, more than a decade before, what was believed then to be strictly phototrophic such as *Cyanobacteria* (*Synechococcus* and *Prochlorococcus*) were found to be capable of assimilating organic molecules (e.g. amino acids), thereby comprising an active component of secondary production and likely heterotrophic metabolism.

We think that, despite the myth that metabolism is, in its holistic meaning, basically mapped out based on cultivated model cellular systems, the improvement of genomic techniques together with the effort in improving culture strategies will reveal still more diverse metabolic pathways that we are not fully aware of but have enormous potential for biotechnology and industry.

Microbial biodiversity: molecular techniques and the need for creative culturing

In the last decade, molecular techniques have dramatically shifted our understanding of the functional diversity found in microbial communities. However, many questions remain as to how this diversity relates to community function. A large portion of environmental sequences are annotated as hypothetical, and even those genes that have an annotation might be misidentified,

particularly since a large portion of current databases are biased towards biomedical, rather than environmental sequences. Furthermore, it is unclear what components of a community's genetic pool are actively expressed, and how that expression varies on temporal and spatial scales. One method, metaproteomics, holds great potential, although current technological challenges restrict its widespread use in the very near future. On the other hand, the technology for isolation and sequencing of environmental RNA (metatranscriptomics) is readily available today. We believe as sequencing costs continue to decrease, the use of metatranscriptomics will become more widespread, offering researchers the opportunity to discern between a community's genetic potential and actual function.

While molecular methods have enabled extensive insights into microbial diversity in various environments, a 'physiological revolution' is upon us that necessitates representative cultured isolates. Having culturable representatives of organisms containing genes found to be of interest from metagenomic surveys provides us the opportunity to functionally annotate the metagenome of the ocean and acquire information that can help refine our models of the biological contributions to geochemical cycles in the oceans. Moreover, such approaches have informed our understanding of microbial physiology and therefore lend themselves as potentially useful tools in our efforts to constrain ecosystem metabolism.

We now have several technologies that have demonstrated success in allowing us to cultivate the 'uncultivable' and a wealth of metagenomic data to draw from as we proceed with our cultivation efforts. Thus, our current challenge is to be creative in how we couple the information we gain from metagenomic studies and physiological investigations of novel organisms to better direct our future cultivation efforts using these technologies. A variety of approaches have been successful for the cultivation of novel organisms from the environment. A now classic example is the use of high-throughput methods that employ a dilution-to-extinction approach, which enabled the first cultivation of members of the ubiquitous SAR11 clade (Rappé *et al.*, 2002).

In the case of these SAR11 isolates, a dilution-to-extinction approach provided a means to eliminate competition by faster-growing organisms. In another example, a diffusion growth chamber was utilized to allow the exchange of chemicals between the chamber and the environment but restricted the movement of cells; this study revealed that isolation-based approaches may impede cultivation success by disrupting natural chemical interactions that occur between organisms (Kaeberlein *et al.*, 2002). Thus, an important consideration for future cultivation studies and studies of marine microbes in general is how the dynamic chemical interactions

between organisms in the marine environment may affect their growth and cultivability.

Small but powerful: aquatic viruses

The significance of viral-mediated processes for the understanding of global biogeochemical processes has become increasingly apparent over the past two decades. But what do we really know about aquatic viruses and their impact, what do we think we know and more important, what do the current methods allow us to think? Viruses are small, highly abundant (Bergh *et al.*, 1989), have great potential to infect several autotrophic and heterotrophic bacteria, archaea and eukaryotes (Proctor and Fuhrman, 1990), and therefore great potential to shape the chemical composition and biological diversity of aquatic communities. By lysing prokaryotic and eukaryotic cells viruses can stimulate ecosystem respiration and nutrient regeneration, alter the transfer of energy and organic matter, and influence genetic exchange among microplanktonic members of aquatic environments. Determining whether cells are removed by grazing or viral lysis has important biogeochemical implications. Cells that undergo grazing can be channelled to higher trophic levels, while cells that undergo viral lysis may funnel more material into the dissolved phase, thereby reducing the flux of sinking particulate organic matter from the surface ocean to the seafloor ('biological pump'), a significant issue in terms of global carbon cycling.

Successful infection depends on the encounter between the virus and the host, partly explaining the fact that some groups of microorganisms and habitats are minimally affected by virus-induced processes. Heterotrophic bacterial mortality due to viral lysis is much better studied than that of phytoplankton and there exist even fewer reports on viruses infecting marine heterotrophic protists. In addition, most data on virus infection are for the marine surface waters rather than subsurface waters or sediments. Assessing (i) the impact of viruses on host populations and (ii) the fate of host cell material released remains a difficult but important challenge that is currently limited by methodological hurdles (Suttle, 2007). Results of viral-induced mortality of prokaryotic and eukaryotic organisms obtained from different approaches (e.g. modified dilution method, transmission electron microscopy) often produce variable and ambiguous results. Thus, we need more straightforward and reliable methods to assess viral-induced microbial mortality in order to include these processes in complex nutrient and energy cycle-based models. Combining existing approaches with advances in flow cytometry or molecular tools might reveal new and promising insights.

Recognition of the pivotal role viruses may play in aquatic ecosystems has undoubtedly been fruitful to our

knowledge of processes in microbial oceanography but still 'the unknown appears to beat the known'. In the past two decades, much research had been devoted to the understanding of marine viruses and we foresee that much more progress will be made in the near future.

Ecological theory and the all-important hypothesis

Microbial processes are essential for ecosystem sustainability and understanding these processes appears to be best achieved by generating a theory that is based on existing observations and subsequent experimental validation. In the face of the new '-omics' age, the integration of novel technologies into classical ecological concepts should occupy a central position for future research in microbial oceanography, and both general ecologists and microbial ecologists need to become more interested in bridging the gap between these two disciplines. The concept of ecological theory applied to microbial oceanography is not new – and yet we still struggle to develop theories to encompass patterns and observations. When it comes to microbial ecological theory: does one apply to all? Are there perhaps multiple theories to explain different environments? Or are we pressed to develop a new, all-encompassing theory? Nevertheless, much can be learned from classical whole-ecosystem approaches, where investigations of the patterns of species abundance, distribution and biomass, community composition and food web structure can yield insight on the feedback mechanisms between marine ecosystems and the atmosphere.

Also, we must always be cognizant of the importance of hypothesis-driven research. Discovery-based science is valuable in and of itself, but proceeding without a clear purpose to meet a relevant conclusion will not propel this field forward. The goals of the study must dictate: Are relevant data collected and what are the relevant data? Perhaps one of the most outstanding hurdles to overcome rests in determining the appropriate scale at which to sample and test based on the particular question of interest. How do microbial oceanographers extrapolate microscale transformations, interactions and diversity to ocean-basin, or even global, processes? Is such extrapolation necessary in the context of a question that seeks to investigate the micro- or even nanoscale?

Continuous monitoring and time-series programmes will be invaluable, since they link changes in physical, chemical and biological aspects of marine systems and facilitate the microbial contribution to the global climate change. Events are more easily digested and described in hindsight, but predicting their impacts on microbial community structure and function, and larger-scale ecosystem and biome impacts will be a real challenge. Our hope is that targeted monitoring and hypothesis-oriented

research, accompanied by modelling efforts that are well informed by sound experimental data, will continue to improve and better focus our predictive abilities, allowing us to make critical decisions concerning the management of our current environment for future generations.

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Real-time evolution observatories in the wild

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Prokaryotic systematics is still lacking a theory-based species concept. The current pragmatic taxonomy of

prokaryotes describes genetically and ecologically diverse taxa as species. This conservative species concept is a drawback in microbial ecology hampering comparisons between prokaryotic organisms and eukaryotic micro- or macroorganisms. Another enormous drawback is of course that the majority of the prokaryotic diversity is represented by undescribed species. Hence, ecologists investigating prokaryotes struggle with the identification of phylogenetically and ecologically coherent taxa. Better insights in microevolutionary processes should be helpful in the progress towards achieving a future theory-based species concept for prokaryotes, because microevolution is resulting in the separation of phylogenetic units, which taxonomists could consider as species taxa. Recent and ongoing advances in DNA-sequencing technology combined with cost reductions will enable deep insights in the microevolution of prokaryotic populations. Observatories will monitor microevolutionary processes in microbial communities by metagenomic approaches in quasi-real time. It does not take a crystal ball to predict that such evolution observatories will (at least initially) not be installed for investigations of marine or soil communities. Extreme rather isolated aquatic systems like hot springs or small saline ponds could be in the focus of such projects. Metagenomic time series data on low-diversity communities inhabiting such systems should enable the observation of gene flow via different routes (conjugation, transformation and transduction) and spreading of fitness-increasing mutations within the most abundant populations. Analysis of gene flow within the community could reveal differences in intensity of gene flow between different coexisting populations. Quantification of the permeability of barriers to gene flow could be another cornerstone to a theory-based species concept. If lucky, the observers will witness immigration of superior competitors, selective sweeps or horizontal gene transfer. Even more interesting as installing evolution observatories at extreme habitats would be the investigation of planktonic communities in natural freshwater ponds characterized by the presence of predominant low-diversity populations strongly contributing to total prokaryotic cell numbers but only consisting of a few genotypes. In ideal cases such ponds would represent ecological islands more or less isolated (in terms of dispersal) from similar habitats. Non-extreme systems are characterized by more diverse ecological interactions (competition, predation, etc.) across more trophic levels. This may result in stronger and more diverse selection forces, which may increase the dynamics of genetic adaptations within prokaryotic populations. In such systems one may even observe bottom-up influences of microevolution events on the diversity and structure of communities at higher trophic levels. Clearly, single research group will not establish evolutionary observatories. These projects will

need intensive cooperation between disciplines like bioinformatics, evolutionary biology, population genetics, ecology, etc. My crystal ball already shows me animated depictions of gene flow and genome evolution in natural multi-population systems, displaying main and minor flows between and within populations via different genetic routes.

Energy emerges

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Beneath the thin skin of our planet's surface, most microorganisms exist under conditions of strict energy limitation. The availability of energy – a universal requirement of life – shapes the distribution, size, composition and activities of microbial populations throughout much of the environment. Such constraints may be especially tangible for life in 'frontier' environments, such as the deep subsurface or systems characterized by physicochemical extremes. In the realm of energy limitation, maintenance, survival or dormancy may largely replace growth as the prevailing physiological state (Morita, 2000). Our ability to characterize the largely unexplored populations living in this state – and, thus, to understand the true nature of Earth's 'silent majority' – depends critically on understanding the adaptation of life to low energy fluxes. Yet, our understanding of microbial physiology, including the mechanistic and quantitative aspects of energy metabolism, is based on model systems in which energy is provided in abundance. A look into the Crystal Ball suggests that, in the years ahead, 'energy' will emerge as a focal point for understanding natural populations that represent the current frontiers in environmental microbiology. But advancement will require the development of new models and new approaches in laboratory microbiology, combined with new technology and innovative approaches for the study of natural populations.

Energy fluxes in nature, particularly in the deep subsurface, may be profoundly lower than those that characterize our common experience, and upon which our understanding of microbial physiology is based. Even when considered on a cell-specific basis, energy flux and metabolic rate may be lower by five to six orders of magnitude in natural populations than in the cultured organisms that serve as models to represent them (Price and Sowers, 2004). For example, phototrophic green bacteria in the Black Sea chemocline live with an *in situ* photon flux of $0.001 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ – 10^6 -fold lower than surface irradiance – leading to estimated mean cell dou-

bling times of 3–26 years (Manske *et al.*, 2005). In deep-sea sediments from ODP Leg 201, measured bulk sulfate reduction rates and cell counts translate to mean cell-specific sulfate reduction rates of $10^{-4} \text{fmol SO}_4^{2-} \text{cell}^{-1} \text{day}^{-1}$ – about one sulfate ion metabolized per cell, per second (H. Røy and B.B. Jørgensen, unpublished). Approximately 10^5 -fold lower than cell-specific rates observed in energy-unlimited pure cultures, this metabolic rate would translate to cell doubling times of 300–3000 years *if* culture-based growth yields are applicable to the natural population. The likelihood that a larger fraction (perhaps all) of the energy of catabolism is partitioned into maintenance rather than into growth in the natural population suggests that doubling times may actually be much longer. How do the mechanistic and quantitative aspects of microbial energy metabolism, as we understand them from the study of model organisms in energy-rich cultures, map to the microbial ecology of systems that provide such dramatically lower energy fluxes?

To understand how energy availability influences microbial populations in the natural world requires that we examine microbial energy metabolism from new perspectives and using new methodology, with a focus on energy limitation. The questions to be addressed are fundamental:

- (i) What are the minimal energy levels and fluxes that can support life? How does the biological demand for energy change as a function of physicochemical environment?
- (ii) Are there biochemical or physiological adaptations that allow organisms to cope with lower energy yields? For example, smaller genomes and less active regulation of metabolism, decreased turnover or enhanced stability of biomolecules, and mechanisms that increase the efficiency of energy conservation and/or retard the futile dissipation of stored energy may all confer such capability. Do such mechanisms map to phylogenetic or metabolic affiliation, such that certain organisms possess an inherent advantage in low-energy settings? Valentine (2007), for example, points out a variety of characteristics that may favour *Archaea* over their bacterial counterparts in settings at the energetic fringe.
- (iii) Are there novel or unexplored energy sources that contribute to, or even dominate, the overall flux of energy in Earth's subsurface environments? Are there energy-harvesting strategies that are not yet represented among cultured organisms?

A combination of culture-based and environmental studies, aided by the emergence of new technologies and new approaches in both areas, is needed to resolve these questions.

Culture-based studies provide control, specificity and relative ease (in comparison with natural systems) in characterizing the physiology and activity of the cultured organisms. But cultures presented with environmentally realistic energy fluxes may grow so slowly that the time required to generate sufficient biomass for study with traditional methods could be unappealingly long. Nonetheless, the development of new culture methodologies that control and limit energy fluxes, and maintain rather than grow biomass, will be crucial to advancement in this arena. The development of analytical and imaging techniques that can perform characterizations on smaller scales (with much less biomass, or even on single cells) will aid significantly in this regard.

Access to highly energy-limited natural populations will expand substantially in the years ahead. Methodological and technological advances that are tailored to such environments – improved detection limits and accuracy for enumerating low cell numbers and for quantifying concentrations, fluxes and turnover of substrates at extremely low levels; techniques for assessing metabolic status *in situ*; and methods for linking genetic (metabolic) identity with biogeochemical function at low activity levels – will dramatically enhance our understanding of life at the energetic fringe.

Thus, energy will emerge, in the years ahead, as a path toward understanding the ‘silent majority’ of microbes that occupy the current frontiers of environmental microbiology.

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Microbial observatories in the sea

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All marine habitats support diverse and dynamic assemblages of microorganisms, including bacteria, archaea, eukarya and their viral and metazoan predators. These

complex communities interact through a variety of genetic, metabolic and ecological processes to sustain life in the sea. Recent discoveries of novel microbes, new metabolic pathways and their intimate interrelations have challenged pre-existing paradigms and have led to a renewed commitment to a comprehensive study of the biology and ecology of marine microorganisms from genomes to biomes (e.g. <http://cmore.soest.hawaii.edu>).

Our general understanding of the structure, function and regulation of microbial communities in the sea has changed radically during the past three decades but is still fragmentary. Currently, we are woefully ignorant of the time–space domains of microbial processes in the sea, in large part because of the extreme magnitudes of scale (seconds to millennia for the temporal domain and micrometre to ocean basin for the spatial domain). Because most marine ecosystems are remote and out of direct human sight, they are sparsely observed and grossly under-sampled; indeed, the scales that matter for microorganisms may be far removed from the scales that our own senses can perceive. Furthermore, anthropogenic forcing of the Earth since the dawn of the industrial revolution has created a ‘no analogue’ new world and we may be altering the ocean habitat in ways that we do not understand. The changing ocean may lead to different, perhaps novel, marine habitats that can select for new microbial assemblages. The lack of comprehensive temporal observation and facilities for long-term field experimentation currently limit and will ultimately restrict our rate of progress in the field of marine microbial ecology, in my opinion. There is no substitute for at-sea observation and experimentation, and no short cuts either. This situation needs to change, and it will improve in the near future according to my crystal ball!

Much of our extant knowledge concerning the role of microorganisms in the sea is based on either laboratory study of selected isolates or the results obtained during oceanographic research expeditions. The latter are, at best, single freeze frames of what are probably full-length motion pictures. Consequently, this expeditionary mode of data collection provides an incomplete account of the dynamic and stochastic nature of microbial life in the sea. Marine microbial community structure and the ecosystem services that they support are complex, time-varying, non-steady-state features of a changing planet, and must be studied as such. This demands a financial commitment for the establishment of microbial observatories for long-term observation of oceanic phenomena, an intellectual investment in the establishment of transdisciplinary research teams and the development of novel instrumentation for continuous, remote observations of marine microbes. Above all, there must be sustained ‘access to the sea’, not in the international political sense of Hugo Grotius’ classic treatise *Mare Liberum* (1609), but in a modern ecological research sense.

Long-term ecological studies are common in terrestrial and limnological research. They are ideally suited for investigation of subtle habitat changes, irregularly spaced stochastic events and complex interdependent phenomena. In 1988, two open ocean observatories were established to track the temporal changes in a variety of microbial processes in response to physical and chemical dynamics of the selected habitats. The two study sites, one in the North Atlantic Ocean near Bermuda (Bermuda Atlantic Time-series Study, BATS) and the other in the North Pacific Ocean near Hawaii (Hawaii Ocean Time-series, HOT), were initially focused on the ocean's carbon cycle from solar energy-based carbon dioxide reduction to particulate matter export and deep-sea carbon sequestration. At the time of their creation, there were no marine analogues of the atmospheric $p\text{CO}_2$ record (the so-called Keeling Curve) and no microbial-based ecosystem models for accurate predictions of carbon fluxes in the sea. To put our ignorance into proper context, *Prochlorococcus* – a tiny marine photoautotroph now recognized as the most abundant plant on Earth – had just been discovered, and the 'marine microbial genomics revolution' had not yet begun (the first full genome sequence of a marine microorganism was not available until 1996).

The design of the sampling and core measurement programme for HOT and BATS was based on the best information at that time; an approximately monthly frequency, ship-based programme lasting for at least one decade was believed to be a minimum investment for establishing the seasonal climatologies and variability spectra for most key physical, biogeochemical and microbial processes. Now, 20 years later, the data from these ocean time-series programmes continue to reveal previously unrecognized phenomena including, but not limited to, novel ecological processes and unpredicted climate-driven biogeochemical connections. These microbial ocean time-series programmes have attracted the interest of collaborators who have benefited from the existing core measurement programmes and from shared and leveraged access to the sea. The new knowledge gained is being used to improve existing ecosystem-based models, and to develop new ones.

At the beginning of the HOT programme, we had no idea that our efforts would facilitate the discovery of novel microorganisms, unexpected metabolic pathways and new ecological insights. We also had no idea that a monthly sampling frequency would be deemed inadequate for all key microbially mediated biogeochemical processes that we had endeavoured to understand. For example, it is now known that stochastic physical processes with time scales of less than 1 month can trigger a pulsed delivery of nutrients, resulting in changes in microbial community structure and a cascade of independent ecological processes. Higher temporal frequency microbe

detection to track these rapid perturbations will require the development of new sensors and sampling procedures since it is impossible to sustain continuous human presence at this remote field location. Significant microbial and biogeochemical changes have also been observed on decadal time scales, possibly triggered by large-scale changes in the coupling of the ocean to the atmosphere (e.g. El Niño) or by secular changes in habitat due to greenhouse gas-induced climate warming. Ultimately, these long-term changes are the result of processes taking place over a broad range of scales eventually propagating into the habitat that impacts microbial processes. Understanding how marine microbial assemblages in different oceanic habitats may evolve over time in response to climate change will require not only a characterization of the microbes' response to physical and chemical changes but also the development of how interactions among microbes contribute to the metabolic plasticity and resilience of the biome as a whole. Prior to the start of the HOT programme, the North Pacific subtropical gyre – Earth's largest contiguous biome – was thought to be relatively stable in time and coherent in space, a habitat supporting a climax-type microbial community that was well tuned to its environment and resistant to change. We now recognize a fundamentally different biome, one that is dynamic, variable and poised for ecological opportunity. The design of future ocean time-series programmes and field experiments can benefit from lessons learned in HOT and related time-series programmes.

In summary, marine microbial communities are genetically gifted, metabolically talented and functionally literate. All microbial and biogeochemical processes in the sea reside in a temporal domain, and each process has a characteristic time scale of variability. A major achievement of the HOT programme is an improved understanding of the time-varying changes in microbial community structure and fluxes of energy and carbon at a key, open ocean site. If the ultimate goal of ecological research is to understand how organisms regulate metabolism and growth, coexist in complex and diverse assemblages, and respond to various environmental forces, then the future research prospectus needs to be comprehensive, ranging from extensive field observations, to well-controlled laboratory studies using model microorganisms, to whole ecosystem experiments. The design and conduct of meaningful field experiments demands an *a priori* understanding of habitat dynamics and metabolic controls, which ultimately requires extensive at-sea observations in advance of formulating the specific research question. The emergent HOT data sets are serving as the basis for the generation of new ideas, improved models, controlled experiments and other forms of hypothesis testing. Future ecological research will focus on the interrelationships among climate,

habitat, microbes and their collective metabolic function. Most important to our success will be continued, indeed enhanced, access to the sea for sustained observation and experimentation. By the next year of the dog, I predict that at least a dozen new ocean time-series programmes will have emerged with a collective focus on the key roles that microbes play in sustaining planetary habitability. These comprehensive, transdisciplinary field programmes will fully integrate physical and chemical oceanography with theoretical ecology and modelling into the wonderful world of marine microbes. It should be a very exciting decade of discovery.

This article is dedicated to the past, present and future scientists, students and technical staff of the Hawaii Ocean Time-series programme on their 20th anniversary (1988–2008) of successful field operation (<http://hahana.soest.hawaii.edu>).

A visit to the paediatrician in the not-so-distant future

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'Open wide and say "Aahh" Timmy', Dr Lemon instructs the child, then swiftly takes a swab sample from his throat. 'That wasn't so bad, was it? Now, I want you to do one more thing and I promise we'll be done: step into the restroom and please pee into this little jar.'

As the child leaves the room, Dr Lemon turns her attention to Timmy's mom who has been sitting quietly in the corner of the room. 'I am delighted to see Timmy mostly on well visits now. . . . I'll send the throat sample away for microbial community profiling and the urine for a metabolite screen. We'll know by tomorrow if the beneficial microbe cream you've been using on his nostrils is still keeping those nasty Staph, Strep and H-flu at bay. . . . From the looks of him, I suspect those wonderful good germs are well-established and continuing to do their work. We will only call you if we detect any imbalances in his microbiota. . . .'

Dr Lemon has the experience to know that just a couple of decades ago the way she would have approached Timmy would have been dramatically different. He could easily have been one of those typical cases of children with recurrent ear and throat infections. At the first symptoms of otitis media she would have prescribed antibiotics, in great part to assuage parental anxiety. Chances are she would have seen Timmy come back many times and eventually amoxicillin would have proven ineffective as evolution played out its drama and resistant microbes took over the child's upper respiratory tract. Now, Dr Lemon knows full well that powerful antibiotics can be held in reserve for use in life-threatening systemic infec-

tions. The wonderful small molecule natural products made by beneficial microbes that routinely colonize the upper respiratory tract can be delivered 'naturally' using the topical probiotic cream she prescribed to Timmy. Early following of Timmy's microbiota by culture-independent methods had provided her with the clues that his particular microbiota put him at risk for colonization by *Staphylococcus aureus* and *Haemophilus influenzae*. Prophylactic treatment with a consortium of microbes known to produce a cocktail of bioactive secondary metabolites almost always rectified the imbalances in microbiota such as she had detected in Timmy. . . . Ever since the use of diverse probiotic approaches became widespread among physicians, the incidence of difficult-to-treat antibiotic-resistant infections had steadily decreased.

If the advances being made today through the study of the ecology of human microbiota do not begin to impact the way physicians treat their patients, we – the community of microbial scientists – will have failed. I strongly believe we will not fail. Recent advances in massive parallel DNA sequencing are already making microbial community profiling rapid and relatively inexpensive. The latest mass spectroscopic approaches allow rapid metabolite profiling. These facts combined mean that we now can begin to move from a human microbial ecology that has greatly focused on determining 'who is there' in a relatively small number of individuals to a human microbial chemical ecology that can ask 'who is there and what are they doing' on very large numbers of individuals in longitudinal studies worldwide. The information thus obtained will give rise to models where the patterns of microbial community composition dynamics and activities may be used to predict clinical outcomes. Couple this to a growing acceptance of the beneficial role of microbes, along with the perennial concern that the 'scorched earth approach' inherent in antibiotic therapy spells ecological disaster for the human microbiota, and you have the stage set for the development of probiotic prophylactic approaches to be applied in the clinical setting.

Experimental community -omics

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With microlitre-sized niches and generation times measured in hours rather than years, microbial communities are eminently manipulable. Microbial responses to system-wide perturbations can be measured on gratifyingly short time frames. Indeed, it was while reading about microbial reactions to manipulated watersheds at the Hubbard Brook Experimental Forest that I was inexorably pulled away from my fledgling career in plant ecology about 25 years ago. And while I was drawn into a world of

organisms I could not see, it was a world of organisms whose collective responses to precise manipulations could be measured in a single afternoon. That was hard to beat.

Molecular biology and genomics infiltrated the toolbox of microbial ecologists over the intervening years, and I was happy to jump on the technique bandwagon. These tools were critical for deconvoluting each part of the collective responses we had been measuring. Yet I also worried that they drew us away from a culture of experimentation. Many molecular studies were highly descriptive, a direct consequence of the laborious and expensive nature of the techniques. And some seemed downright anecdotal: a single sample from one location at one time was presumed to represent a microbial community more complex and dynamic than any of us has yet been able to imagine. The home-grown tool of metagenomics, while the most exciting of all, was also the most egregious in promoting unreplicated descriptive science. Since even one metagenome required a major financial commitment, we began collecting solitary community genomes in the hopes that they could shed light on fundamental questions about the organization and regulation of microbial communities. They cannot.

There is no doubt that the early metagenomic studies revealed truly amazing insights into microbial communities purely by providing descriptions of genes harboured in unknown and uncultured microbes (Handelsman, 2004; Delong, 2005). But description alone can only go so far, and in the upcoming era in microbial ecology, it will not have to. The availability of inexpensive sequencing in a size range ideal for comparative analysis of unassembled data is increasing daily; the development of cyberinfrastructures to archive and analyse hefty environmental sequence data sets has begun (Moran and Amann, 2008). Over the next 5 years, we can expect that survey metagenomics will give way to experimental metagenomics, and we will routinely measure replicated responses at the gene level for microbial communities perturbed in precise and repeatable ways. The rise of hypothesis testing through experimental metagenomics will be joined by similar experimental approaches using the developing techniques of metatranscriptomics and metaproteomics. To be sure, -omics-based hypothesis-testing in microbial ecology is already beginning, such as with recent studies comparing the genomes of bacterioplankton assemblages able to metabolize different components of marine dissolved organic matter (Mou *et al.*, 2008), and tracking changes in community gene expression in direct response to manipulated CO₂ concentrations (Gilbert *et al.*, 2008). Thus after a requisite period for assimilation, the -omics approaches will soon be joining the rest of microbial ecology in a highly

experimental framework that together will provide unprecedented depth of understanding of microbial processes. If I were not a microbial ecologist, I would be envious.

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Incredible anaerobes – more bioenergetic surprises to come

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Strict anaerobes are truly incredible. They can make a living from various, often unusual, substrates and lots of them live at the thermodynamic limit of life. Consider, for example, that methanogenesis from acetate does not yield more than one-third of an ATP molecule. Researchers have focused over the decades on the elucidation of the pathways that anaerobes employ to make a living and, like the princes of Serendip, made incredible findings on the road: novel enzymes with novel reaction mechanisms and unprecedented metal and cofactor content, as well as novel bioenergetic principles and coupling sites. For example, the pathways for methane and acetate formation have been solved and we have a good understanding of how cytochrome-containing methanogens conserve energy. However, we still do not know how methanogenic archaea without cytochromes, or the entire group of the environmentally so important acetogenic bacteria, conserve energy. In every class in which I have to teach the anaerobic way of life, I see students that are fascinated about the subject but astonished at the same time about the lack of information on the bioenergetics of these two groups. By the way, that also holds true for sulfate reducers, whose bioenergetics is only poorly understood. Meanwhile, the golden age of anaerobic biology still continues: novel metabolic capabilities, like anaerobic

methane and ammonium oxidation, as well as anaerobic degradation of aliphatic and aromatic hydrocarbons, were discovered in the last years and are being unravelled now. These bugs will give us even more bioenergetic problems to solve.

Genome sequencing puts us into a position to unravel the lifestyle of microbes by a bioinformatic approach. However, we should all keep in mind that all we can get from these studies is a 'molecular hallucination' of a lifestyle. Nonetheless, genome sequences help to find out what is NOT there and sometimes give ideas about what is there that may lead to the discovery of new bioenergetic principles. Two recent good examples: the genome sequence of *Clostridium kluyveri* implicated a way to explain an old question: how clostridia couple butyrate fermentation to hydrogen production. Apparently, by a new mechanism called 'electron bifurcation' (Herrmann *et al.*, 2007, Li *et al.*, 2007). There, the exergonic reduction of crotonyl-CoA with NADH as reductant is used to drive the endergonic reduction of ferredoxin. The latter is then used as a driving force to reduce protons to hydrogen. This pathway is apparently similar to the electron bifurcation in the *bc₁* complex. This discovery may shed new light on other mysteries. For example, the still not solved mechanism of energy conservation in cytochrome-free methanogens. There, the FAD-containing subunit of the heterodisulfide reductase may catalyse a similar reaction (Thauer *et al.*, 2008). This would readily explain the coupling between exergonic and endergonic reactions in this group of microbes and I am convinced that we will see a lot of development in this area in the future.

The genome sequence of *Clostridium tetani* revealed another unusual feature: a gene cluster (*rnf*), whose predicted proteins have similarities to a long-known enzyme, the Na⁺-translocating NADH quinone reductase from marine vibrios. What is this enzyme doing in clostridia? Brüggemann *et al.* (2003) suggested that it couples the exergonic electron flow from reduced ferredoxin to NAD, with electrogenic Na⁺ transport across the cytoplasmic membrane, thereby conserving energy in form of a transmembrane Na⁺ gradient. Genome sequences tell us that the *rnf* genes are widespread in microbes, aerobes as well as anaerobes. The Rnf complex is a new coupling site in biology, and especially important in anaerobes that employ the complex to harvest a little energy from the difference in redox potential between ferredoxin and pyridine nucleotides (about 100 mV, just enough to pump out one Na⁺) (Müller *et al.*, 2008). Although the yield seems low, it actually makes a major fraction of their overall energy yield. Recent data suggest that Rnf may also be the long-sought coupling site in cytochrome-free acetogens (Müller *et al.*, 2008). Clearly, more work is required to prove or disprove a role of the Rnf complex in ion

transport, but we can be certain of a major advance in this direction in the near future.

The road is paved to deepen our understanding of bioenergetics of anaerobes. It is paved with biochemistry and genomics but there are still major holes in the road. We need to develop genetic systems for more anaerobes to address the physiological role of these new enzymes. The genetic system (always together with the appropriate biochemistry) will also open the door to a new field: how anaerobes interact with their environment, how signals are sensed and how the signal is transduced as far as protein or gene activation.

Enough now, my crystal ball is running hot, too much smoke, only little to see on the ground; we can all be excited, the future will bring many more surprises from our old friends, the incredible anaerobes.

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Sulfate-reducing bacteria: a deep biosphere–early life connection?

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Sulfate reducers are a unique physiological group of microorganisms, mainly belonging to the domain *Bacteria* with only two representative genera within the domain *Archaea* (*Archaeoglobus* and *Caldivirga*), sharing the ability to use sulfate, SO₄²⁻, as terminal electron acceptor for their growth. Sulfate-reducing bacteria (SRB) are present in all the terrestrial and subterrestrial ecosystems

(Fauque and Ollivier, 2004). Indeed, besides soils, digesters and other common habitats, SRB thrive in extreme environments and their activity has been demonstrated at temperatures and salinities as high as 100°C and 25% NaCl, respectively, and at pH of 4 or 10, thus suggesting that their respiratory systems can adapt to almost all the drastic physicochemical conditions that they may encounter, in particular in hot and/or saline deep environments within the Earth.

Determining who are the dominant actors of the deep Earth biosphere remains an unsolved challenge. Are sulfate reducers a main component of these ecosystems? Indeed, one of their important metabolic features is the ability for most of them to grow using H₂, thus making them good candidates for lithotrophy in the deep crust of the Earth, as reported for thermophilic *Desulfotomaculum* spp. pertaining to the *Firmicutes* (Moser *et al.*, 2005). Interestingly, using environmental genomics and metagenomics, a similar type of uncultivated sulfate-reducing sporulating bacterium (*Candidatus Desulforudis audaxviator*), also belonging to the *Firmicutes*, has been identified as the major inhabitant of a 2.8-km-deep gold mine in South Africa (Chivian *et al.*, 2008). All the processes necessary for life, including energy metabolism, carbon fixation and nitrogen fixation, are encoded within its genome, thus suggesting that it could survive and even grow in complete autonomy within the porosity of deep hot rocks. Similar energy production based on H₂/SO₄²⁻ has been observed for thermophilic SRB representing the phylogenetically deepest-branching bacterial groups and consisting of the genera *Thermodesulfatator* and *Thermodesulfobacterium*. Indeed *Thermodesulfatator indicus*, which has been recently isolated from deep-sea hydrothermal vents, was shown to almost exclusively use H₂ in the presence of sulfate (Ollivier *et al.*, 2007). Similarly, *Thermodesulfobacterium* spp. recovered from oilfield waters have been reported to oxidize a limited range of substrates including H₂ (Ollivier *et al.*, 2007). It is therefore clear that SRB play an important role in the deep biosphere together with *Methanoarchaea* and homoacetogenic bacteria.

It is somewhat natural to make a connexion between the anoxic ecosystems of the deep biosphere and the early life, since atmosphere and hydrosphere were devoid of O₂ during the first 2.2 billion years of Earth history. Prokaryotic fossils from that ancient time are basically inexistent, or at best controversial and poorly informative, but the fossilized stable isotopes signals can provide some useful informations. Bacterial sulfate reduction is accompanied by an enrichment of the produced H₂S or sulfides into the lighter S isotopes and isotopic evidences of SRB in the present-day deep biosphere have been documented (e.g. Fardeau *et al.*, 2009). Isotopic analysis of microscopic sulfides in 3.47 billion-year-old barites from

Australia indicated microbial sulfate reduction, thus suggesting that the sulfate-respiring process might have been an ancestral metabolism (Shen *et al.*, 2001). The univocal biogenic character of this signature remains however to be demonstrated since abiotic hydrothermal processes may provide similar isotopic signals. The isotopic record of archaean rocks nevertheless suggests that biological sulfate reduction occurred, and that the relatively low fractionations observed for sulfur isotopes in these old samples are due to low global sulfate contents in the archaean oceans (Habicht *et al.*, 2002). As a matter of fact, it is not easy to maintain large amounts of SO₄²⁻ under strictly anoxic atmospheres. In this context, the recent suggestion, again on stable isotope grounds, that early archaean microorganisms produced sulfide from elemental sulfur by disproportionation rather than by sulfate reduction (Philippot *et al.*, 2007), is of great interest. To our knowledge, with the exception of one non-SRB facultative anaerobe, only SRB are known so far to perform this reaction (e.g. *Desulfocapsa sulfoexigens*). In this respect, SRB might have been in a direct line of descent from ancestral Sulfur-Disproportionating Microorganisms (SDM), the emergence of SRB possibly responding to the large delivery of SO₄²⁻ from sulfur disproportionation and/or to a progressive oxidation of the atmosphere/hydrosphere ca 2.7 billion years ago. Following that line of arguments and going back and forth between deep biosphere and early life problematics, an interesting hypothesis would be that SDM, together with SRB, are important contributors to the present-day deep biosphere.

A primary biogeochemical importance of SRB is their contribution to sulfide production. Indeed, sulfides are powerful antioxidants which play a pivotal role in a large variety of aerobic and anaerobic organisms (Lloyd, 2006). In this respect, SDM and SRB, as active sulfide producers, might have been of significance in life adaptation to a progressively oxidizing atmosphere and possibly play an important ecological role in oxidation/reduction gradients in the present-day deep Earth. Moreover, H₂S outgassing from geochemical systems leads to pH increase and thus to potential solid carbonate precipitation. The role of SRB in the genesis of dolomite, a major carbonate rock, has been suggested under hypersaline conditions (Vasconcelos *et al.*, 1995), and the link between sulfate reduction and solid carbonate production has been documented (Aloisi *et al.*, 2006). Correlations between SRB activity and dolomitization peaks could be searched in the ancient Earth. In this regard, an unsolved challenge is to determine whether solid carbonates produced by SRB do really exist in the Earth subsurface. This question is of particular importance in the context of projects of deep CO₂ geological storage. The possible acceleration by SRB of the transformation of CO₂ into solid carbonates is one of

the important issues in carbon capture and storage research. A documented association between solid carbonates and SRB indeed occurs in the biogeochemical process of Anaerobic Oxidation of Methane (AOM) at shallow and deep water cold seeps, and in ubiquitous methane sulfate transition zones of the sediments (Knittel *et al.*, 2005). In these systems, SRB, which have not been cultivated yet, are associated to methanotrophic archaea. The existence of AOM recorded in 2.7 billion-year-old rocks from Australia has recently been suggested on stable isotope grounds (Thomazo *et al.*, 2009). More confirmations are needed but one can suggest consortia of methanotrophic archaea and SRB as an interesting research objective in the present-day deep biosphere.

Thus, all the information that we have got in recent years is convergent with the idea that SRB are active contemporaneous actors in biogeochemical processes existing in terrestrial, but also in deep subterrestrial ecosystems. Stable isotopes suggest their importance both in deep underground and in the oldest geological records. Sulfate-reducing bacteria probably play a determinant role in modulating the fluxes of CO₂, H₂, CH₄ and H₂S coming from the deep Earth. Their metabolisms may have deep implications for deep geological storage. For sure, it is clear that much more attention should be paid to their microbiology, physiology and metabolism, which are far from having delivered all their secrets, in order to get a better understanding of their impact not only on biogeochemical cycles, but also on the life–Earth co-evolution.

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An interview with the Magic 8-Ball on microbiology in the 21st century: clairvoyant prognostication or stochastic guesswork?

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When we were first contacted by Ken Timmis to provide our input for the bi-annual 'crystal ball' issue of *Environmental Microbiology*, we immediately realized that we lacked such a convenient forward-thinking device in either of our laboratories. However, having both grown up in the 1950–1960s, we were familiar with another instrument of foretelling the future, namely the Magic 8-Ball, originally manufactured by the Alabe Crafts Company. Luckily, we managed to secure an appointment for an interview with the venerable 8-Ball, as its predictive services are currently in great demand these days by both high level government and corporate officials around the globe following the collapse of international financial markets. What follows below is a transcript of an interview quickly arranged over cocktails in the First Class Departure Lounge of the San Francisco International Airport. The 8-Ball was summoned in great haste by world leaders to attend the G-20 Economic Summit in Washington, DC.

Q: First, we would both like to thank you for making yourself available on such short notice. While the topic of the direction microbiology's future is important to many scientists, it is not as critical as that of the current international economic crisis. Do you find it difficult to bounce between these two disparate disciplines?

8-Ball: *No.*

Q: Well, getting down to the brass tacks now. There have been some exceptional technical breakthroughs in microbiology over the past few decades, most notably in the realm of rapid DNA genome sequencing of not only individual microbes, but entire microbial communities as well. When coupled with advances in computer algorithms and mass spectrometry it has spawned the allied fields of 'proteomics' and 'metabolomics'. Clearly these latter two disciplines can also be extended to the study of complex, mixed microbial communities be they found in benthic aquatic 'mats', within the gastrointestinal tracts of animals, or within infected diseased tissues. Do you think that this will be the major direction for microbiology in this new century?

8-Ball: *You may rely on it.*

Q: We are concerned about this development. Not so much from the perspective of exploiting the new technologies to achieve better and more comprehensive understandings of microbial processes, but by the fact that they are quickly becoming the only area of emphasis. If this trend continues it could ultimately displace and eclipse more traditional forms of microbiological investigation, such as culturing of novel species and defining their physiology and biochemistry. Would you agree with our concern?

8-Ball: *Signs point to yes.*

Q: But this could even supplant traditional bench-top biochemistry. No more mass culturing, column purifications, enzyme assays, crystallizations. No EPR spectra of metal(oid) reaction centres, just *in silico* modelling. These fields could also stagnate. Would you agree?

8-Ball: *Yes, definitely.*

Q: But this is not a good thing. After all, we don't even know the function of most of the operons in the genomes already fully sequenced, and many of these annotations just infer function from DNA sequence homology with known genes. It's the old case of 'garbage in/garbage out' when relying only on computer databases in this case, and similar homology does not mean similar biochemical functionality. We get the feeling that if we go too far down this path, we will lose expertise in the experimental area of testing these hypothesized functions, let alone discovering new ones from new microbial sources. Would you agree?

8-Ball: *Better not tell you now.*

Q: You're hedging!

8-Ball: *Concentrate and ask again.*

Q: OK, OK, we get it. We have to ask you a specific question. Well, this may just be a case of the pendulum currently swinging too far in one direction. There is something deeply satisfying about isolating a novel organism that displays novel biochemical properties, and then conducting further investigations using a number of available experimental approaches to better define how this novel process works mechanistically, and what other microbes out there have this facility. We do not think you can truly achieve this relying only on complex database comparisons. In other words, 'to know it you got to grow it' to recapitulate Derek Lovley's mantra. At some point individual researchers that rely heavily on *in silico* approaches may begin to feel dissatisfied using this route as a sole avenue of investigation. This could be from internal personal motivation or perhaps from external factors like subtle shifts in granting agencies and journal manuscript acceptances. Perhaps in 10 years' time there will be a strong swing back to embrace more traditional (but updated) classical approaches to microbiology and biochemistry as a means to enhance genomic databases. Do you predict that this will eventually occur?

8-Ball: *Ask again later.*

Q: Rats!!!! OK, it's now 'later'. Same question, so what's your current answer?

8-Ball: *Reply hazy, try again.*

Q: Give us a break! We are losing patience, and I see that they are calling your flight for boarding. So, while there is still time, will you please give us a response to our important question? We really are eager to know if this will occur.

8-Ball: *Outlook not so good.*

Q: So you don't think that there will be a shift back to using classic approaches any time soon?

8-Ball: *My sources say no.*

Q: Oh, you mean there will be a resurgence of classic approaches in the future?

8-Ball: *It is certain.*

Q: Well, thank you very much for your deep insights and for sharing your time. Don't worry about your bar bill, Stolz will pick up the tab. Have a good and safe trip. By the way, can you give us any stock market tips?

8-Ball: *My reply is no.*

Note: This contribution is a US Government work and is in the public domain in the USA.

Microbes are not(?) platinum

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A geochemist colleague whom I shall not name likes to provoke microbiologists into friendly arguments by saying 'A microbe is like a piece of platinum'. The essence of this

statement rests in the supremacy of thermodynamics: the microbe catalyses a reaction that inherently is favourable – its enzymes merely are catalysts for manipulating the rate. To geochemists, this black-box description of environmental microbiology illustrates an accurate principle of physical chemistry. Of course it also wholly ignores the workings inside the box.

'Inside the box' is what fires up environmental microbiologists. The diversity of a microbial community, the specific metabolisms regulated by its members, and the mechanisms of inter- and intracellular actions are keys to a deeper understanding of the guiding principles of microbiology and are the subjects of most articles published in *Environmental Microbiology*. Given that most days I claim to be both a geochemist *and* an environmental microbiologist, the above philosophy says I should be at war with myself. However, my colleague's implicit claim that the microbes themselves do not matter is not intended to be a pejorative, it is a challenge: prove that the contents of the box *do* matter.

Philosophically, the answer is easy. It is precisely the ability to differentially manipulate rates of reactions (including linking unfavourable ones with favourable complements, as in syntrophy) that sets microbes apart from minerals. Strict abiotic reactions cannot begin to compete with the redox complexity that microbes achieve over minute spatial and temporal scales. These net community-driven rates of redox processes determine how much reducing power ultimately is buried within sediments – imposing a microbial control on the oxidation state of the atmosphere. You and I woke up this morning and could breathe: for this we must thank the microbes, and not the autotrophic ones that produced the oxygen, but rather the heterotrophic ones that could not respire the residual organic matter fast enough.

Although it is easy to make qualitative arguments for the importance of microbes in maintaining redox disequilibrium, there remain many practical challenges to showing how, exactly, microbes control the inevitable slide towards minimum free energy. My crystal ball tells me that in the upcoming year, new efforts to quantify the connection between metabolic diversity and community diversity will reign. By this, I mean that investigators will step beyond the new, ultra-high-throughput pyrosequencing strategies for cataloguing 16S rRNA microdiversity. Counting ribotypes is not enough. The dominant (but not necessarily whole) genome complement, and in particular, the expressed proteome, must be linked to the diversity of redox processes performed within a given sample. To date, it remains unknown whether total metabolomic diversity correlates with diversity of ribotypes, but the answer to this question is of fundamental importance to geochemistry. How many species, how many metabolisms and how many electron transfer steps occur within a

given community? And most importantly, do these numbers vary directly or inversely with the rate at which carbon and energy metabolism slows down? New studies will illuminate the factors that determine the amount of untapped electron density that remains in a community's final resting state. This will include the importance of *gross* versus *net* metabolic turnover and coupling (or uncoupling) to ribosomal diversity.

Today it is possible to watch a radiotracer pass through a single cell, look at isotopes and biomarker lipids on nanometre scales, and catalogue gigabases of total DNA sequence from dozens of different environments. But in the future, the most successful projects will be the ones that most correctly match the resolution of the analytical resource to the nature of the question. The rebuttal to the 'platinum' argument will be quantitative and will be made at many scales.

Synthetic communities

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Recent technological advances, most notably in sequencing technology, have both amazed and confounded many environmental microbiologists. The rate of proliferation of genome sequences and metagenome data sets far exceeds available analysis resources. The astrophysicist Sir Arthur Eddington once said 'Not only is the universe stranger than we imagine; it is stranger than we can imagine'. Environmental microbiologists are now peering into a microbial cosmos of exceeding complexity, one that certainly stretches the imagination, and there is pressing need to constrain and bring experimental context to unabated sequence proliferation. We can expect that advancing technology will soon provide for assembly of metagenomes into genomes, reducing the number of permutations of gene content within the basic units of life – the cells. That will help. But what next? In order to fully understand a genome, I suspect there is no escaping the need of going to the environment or to some reasonable approximation of the environment. That is to say, to fully understand the functions encoded by a genome – and therefore predict the range of cellular activities of environmental relevance – it will be necessary to go to where the genome lives and evolves. The laboratory pure culture is far removed from environmental relevance.

Recent advances in measuring the stable isotope composition of single cells in the environment are certainly a major step in this direction (Orphan *et al.*, 2001). I suggest that another part of the answer to the 'what next' question is the development of experimental 'stepping stones' (bridges may not be the correct metaphor) to connect the experimental utility of laboratory culture with processes

occurring in the much more poorly constrained environment. One such stepping stone is the construction of synthetic communities that incorporate environmentally relevant interactions, reconstructing simple food webs and syntrophic interactions. There has been recent progress in this area and an associated recognition of the importance of spatial organization in determining process and community stability (e.g. Kim *et al.*, 2008; Stolyar *et al.*, 2008; Weibel, 2008). Such synthetic communities significantly increase biological complexity, but remain amenable to molecular characterization, offer the opportunity for genetic modification, provide a controlled environment in which to study the evolution of symbiosis, simplify measurements of single cell activity and local structure, and to some extent can be described by mathematical models. A classic two-tiered trophic interaction is the obligate coupling between hydrogenotrophic methanogens and fatty acid-degrading bacteria (syntrophs) to affect the final steps of organic matter mineralization in anoxic habitats. This environmentally significant interaction can be represented by simple two-member laboratory communities, as first described by Marvin Bryant (1967). Recent studies within The Department of Energy GTL programme has shown that some electron transfer systems appear to be dedicated to syntrophic growth (C. B. Walker, Z. K. Yang, Z. He, S. S. Stolyar, J. Jacobsen, J. A. Ringbauer, Jr, J. D. Wall, J. Zhou, A. Arkin, and D. A. Stahl, unpublished). The possible involvement of electrically conductive connections between the populations described by Gorby and colleagues (2006) points to novel mechanisms for metabolic coupling (and gene function) that may not be amenable to characterization in pure culture. Other examples of simple systems are the naturally occurring phototrophic consortium described by Glaeser and Overmann (2003) and the synthetic community of five developed for the breakdown of cellulosic feedstocks (Kato *et al.*, 2005). I apologize for any omissions I have made in this short piece, and will conclude with another possibly relevant quote, not from a scientist but from the abstract painter Georges Braque (1882–1963) – ‘I do not believe in things, I believe only in their relationships’.

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Anchoring (environmental micro)biology in physics-based theory

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In his contribution to the crystal ball 2 years ago, Tom Curtis pleaded for theory in biology. Even though new and more sophisticated technology will increase our ability to ask and address biological questions, they will merely augment our wonders of (microbial) life, rather than our understanding of it. We think he is absolutely right. We now have the technological ability to manipulate and look at many levels in molecular detail, from the noisy single-molecule level, to the global molecular level by -omics technologies to finally the whole population level through metagenomics. It does not take a crystal ball to predict that we will be flooded by more data, and data of new types that will undoubtedly be predicted by some of our colleagues somewhere else in this issue. The functional genomics and metagenomics studies of last years, however, show that they hardly lead to true understanding of biological function, but rather very often to an admittedly impressive list of hypotheses, leads and piles of numbers to lure at. Studies that have led to truly new understanding often showed a clever combination of new experimental technology with rigorous modelling and theory development, as Curtis suggested. These relate especially to single-cell, and even single-molecule analyses, with far-reaching consequences on how we should look at a microbe today: it is a crowded, noisy place, and heterogeneity is everywhere, from spatial heterogeneity

within the 'bag of enzymes' to phenotypic heterogeneity at the population level.

So what would the theory be about, and what would be the general characteristics of such a theory? To us one of the most fundamental questions in microbiology is about the adaptive potential of microorganisms. Its theory will differ from any theory in physics as its reliance on laws will be much less strict. The second 'law' of biology is natural selection, the first one being that all organisms carry something like DNA. Like the second law in thermodynamics its biological counterpart gives the arrow of time. In biology, every new adaptation – benefit – comes at a cost. Both the benefits and costs, together determining fitness, however, may have complicated physicochemical or ecological origins. It is also not to be expected that adaptations may prove the optimal solution as they are contingent on pre-existing network structures that are simply moulded by mutations – mere chance. Nonetheless, historical and physicochemical constraint-based optimality is a powerful concept that may offer interesting hypotheses, but one that should be handled with caution as it may lead us in the wrong direction.

During our yearly Christmas drinking opportunity, we were discussing the nature of the constraints that would play a role in a biological theory of adaptive responses. For starters, we concluded that protein economy is strongly controlled. Proteins are costly for a number of reasons. For instance, they occupy valuable space in the cytosol. The average distance between macromolecules turns out to be of the order of their radius: the cytosol is packed! This protein crowding also has a benefit, as it contributed to preservation of the nucleoid packing and efficient DNA segregation during cell division. But why not make a larger cell? Also this brings about costs associated with the construction of a larger periplasmic and membrane environment, and constraints related to the possible impact on cell volume to surface ratios, impact of distance on the rate of signal transduction, and the like. Then why are proteins so big? Enzymes accelerate reactions by offering a local environment in their catalytic site that has a reduced activation energy barrier for their cognate reaction. It turns out efficient catalysis is in part rooted in physical considerations dealing with the need for accurate positioning of chemical groups in the active site that may require large and, therefore, costly proteins.

So a biological theory of adaptive responses could predict that protein costs cause natural selection to sieve out cells that manage with lower macromolecule levels. In that regime, the inherently stochastic nature of processes may subsequently become a hindrance. As the synthesis and degradation reactions of, for instance, mRNA, will never be in sync, as it relies on random

walks of the catalysts, some cells may experience a number of consecutive transcription events without any interruptions by a degradation event. Other cells may experience the opposite. This means that, on average, cells may be identical but on short time scales differ substantially in their macromolecular make-up. Such molecular noise has indeed been measured at the single-cell level, leading to the awareness that regulatory networks typically rely on a few molecules and may become error prone. Typically, one finds a coefficient of variations of mRNA levels across members of an isogenic cell population of the order of 30%, meaning that approximately one-third of all cells will vary more than 30% from the mean level. When the correlation times between fluctuations span several generation times single cells may adapt to noise rather than meaningful information. And so, theory needs to deal with important questions such as to what extent does noise pose constraints on the evolution and adaptation of microbes? One easy way to deal with noise would be to increase the number of molecules as this would reduce noise through averaging, but as we saw, there are also costs associated to these molecules, especially if they are proteins. A 'near' envelope calculation shows that prokaryotic two-component signalling can function reliably and fast with a minimum of about 50 molecules per cell, which is about right for many such systems. The response time is then fast enough, so that any fluctuation in the number of sensor or response regulator would not affect the search time.

And so, at the end of the Christmas party, we concluded that much progress in understanding will be gained by addressing microbial life from the perspective of quantitative experimentation, insightful simulations and most importantly, genuine biological theory rooted in 'laws of biology' and constraints of physics and chemistry.

Protists and the rare biosphere

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Ecology of microbial eukaryotes is a burgeoning field, principally because the introduction of genomics tools promises to reveal the true diversity and phylogeny of protists through the discovery of species typically missed by classical approaches. The 18S rRNA gene surveys of protistan richness started only a few years back (López-García *et al.*, 2001; Moon-van der Staay *et al.*, 2001), but they have already covered a significant portion of the biosphere. These molecular surveys have discovered protistan novelty at all levels of taxonomic hierarchy, and also

indicated that the species richness of protistan communities may be so high as to border on being intangible. There is no 18S rRNA gene survey of any community that is reasonably complete; instead, even the largest ones appear to be no more than small samples from apparently endless lists of species present at any locale studied. This richness may be so substantial that even high-throughput sequencing approaches, such as 454 pyrosequencing technology, may not capture all the molecular species in a sample: as several ongoing studies indicate, larger sequencing efforts simply result in a larger number of detected species, with no apparent saturation of accumulation curves. The total sample richness may be around 500 protistan species per litre of water or gram of aquatic sediment (Jeon *et al.*, 2006; Stoeck *et al.*, 2007), of which the majority seems to be represented by just a few cells per sample. This is not unlike the dominance of rare species in prokaryotic communities, termed 'rare biosphere' (Sogin *et al.*, 2006). This prompts the following questions: what is the meaning and function of such enormous diversity of rare protistan species, and what maintains such diversity? We see three distinct possibilities.

First, the phenomenon of rare species may be only apparent, and the long tail of rare species in the community frequency distribution may actually not exist. If dead cells and free DNA can persist in the environment, most communities may be flooded with dead – but amplifiable – DNA of diverse geographic origin. Factors of purely physical and chemical nature would maintain this pseudodiversity, which would of course play no functional role in the community. If so, the protistan (and prokaryotic) communities may prove much less complex than is currently thought. This possibility is rarely discussed even though species from the 'rare biosphere' have actually never been seen: their presence is simply assumed because their rRNA gene sequences are detectable. There are two obvious ways to check this possibility: first, by focusing on rRNA species instead of rRNA genes, and second by developing a protocol for DNase pre-treatment of environmental samples.

Second, the rare species may be perfectly alive and functional, just very inabundant. Being continuously rare may have an interesting implication. For a protist, one litre is a very, very large volume, and so is the convoluted network of interstitial channels in 1 g of sediment for the bottom dwelling protists. If such volumes are inhabited by one to few cells per species, then kin cells may rarely – if ever – meet. The term 'population' could then be applied to such cells only metaphorically, as each cell would in reality represent a lineage quite independent of other such lineages. Two related cells from such a 'population', even though co-occurring in the same sample, could then be separated from their common ancestor by many generations, and be detectably different genetically. Note that this

means rare microbial species may not need geographical barriers for speciation: life in the diluted environment would by necessity make them allopatric. Note also that this scenario predicts a high level of microheterogeneity – a large number of minutely different genotypes – in environmental samples, which is of course precisely what rRNA gene surveys universally detect in microbial communities, both pro- and eukaryotic. To account for the observed diversity of rare biosphere, this scenario does not require high dispersal rate of microbial species. What it does require in order to explain the maintenance of the high community richness, and its genetic microheterogeneity, is long generation times for the majority of species. One way to check this possibility is *in situ* studies of growth rates characteristic of the 'rare biosphere'.

The third possibility is in some ways an intermediate between the two scenarios above. The rare species may be represented by living but metabolically inactive cells. This might be possible if the majority of species detected by rRNA gene surveys are dormant. Having few nutritional and energy requirements and being tolerant of many environmental stresses, dormant cells could survive for a very long time and travel long distances. As in scenario 1, high richness of the given community could be explained by multiplication in one locale, and dispersal to another. Genetic heterogeneity could then result from the co-occurrence of dormant cells arriving from different centres of origin. Of course, if the dormant cells do not eventually find conditions permitting their growth, they will die, meanwhile being functionally irrelevant to any environment through which they travel. Checking this scenario is challenging because it is difficult to tell the difference between a dead cell, and a cell that remains dormant only because we do not know how to induce its growth.

The three described scenarios are not mutually exclusive, and the composition of the 'rare biosphere' may originate from more than one source (including artefactual ones). Assuming that at least some species from the pool of the rare ones are viable, what would be the ecological role of such species? An exciting possibility was suggested previously (Pedrós-Alió, 2007): the rare species may be the 'seed bank' for new growth. The community response to environmental changes might be a population decline in abundant species, and increase in the selected rare ones, perhaps to the complete reversal of the dominance structure. This is an important scenario because it would explain how an ecosystem could continuously function even if faced with environmental change – whether gradual or dramatic. The role of rare species as a seed bank could be tested by challenging communities with known species structure, monitoring the changes, and learning about autecology of the key players as they replace each other. Revealing the true nature of the rare biosphere, be it protistan or prokary-

otic, will be a major research effort in coming years as it will help us understand the mechanism(s) that create(s) and maintain(s) biodiversity and ecosystem function(ing) on our planet.

Since the phenomenon of rare species only became apparent recently, these and other hypotheses on the extent and role of their diversity remain to be tested. The need for such testing promises that, for the microbial ecologist, the next few years will be very interesting indeed.

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Getting closer to the real life

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The golden era of bacterial genetics from the 1950s to the 1970s was a triumph of reductionism. The complexity of biology was scaled down to single-locus genotype–phenotype studies in controlled model systems on the bench that led to the formulations of the paradigms of molecular biology (Jacob, 1988).

Next-generation sequencing technologies (Shendure and Ji, 2008) will allow a rollback towards the wild. Whereas microbial ecologists in the pre-molecular biology era had to rely on phenotypic assays when they assessed the polymicrobial communities in a habitat, today's environmental microbiologists – like dedicated stamp-collectors – can impress their peers with large phylogenetic 16S rDNA trees that contain many novel Operational taxonomic units that yet did not make it into Bergey's manual.

Direct high-throughput sequencing of microbial metagenomes is the next step so that any inherently

biased amplifications of the specimen by PCR or cloning are bypassed. The quantitative composition of a microbial community can be determined from the millions of short sequence reads that are generated by the sequencing-by-synthesis technologies. The taxon-specific usage of short oligonucleotide words and the dictionary of taxon-specific longer oligonucleotide words are extracted from the growing encyclopaedia of completely sequenced microbial genomes. Strains, taxa and genera are identified from the mapping of primary sequence data onto the encyclopaedia of genomes, from the search for diagnostic words within short strings (15-mers to 100-mers) and from the analysis of oligonucleotide usage in longer strings (100–1000 bp of sequence). The software and algorithms for the handling, assembly and analysis of 10^8 – 10^{11} bp of primary sequence are currently being developed. An example from the own lab may allow to illustrate the power of the new algorithms. A dictionary of marker oligonucleotide words was compiled from more than 700 completely sequenced bacterial genomes. The subsequent search for these informative markers in the 150 Mb metagenome of the 'Global Ocean Sampling Expedition' (Yooseph *et al.*, 2007) took just 2 min on an ordinary PC including the implementation of the sequence data, the search and the generation of an output file with a quantitative estimate of the taxa present in the sequenced sample.

Similarly, the gene expression profile of the community can be assessed by direct sequencing of the transcriptome (Urich *et al.*, 2008). A novel, yet unexplored aspect will be the interspecies modulation of gene expression. In other words, we shall learn within the next years about the mutual interactions of microbes that are co-inhabiting the same niche.

In summary, next-generation sequencing technologies and the concomitantly developed novel bioinformatic tools will revolutionize the analysis of natural microbial communities. DNA sequence and mRNA expression data will continue to grow exponentially within the next years. Proteomics and metabolomics will also improve their performance in terms of sensitivity, specificity and coverage, but due to the more heterogeneous nature of the compounds at a much lower rate.

Technology for single-cell analysis of microbial communities will probably also evolve in the near future. Single-cell identification by *in situ* hybridization techniques has recently been linked with secondary ion mass spectroscopy to allow the simultaneous phylogenetic identification and quantification of metabolic activities of single microbial cells in the environment (Musat *et al.*, 2008). In parallel, iterative chip-based cytometry has been developed for the analysis of minute samples within the field of immunology (Hennig *et al.*, 2008). Living cells are self-immobilized within microfluidic chips and then stepwise

explored with a virtually unlimited number of intracellular and surface markers by automated epifluorescence microscopy. The author is anxious to see whether this new and rather inexpensive technique will cross-fertilize microbiology within the next years. The generation of a toolbox of antibodies for the robust immunophenotyping of bacterial populations will be a major challenge. A more obvious application will be *in situ* studies of the interaction of microbes with immune effector cells that already by now can be differentiated by chip-based cytometry with a plethora of marker antibodies.

François Jacob (1988) views science as 'the most elevating . . . revolt against the incoherence of the universe'. Hence, let us carry on this carry-on.

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Exploring the vast genetic wilderness: dsDNA viruses

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My reflex reaction at the honour of being asked to speculate about the future was to take a nostalgic trip down memory lane. This year marks the 20th anniversary of the seminal discovery of high viral abundance in the ocean (Bergh *et al.*, 1989). In this time we have established that viruses (especially the dsDNA type) are omnipresent and abundant in natural environments; that the process of viral lysis makes significant contributions to global biogeochemical cycles; and influences, directly and indirectly, the composition of co-existing microbial host communities. Now that our view of the forest is clear,

albeit with a healthy degree of uncertainty, the coming decades of viral ecology research promise to add details at the scale of virus–host interaction. Undoubtedly ensuing discoveries will alter our view of the synecology of specific host groups, and because of the ubiquity of viruses, will have implications for our understanding of global biogeochemical processes.

An elegant prelude to this prediction was the discovery that the genome of many phages infecting marine *Synechococcus* spp. and *Prochlorococcus* spp. contain *psbA*, a gene encoding the D1 protein of the photosystem II reaction centre (Mann *et al.*, 2003). Follow-on work confirmed our collective suspicions that indeed cyanophage *psbA* is expressed during infection and serves to maintain oxygenic photosynthesis during phage replication (Lindell *et al.*, 2005). Back of the envelope calculations based on host activity and estimated viral production rates predict that as much as 5% of global oxygen production can be attributed to cyanophages. What other genetic and physiological surprises will phages present in the ensuing decades? Certainly the possibilities extend far beyond their well-known ability to transform bacteria into pathogens.

Recent metagenomic surveys indicate that viral assemblages contain sequence homologues to functional genes across a broad range of metabolic categories, with the frequencies of occurrence similar to those seen in microbial metagenomes (Dinsdale *et al.*, 2008). The degree to which these metabolic genes are linked either to viral life cycle, as is the case for *psbA*, or as a signal of discriminate transduction mechanisms is unknown. However, this finding should serve as a collective 'shot across the bow' that we need to think even more broadly about the influence of viruses on the business of microbial communities. And these are only the genes we know.

It is a common tendency of human beings to regard the unknown as unapproachable or, at worst, unimportant. The one consistent message from viral metagenomic and genomic investigations has been that we know a lot less about the universe of genes on earth than previously believed. As these studies attest, the majority of sequences within a shotgun library from a natural viral assemblage (or genes within a dsDNA viral genome) either are completely novel with no homologue among previously reported sequences, or are homologous to only other uncharacterized environmental sequences (Wommack *et al.*, 2008). Moreover, for the minority of viral sequences that do show significant homology to 'known' genes within the GenBank nr database, the most common gene categories are 'hypothetical or unknown protein'.

Because dsDNA viruses are the most abundant microbes on earth with an equally impressive reproductive rate, means that this collection of unknown and novel genes are quite possibly the most actively replicated (and

expressed) genes on the planet. Thus, the opaque view of viral genetic diversity poses a number of philosophical and technical challenges that will shape the coming third decade of viral ecology research. Primary among these are the need to intelligently categorize unknown viral genes according to inherent sequence properties, metadata and phylogenomic/evolutionary models, and to subsequently develop rational means to prioritize those gene families for further characterization. Most of these gene families will not contain a single member allele within a known, cultivated virus. Because of this, more established techniques for functional characterization will be unavailable and the community will initially need to look to more phenomenological data (e.g. the occurrence, abundance and diversity of viral gene families within a range of environmental contexts) to form hypotheses on the potential function of unknown viral genes. Alternatively, clever new approaches to functional prediction may be developed through advancements in structural biology, bioinformatics and molecular genetics. In essence, meeting these challenges through the study of natural assemblages of dsDNA viruses will offer the first best test case of a true gene-ecology approach to understanding biological systems.

Practical benefits of the 'ecological' characterization of viral genes will be the long-sought ability to dissect and deconstruct viral impacts within complex microbial assemblages. While we understand that the daily turnover of whole viral assemblages is likely due to the active production of only a finite and ever-changing subset of potential viral–host pairs, with present tools it is impossible to identify these actively replicating viruses. Broad-scale understanding of linkages between specific genes and specific groups of viruses should provide the tools necessary to quantify viral impacts on particular host groups. Alternatively, we may find that indeed genomic mosaicism extends throughout the viral world and that such specific linkages to viral groups are rare (Hendrix *et al.*, 1999). Instead, we may uncover genes and groups of genes that are tied to specific environmental contexts, life cycle characteristics and ecological strategies among dsDNA viruses. Any one of these scenarios promises to fundamentally change our appreciation of the role of viruses within the biosphere. Let the search begin!

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From microbes to global environmental change: thinking big about small things

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It is an exciting time for the field of marine microbial ecology, as the previous Crystal Ball features have eloquently discussed. It is also a sobering time, as we face some of the most wide-ranging global environmental problems that humans have faced. In thinking about the future of microbial ecology, I envision a renewed energy and focus on measuring and estimating rates of microbial processes in a biome and global context. The processes and activities of microorganisms are the critical aspects of microorganisms that shape our environment.

Within the last two decades, aquatic microbiology has benefited from applications of molecular biology and (meta)genomics and many other present and future '-omics'. We now have capabilities for amplifying and sequencing genes and genomes which provides us information on microbial diversity and the phylogenetic identification of even uncultivated microorganisms. There are now many completely sequenced microbial genomes and microbial community metagenomes that have provided completely new information on the biological capabilities of organisms. We have various descriptions of microbial diversity, some with extraordinary claims of incredible microbial diversity documenting sequence diversity of short sequences of DNA in variable regions of rRNA. New microbes have been brought into culture using a variety of clever techniques. These advances have provided us discoveries and new information on microorganisms in their environment, their capabilities, and generated hypotheses on their roles in nature. Although we are acquiring information on basic microbiology at an unprecedented rate, there are big questions on how this information relates to the function of microbial communities in the environment, and the relationship of diversity to ecosystem structure, function and stability.

At no time in my career has the understanding of the functioning of the microbiome of the planet been more

important than now, at a juncture of environmental change potentially endangering human survival in Earth's biosphere. In my opinion, we need to begin to implement and develop methods directed towards understanding microbial function in the environment even more than we have before. It is important, in addition to documenting diversity and uncovering new proteins and pathways, that we begin to understand what the relevant levels of microbial diversity are for ecosystem function and change and how to measure and predict them over large spatial and temporal scales.

Current developing technologies in single-cell genomics and physiology approaches and isotopic techniques are beginning to link genetics with phenotype, but in the mean time, we need to deploy routine assays for monitoring microbial structure and function in the oceans now, if we ever hope to be able to detect change, or predict the effects of change. We do not know how to monitor genetic diversity at the right genetic resolution to monitor the ecologically significant effects of global change. There is a fundamental disconnect between our understanding of what microorganisms can do, and how they function at ecosystem and biome levels. There is also a disconnect between understanding what microbial diversity means for microbially mediated processes and functions in the environment. And finally, the scale of the environment necessitates implementation of approaches and instrumentation that allows the collection of data at time and space scales that facilitate large-scale correlation with physical and chemical conditions. Science, including microbial ecology, is driven by what we can do, not necessarily what we want or need to do. From my perspective, it is important that we begin to implement more monitoring and measurement programmes to understand the global biogeochemical cycles and microbial transformations at biome and global scales. This vision requires a focus of research on questions of function in an ecosystem context, and more importantly development of instrumentation that facilitates high-resolution sampling. The ocean is large, and our sampling opportunities are small. We need equipment and techniques that allow us to gather microbial process and function information in order to understand how microorganisms are linked to the dynamics of the current environment, and how they will change under future global change scenarios.

In the crystal ball metaphor, we can envision a future where microbial communities in the oceans are being monitored by remote devices, autonomous vehicles and shipboard devices. Such visions have been reviewed recently (Paul *et al.*, 2007) and discussed at a number of workshops over the years (EPA Ecogenomics Indicators for Water Quality Assessment, Kansas City, KS, May 2002; US National Oceanographic Partnership Program, Ocean Ecogenomics Workshop, Washington, DC, 7–8 March

2005; Workshop on the Implications and Opportunities of the Marine Genomics Revolution, BIOS, Bermuda, 28–30 October 2007). There are technologies that provide platforms for implementing enzyme, protein, DNA, mRNA and rRNA assays remotely (the Autonomous Microbial Genosensor and the Environmental Sample Processor; Paul *et al.*, 2007), and even perform experimental isotopic incubations (<http://www.who.edu/page.do?pid=8415&tid=282&cid=11376>). Detection strategies can also be implemented on shipboard equipment, to facilitate biological measurements closer to the scales with which chemical and physical variables are measured.

However, the microbiological targets for such technologies have not yet been well defined by the microbiology community. In my vision, instruments will be equipped with sensors that measure key metabolic parameters of the sea, at appropriate genotypic and phenotypic levels. We can target many things, bacterial groups, various rRNA or metabolic genes, but what will those targets tell us? Some even envision DNA sequencing *in situ*, although it must be remembered that many key players, or keystone microbes and proteins, are some of the least abundant in the microbial community, and that without targeting, sequencing efforts only glean the most relatively abundant organisms and randomly sequenced genes. I foresee many molecular biological targets that could provide us the temporal and spatial microbial information that allow us to detect ecosystem trends in time, and to extend observations spatially at biome and global scales. Examples might be nitrogen fixation, but also a variety of other nutrient utilization genes, or physiological stress markers.

Microbiological sensors can be used to target different components of the microbiota (major phyla, major metabolic pathways, pathogens, etc.) and potentially to provide information on the physiological status or even growth rate of *in situ* populations (gene expression of stress proteins, induced proteins, nitrogen and phosphorus metabolism genes). Sensors can be used to target key species in ecosystem function, or microorganisms whose presence indicates an ecosystem state, analogous to the canary in the coal mine. Tools can be developed to assess diversity, but the relevant scale (phylogenetic groups or microdiversity) must be determined prior to implementing a monitoring programme. Molecular biology tools can be used in a variety of ways to assay microbial community characteristics by targeting microorganisms that carry out specific key biogeochemical functions as well as assessing the physiological status of microbial communities and populations. The information for such sensors can be derived from knowledge of genetics of processes (e.g. functional genes such as nitrogenase for nitrogen fixation), or from key targets that are identified as useful markers of microbial community properties from environmental shotgun sequencing surveys. Although it is easy to

visualize (at least in a crystal ball) how instrumentation might yield high-resolution microbial community and process information, there is much work to be done to (i) identify useful targets and (ii) perform the relevant biological experiments and environmental tests to verify utility and design sampling strategy. What are the targets to tap the pulse of the microbial community? What are the taxa that need to be targeted? Do organisms or gene expression need to be measured to provide useful information? Are genes expressed in meaningful patterns, i.e. do diel cycles need to be measured or the responses to nutrient additions? There is a long road ahead to realize the vision of monitoring and measuring microbes in action, and we need to begin this process now, if we hope to be able to learn how microbes and microbial processes affect and/or are affected by global environmental change. Although

some of this research might not be as exciting (initially) as making new discoveries, ultimately, it might be some of the most significant and important work that microbial ecologists might do: if it is not too late. Time is of the essence if we are to understand the roles of microbial processes within the time scale of anthropogenic global environmental change.

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Reference

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