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Microbial activity in biogeochemical gradients – new aspects of research

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ABSTRACT

In April 2004, the German Research Foundation (DFG) and the National Science Foundation (NSF) co-organized a meeting for US and German junior geoscientists in Washington DC. As an outcome of this Research Conference titled 'Earth, Fire, Water, Air and Life', a follow-up workshop took place in May 2005 at the Eberhard-Karls University of Tuebingen in Germany. This workshop covered new aspects of research to improve the understanding of steep biogeochemical gradients covering pH changes, redox zones, as well as solute and particulate concentration variations in aqueous systems. Detailed understanding of biogeochemistry in this context delivers new fundamental aspects in interdisciplinary research. Such work is also urgently needed to control ever-increasing scarcity of water that is to large parts driven by decreasing water quality. Research ideas on gradients in a biogeochemical context that were discussed by a subgroup of biogeochemists during that workshop are summarized and presented here.

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INTRODUCTION

Micro-organisms interact with the physical environment in ways that are fundamentally different from macro-organisms. Cells at the micrometre scale live in a world of Brownian motion where molecular diffusion controls access to and dissemination of substrates and products through concentration gradients. While this concept is generally recognized, the ramifications may not always be immediately apparent to microbiologists and biogeochemists. Outcomes of the physical realities of the microbial world are that (i) *scale* becomes of central importance and (ii) high resolution *analytical techniques* are needed to quantify physico-chemical conditions and microbial processes at appropriate scales.

Quantification of biogeochemical gradients on a high spatial resolution will lead to a better understanding of chemical reactions and reaction pathways that occur in the environment, in particular directly at the interface of micro-organisms and minerals. The goal of this summary is to outline some of the major research questions and to give examples of modern approaches to answer these questions.

The role of scale in models and thermodynamic calculations – bulk vs. cellular scale

Gibbs free energy (ΔG_r) can be calculated to assess whether or not specific redox reactions are potential energy sources for micro-organisms, thereby establishing the landscape on which microbial ecology is played out. For such calculations, the conditions relevant to microbial bioenergetics (e.g. activities of substrates and products, pH) are those that exist *inside* the cell membrane, but the biogeochemical measurements on which such calculations are usually based reflect conditions in the bulk, extracellular medium. Because steep and rapidly changing concentration gradients are established near cells, the use of bulk measurements may lead to substantial uncertainties in energy determinations. The use of high resolution analytical tools, such as microelectrodes, can help improve thermodynamic

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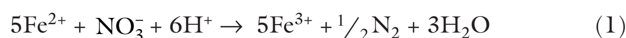
calculations by providing concentrations resolved at the scale of tens to hundreds of micrometres. However, metabolite concentration gradients surrounding a single cell may be established on even smaller scales. Especially in cases of rapid metabolism or low metabolite concentrations, the differences between intracellular and extracellular conditions may be great. In such cases, thermodynamic calculations based on bulk concentration measurements could significantly misrepresent the biologically relevant energetics of a system.

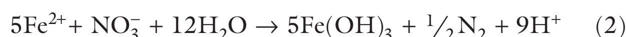
Indeed, the need to consider gradients and mass transport at cellular scales may help explain in part why an apparent thermodynamic advantage, as determined by bulk-scale measurements, does not always translate into competitive success in microbial ecology. As an example, based on bulk measurements, it has been shown that many hydrothermal systems feature >100 energy-yielding organic and inorganic redox reactions (Amend *et al.*, 2003, 2004; Rogers & Amend, 2005; Shock *et al.*, 2005). However, most of these reactions have not been shown to support microbial metabolisms. This may suggest that either the corresponding organisms have yet to be identified, or that certain physiological or kinetic factors prevent the organisms from harvesting the potential energy. It is also possible that the reaction energetics are demonstrably different on the cellular scale compared to the bulk scale.

The potentially dramatic impact on bioenergetics of metabolite dynamics in the intra- and circumcellular domain is illustrated by the example of microbial Fe(II) oxidation at neutral pH. It has been shown that under anoxic conditions at pH 7, Fe(II) can be converted to Fe(III) chemolithotrophically with nitrate as the terminal electron acceptor (Hafenbradl *et al.*, 1996; Straub *et al.*, 1996; Edwards *et al.*, 2003), or phototrophically (Widdel *et al.*, 1993). Under (micro)oxic conditions, Fe(II) oxidation can also be promoted biologically with O_2 as the terminal electron acceptor (Emerson & Moyer, 1997). At circumneutral (or alkaline) conditions, Fe(III) is poorly soluble, and in the absence of Fe chelators, it normally precipitates as ferric (oxy)hydroxides (Emerson & Moyer, 1997; Kappler & Newman, 2004). The energy available from Fe(II) oxidation is critically dependent on the speciation and concentration of the reactants and products in or near the cells. This can be seen by comparing values of ΔG_r of the reactions



$$5Fe^{2+} + NO_3^- + 6H^+ \rightarrow 5Fe^{3+} + {}^1/_2 N_2 + 3H_2O \qquad (1)$$

and



$$5Fe^{2+} + NO_3^- + 12H_2O \rightarrow 5Fe(OH)_3 + {}^1/_2 N_2 + 9H^+ \qquad (2)$$

at 25 °C, 1 bar, pH 7, 4 mM Fe^{2+}, 10 μM Fe^{3+}, and 10 mM NO_3^-. Reaction (1) yields −54.6 kJ mol^{-1} nitrate compared with −381.3 kJ mol^{-1} nitrate for reaction (2). How, where, and in what form the Fe(III) precipitates must therefore exert an enormous influence on the nature of energy flow and therefore on the ultimate role the organisms of interest play in microbial community ecology and biogeochemistry.

In addition to energetic constraints imposed by cellular scale metabolite gradients, further impacts on growth and metabolism may occur due to the limitation in the availability of trace nutrients in micro-environments surrounding cells, or from competition for substrates among cells in aggregates (for example, in biofilms). In complex systems that are typical for natural environments, measurements of bulk concentrations do not represent the small-scale conditions that are actually controlling the microbial activities (Fig. 1). The observations (e.g. electron acceptor profiles in sediments) describe the overall result of the microbe's activities; however, the interactions and processes happen on a micrometre- or even nanometre-scale. Additional approaches may help bridge the gap between macroscopic observations and microscopically occurring processes. For example, modelling of the gradients of metabolites that are present on a micrometre-scale around a cell could help better understand macroscopically observed profiles of metabolites. This, in turn, could help explain why a certain process is selected and catalysed over other processes that theoretically would yield more energy to the microbes. Such studies require close collaboration of numerical modelers and microbiologists.

Measurements of nano-/microscale gradients

Geochemical measurements by microelectrodes and biosensors

Measurements of the chemical and physical parameters at scales relevant to what microbial communities experience are not a trivial problem. Impressive work has been done in the development of an array of electrochemical microsensors that can be used primarily for the measurement of inorganic compounds (see, e.g. Kuhl & Revsbech, 1998; Gieseke & de Beer, in press). These include potentiometric ion-specific electrodes for K^+, Na^+, Ca^{2+}, Cl^-, HS^-, HPO_4^{2-} and pH and amperometric electrodes, e.g. for O_2, NO_3^- and H_2. Nonspecific electrodes that use scanning voltammetry have proven useful for measuring S-compounds, as well as for Fe and Mn (Brendel & Luther, 1995; Taillefert *et al.*, 2000). The environmental use of electrochemical sensors requires that they be small enough to resolve at the micrometre scale, sensitive to low micromolar concentrations, reliable over long time periods, have rapid response times, and are both relatively immune to electrical noise and robust enough to work in heterogeneous environments.

Many studies have demonstrated the use of microelectrodes in microbial ecology (Revsbech & Jørgensen, 1986); for illustrative purposes a couple of examples are cited here. One such study determined the microscale distribution of nitrate, nitrate assimilation, nitrification and denitrification in a diatom-inhabited freshwater sediment (Lorenzen *et al.*, 1998). The high-resolution profiles indicated a stimulatory effect on nitrification by illumination that was caused by photosynthetic O_2 production, while no nitrification occurred during darkness. This

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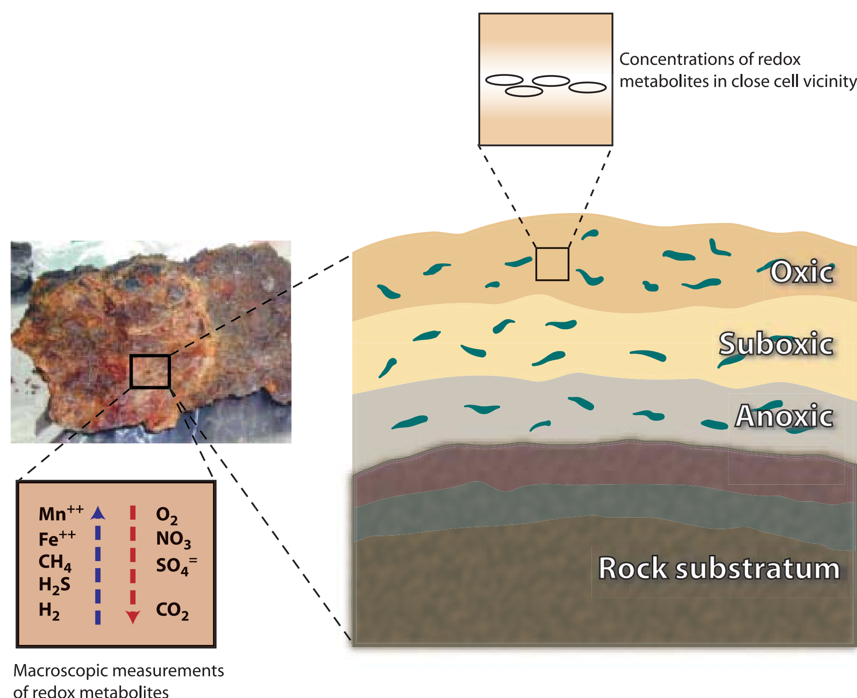

Fig. 1 Microbial biofilm attached to a rock surface: the macroscopically measurable concentrations of metabolites do not necessarily represent the actual conditions present in close cell vicinity.

work also revealed the tight coupling between nitrification and denitrification during illumination, and suggested there were substantial rates of NO_3^- assimilation during illumination. Another study coupled cell-specific molecular probes, amperometric O_2 microelectrodes, and a nitrate-specific microbiosensor to analyse a nitrifying biofilm community (Schramm *et al.*, 1996). The unprecedented spatial resolution offered by this combination of techniques revealed the biofilm to be a tightly structured community where nitrification occurred in the upper 50 μm of the biofilm and was coupled with the highest populations of nitrifying bacteria, although the nitrifiers were present at lower numbers throughout the biofilm. Combining these quantitative techniques allowed cell-specific nitrification rates to be determined in a natural biofilm community (Schramm *et al.*, 1999).

Biosensors are analytical devices able to convert a biological response into an electrical signal. Biosensors may either couple cells or enzymes to electrochemical sensors, or utilize microbes that have been genetically altered to include reporter systems for specific compounds. The biological response that is converted into an electrical signal can be either heat (calorimetric), charge (potentiometric), electrons (amperometric) or light (optical). Although these methods are not yet in routine use, the use of biosensors is growing and represents an area of promise. Biosensors for testing for arsenic pollution were recently developed using the natural resistance mechanism of a nonpathogenic laboratory strain of *Escherichia coli* against arsenite and arsenate coupled to three reporter proteins: bacterial luciferase, beta-galactosidase and green fluorescent protein (GFP) (Stocker *et al.*, 2003). Arsenite concentrations at 4 μg of As L^{-1} and thus far below the current drinking water limit (10 μg L^{-1}) were measured in arsenite-amended water. Arsenite was quantified in the field with a system that contained beta-galactosidase, producing a visible blue colour at arsenite concentrations above 8 μg L^{-1}. This technology used sensor cells dried on a paper strip that were placed in an aqueous test solution resulting in measurable colour development. These biosensor cells offer significantly better accuracy over commonly used chemical test kits; furthermore they could, potentially be coupled with microelectrodes to determine As concentrations on a very high spatial resolution.

Determination of cell-scale gradients using genomic and proteomic methods

While microsensors can quantify spatial gradients down to tens of micrometres, this still does not resolve conditions at the cellular level. The use of molecular-based sensing methods for *in situ* monitoring of cell-scale gradients offers promising new tools. A pH-sensitive derivative of the green fluorescent protein, designated ratiometric GFP, was recently used to measure the intracellular pH in bacterial cells (Olsen *et al.*, 2002). The ratiometric GFP protein was expressed in both Gram-positive and Gram-negative bacterial cells by inserting the corresponding gene downstream of the P32 promoter in the chloramphenicol-resistant expression vector pMG36c, which replicates in both cell types. The ratiometric GFP was

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obtained by introducing specific amino acid substitutions to the chromophore, causing the resulting protein to alter its excitation spectrum according to the pH of the surrounding environment. In cells expressing the ratiometric GFP, the excitation ratio (fluorescence intensity at 410 and 430 nm) was correlated to the pH, allowing fast and noninvasive determination of the internal pH. It is possible that in the future similar techniques could be used to determine concentrations of organic and inorganic metabolites in close cell proximity. One important problem that remains to be solved with these GFP-type sensing systems is their inability to dynamically modulate the response of the reporter as conditions change, for example, as a signal molecule increases and then decreases. There are many other gene reporter-based systems that have been developed for assaying a wide range of environmentally relevant chemicals, see Kohler *et al.* (2000) for a review.

Another molecular approach that does not require any 'engineering' of the cell, but does require knowledge about the sequence of a specific gene, is the direct quantification of gene expression from environmental samples. One example of this is quantifying expression of a respiratory reductase for arsenate by Malasarn and coworkers (Malasarn *et al.*, 2004) which turns out to be a sensitive assay for environmental arsenate concentrations. Direct analysis of RNA can also be done within cells using mRNA-specific probes and fluorescent *in situ* hybridization techniques (Pernthaler & Amann, 2004). Both methods allow 'snap-shots' of potential activity, which can be linked back to the gradient, that the cells experienced prior to sample isolation. By correlating expression of these reporters in laboratory-controlled experiments with concentrations of the stimulant of interest, one could calibrate the environmental results to approximate the concentration of the stimulant sensed in the environment by the reporter strain.

One major constraint to the molecular-based sensing approach is that it requires prior knowledge of the genes encoding the function of interest. In some cases, researchers may be interested in a process for which neither the organism nor the genes that confer the metabolism of interest are known. As our knowledge of genomics and gene-families advances, it may become possible to infer functionalities and design sensor-type assays even without having a specific organism and/or gene in hand. Another major challenge is the speed of analysis. Many current molecular techniques take hours to yield results, even in controlled laboratory conditions; for many environmental applications, it would be better to have an improved temporal resolution. Finally, preservation of environmental samples for these molecular approaches is important. For example, preservation of prokaryotic RNA while maintaining cellular integrity is an especially challenging problem.

APPLICATION EXAMPLE AND OUTLOOK

An important area where the macro- and microscale meet is in hydrogeological models of processes like groundwater flow and, more specifically, transport of organic contaminants. While these processes occur at scales of metres and kilometres, natural attenuation of the contaminant is ultimately controlled by microbial processes that are happening at the scales of micrometres and millimetres. Gradients of electron acceptors and donors measured in groundwater systems typically expand over several decimetres up to a few metres. Typical time scales for a contaminant to travel within a groundwater plume are months to years. Closed form analytical solutions and numerical models often are based on the assumption of fast, i.e. instantaneous reactions relative to the movement of the groundwater plume (Cirpka *et al.*, 2005; Liedl *et al.*, 2005). This results in steep concentration gradients but extremely low concentrations of electron donors and acceptors at the fringes of the plume where most bioactivity occurs in the model (i.e. the model exposes the micro-organisms to zero concentrations). Some questions that arose during the workshop discussions with regard to contaminant plumes were: what role does chemotaxis play in attenuation of contaminant plumes, and is it possible to develop bioremediation schemes that enhance the dissemination of beneficial microbes by chemotaxis? Furthermore, how do the temporal and spatial scales over which microbes exert influence play a role in larger scale physical/chemical gradients, for example spatio–temporal changes or oscillations of concentrations or biomass in contamination plumes?

The development of new microsensors as well as the use of molecular marker molecules for small-scale characterization of physicochemical conditions in combination with modelling processes on a cell-scale will improve our future understanding about microbial activities in the environment. Interdisciplinary research is needed bringing together analytical chemists (especially for sensor applications), hydrogeologists, geochemists, microbiologists and numerical modelers to reconcile scales and processes.

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