FIRST STEPS INTO THE 'SHALLOW BIOSPHERE'

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Introduction

The analysis of biogeochemical gradients and the revolution of molecular biological techniques made evident that the extent of the biosphere on Earth is much larger than thought before. This holds true on a spatial scale: Living prokaryotes were detected down to more than 800 m below the sea floor (PARKES *et al*., 2000) and even deeper in subterrestrian basaltic rocks (SZEWZYK *et al*., 1994). Yet more surprising appears the survival of the 'deep biosphere' with respect to time scale: Sediments with living prokaryotes may be several million years old (FREDERICKSON *et al*., 1995; COOLEN *et al*., 2002). The mean generation time of prokaryotic populations in the 'deep biosphere' seems to be in the range of hundreds or thousands of years (WHITMAN *et al*., 1998). Such slow processes can hardly be reproduced in the laboratory. Our knowledge about subsurface microbial communities is therefore very limited, although they may harbour a large portion of the global biomass (WHITMAN *et al*. ,1998).

In sediments, the microbial activities and cell numbers generally decrease with depth on a logarithmic scale. PARKES *et al*. (1994) found the following relationship as an average of several marine sediments (with mbsf for meters below sea floor):

log (microscopic count) = 8.06 - 0.715 log (depth in mbsf)

This means that in 1 m sediment depth there are about 10^8 prokaryotes per cm^3 detectable by means of fluorescence microscopy. At the surface (at 0.01 m), the number would be higher by 1.4 orders of magnitude, and at 100 mbsf there would still be a count of about $10^{6.6}$.

Thus, the majority of the sediment microbiota resides in the layers between several centimetres and a few metres depth. In these layers the environmental conditions are basically different from those at the surface. Therefore, specifically adapted microbial communities can be expected, for which we use the term 'shallow biosphere'. Although, the 'shallow biosphere' is located rather close to the surface and is relatively easy to access, many of the activity parameters are already hundredfold lower than at the surface.

Therefore, studying the 'shallow biosphere' not only appears as the perfect job training - it aims to elucidate a major part of the sediment microbiota and geochemical processes. In a joint venture of geochemists and microbiologists we are investigating the upper metres of tidal flats between the North Sea island of Spiekeroog and Neuharlingersiel on the East-Frisian coast (RÜTTERS *et al*., 2002a). The analysis of geochemical parameters and tracers is combined with microbiological and molecular biological approaches (Fig. 1).

Cultivation approaches

A major part of our efforts is directed to develop and improve techniques that promote growth of sediment bacteria and increase the sensitivity of their detection (Fig. 2). The molecular biological analysis of microbial communities cannot provide satisfying information about the ongoing processes. Therefore, we not only measure chemical gradients and microbial activities, but study sediment bacteria, too. We try to obtain pure cultures, or at least enrichments with growing cells. Those enable us to understand the adaptations and processes. Novel cultivation techniques include:

- The use of low substrate concentrations which avoid a substrate-induced death of starved cells.
- The use of gradient cultures with substrate concentrations increasing slowly from zero. This allows the cells to find their favourite place.
- The use of media with substrate mixtures that support growth of a large number of different bacteria. Besides defined media, sediment extracts with unknown composition are used.
- The addition of particles instead of using clear liquid media. Iron sulfide concomitantly provides a mild reducing agent.
- The addition of background bacteria which promote syntrophic relationships.

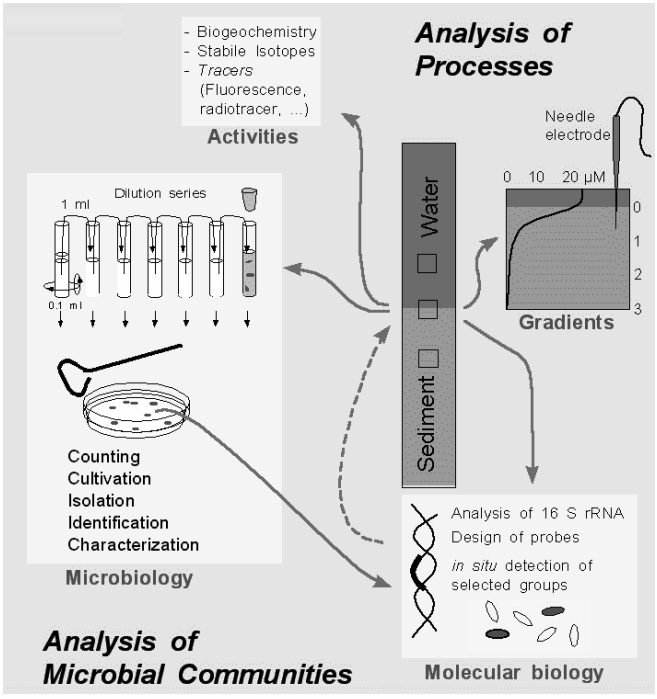

Fig. 1. Combined microbiological and biogeochemical approaches for the study of sediments.

For several reasons, the new cultivation methods require novel and very sensitive growth analysis techniques. First, the media applied allow only the formation of a relatively low level of biomass. Second, it is very likely that many of the indigenous shallow biosphere bacteria grow only to relatively small population sizes, even if substrate is available in excess. Third, the presence of particles hampers conventional growth analysis. Facing these difficulties, we are analysing single cells by fluorescence microscopy with various dyes (Fig. 2) instead of measuring turbidity as growth parameter. The growth analysis is accompanied by molecular biological studies allowing a comparison of the microbial groups in the cultivation assays with the natural community (WIERINGA *et al*., 2000).

Molecular biological approaches

Molecular biological approaches include DNA as well as RNA analysis. This allows to identify the phylogenetic groups. DNA of selected groups can be quantified by means of real-time PCR. Whereas the DNA amount and sequences indicate which groups of prokaryotes are present, RNA analysis could provide additional information about the part of the microbial communities, which is metabolically active. The number of ribosomes and thus the RNA content of the cells is known to increase with the cell activity. A further problem is the fact that DNA bound to sediment particles can

survive over a very long period of time outside of living cells (COOLEN *et al*., 2002). Therefore, a discrimination between living cells and subfossil DNA is necessary. Part of the extracted DNA from sediments may originate from spores, which again have a low content of RNA.

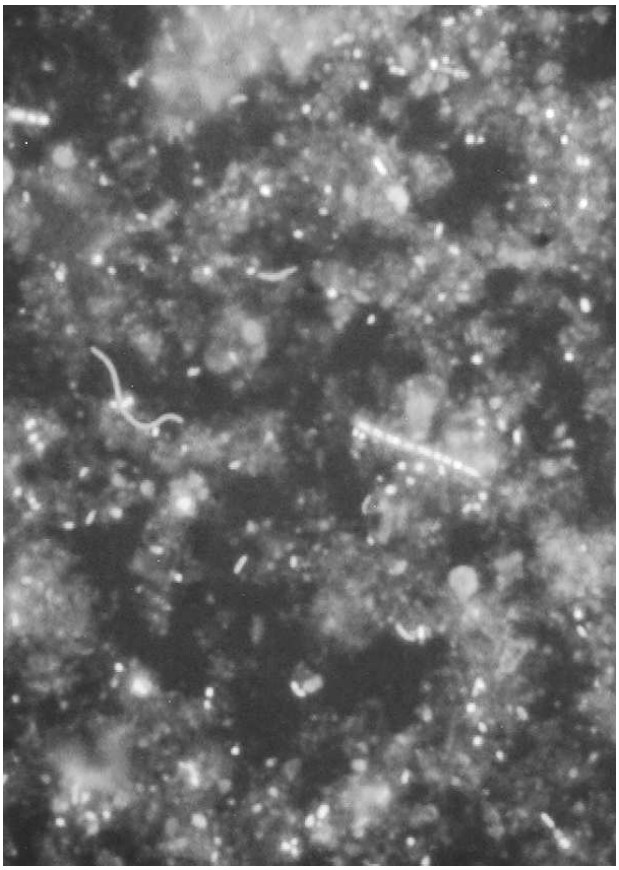

Fig. 2. Epifluorescence microphotograph of surface sediment from Neuharlingersiel, stained with SybrGreen II, a dye that reacts with DNA and RNA. Within 1 m of depth the numbers decrease by a factor of 100.

Geochemical approaches

A set of inorganic and organic chemical parameters is analyzed regularly. The lipid composition of sediment cores is used to distinguish the source of organic matter, which in our study area could be derived from marine algae, terrestrial plant or peat. As indicator for living biomass intact phospholipids were extracted from the sediment and analysed (RÜTTERS *et al*., 2002b). These compounds are known to be degraded within hours after cell lysis. Furthermore, the extraction of intact phopholipids does not include spores. Phospholipids can be seen as a marker of vegetative cells. The specific composition provides additional information about the groups of active bacteria.

In many sediments, microbial activities can be derived from the analysis of gradients. Tidal flats are difficult in this regard, since there are various effects causing rearrangement of the sediment. However, radiotracers (^{35}S-labelled sulfate) and fluorescence-based activity measurements (based on methylumbelliferyl substrates) can be used to assess microbial activities.

Conclusions

It turns out that the 'shallow biosphere' in tidal flats represents a dynamic system, which is still challenging with respect to its scientific analysis. However, the joint venture of geochemists and microbiologists allows to unravel many of its secrets.

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