CULTIVATION OF BACTERIA FROM A SIX METER LONG CORE FROM AN INTERTIDAL SEDIMENT

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Introduction

Hitherto, several microbiological investigations dealt with intertidal mud flats. But, by far the most of them examined only the uppermost few centimetres of the sediment in detail (LLOBET-BROSSA *et al.,* 1998). In spite of this lack of microbiological data, the presence of active microbial populations even in deeper layers of intertidal areas could be deduced from biogeochemical data (THOMSEN *et al.,* 2001). The ecology and the composition of these microbial communities down to several meters below the sediment surface, here referred to as 'shallow biosphere', is still largely unknown. But for the investigation of physiological adaptations of these microorganisms pure cultures are needed.

The aim of the present study is to unravel the community composition and the ecology of the 'shallow biosphere'. This includes quantification of different microbial physiological groups and correlation of their abundance with the physicochemical gradients, as well as the isolation of *in situ* relevant organisms for ecophysiological characterisation. Therefore, the first step is to establish reliable cultivation methods for enumeration and isolation of pure cultures. These new cultivation assays have to be compared and evaluated by comparison of the enrichment cultures with the natural communities by molecular methods. Here we report on the first analysis of a 550 cm long sediment core from Gröninger Plate (East Frisian Wadden Sea) taken in June 2002.

Materials and methods

Counts of viable microorganisms were determined by the most probable number (MPN) method. Samples were serially diluted in 96-well plates using a mixture of monomeric compounds (20 different amino acids, C_1-C_6 fatty acids, C_1-C_4 *n*-alcohols, lactate, malate, fumarate, succinate, and glucose) as growth substrate. Each substrate was added at low concentration (100 µmol·l^{-1} or less). The inoculation scheme is shown in Fig. 1. MPN dilution series were incubated under oxic or anoxic conditions. For the latter the AnaeroCult A mini system (Merck) was used. Alternatively to dilution series, cultivation assays in gradient tubes were inoculated. Gradient tubes allow a slow increase in substrate concentrations from the natural level. All gradient tubes were incubated under anoxic conditions. Total cell counts in sediment samples were determined by epifluorescence microscopy after staining with DAPI. For all cultivation assays, artificial sea water was used (SASS *et al*., 2001). Oxic growth medium was buffered with HEPES. Anoxic growth medium was bicarbonate-buffered and reduced by the addition of FeS.

Comparison of bacterial types obtained in the different cultivation assays with the natural sediment community was performed by PCR (polymerase chain reaction) and DGGE (denaturating gradient gel electrophoresis). Total DNA was extracted from sediment, sediment incubated in gradient tubes, and liquid cultures (MPN series), purified, amplified with the eubacterial primers (GC 357f, 907r), and used for DGGE.

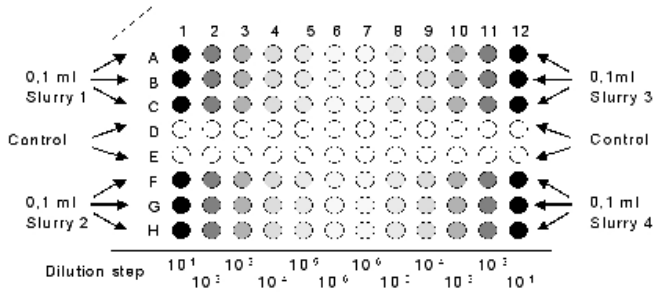

Fig. 1. Inoculation scheme of MPN series in a 96-well plate.

Physicochemical gradients

In June 2002 the oxygen penetration depth in sediment at the sampling site Gröninger Plate was 2.5 mm. The pH showed minor differences along the upper 400 cm. Below, a slight pH decrease from 8.4 to 7.8 was found (data not shown). Free sulfide in the porewater was detected from 40 cm depth downwards and reached a maximum of 0.17 mmol·l^{-1} at 300 cm depth. Porewater sulfate concentrations declined from 32 mmol·l^{-1} at the sediment surface to a concentration of 4.4 mmol·l^{-1} at 60 cm depth. From 200 cm depth downwards sulfate concentrations increased slowly again (Fig. 2). The sulfate profile was not reflected by the chloride profile. At the surface 540 mmol·l^{-1} chloride were detected. In deeper layers chloride concentrations ranged between 500 and 520 mmol·l^{-1}. The temperature decreased from 20.5℃ at the sediment surface to 11.2℃ at 560 cm depth.

Enumeration of microorganisms

Total cell counts in the sediment decreased exponentially from around 10^9 g^{-1} sediment at the surface to about 10^7 g^{-1} sediment at 500 cm depth (Fig. 2). Viable counts reached up to 0.1 % of the total cell count at the sediment surface (Table 1). Cultivation efficiency was similar under oxic and anoxic conditions. However, even at 500 cm depth higher viable counts were obtained under oxic than under anoxic conditions, indicating that most of the organisms living in anoxic sediment layers are facultative anaerobes.

Table 1. Cultivation efficiency (given as percentage value of the total cell counts) of viable counts obtained by the MPN procedure in growth media supplied with a mixture of monomeric compounds (amino acids, carbohydrates, fatty acids, lactate, malate, and others).

Sediment depth [cm]	Oxic incubation	Anoxic incubation
0.5	0.11 %	0.05 %
5	0.03 %	0.02 %
50	0.67 %	0.61 %
500	0.02 %	0.001 %

From the highest positive dilutions of the different MPN series subcultures were taken for the isolation of pure cultures. So far, 25 isolates were obtained under oxic conditions and are currently under investigation. In addition, fifty anoxic isolation assays were inoculated from MPN series and gradient tubes.

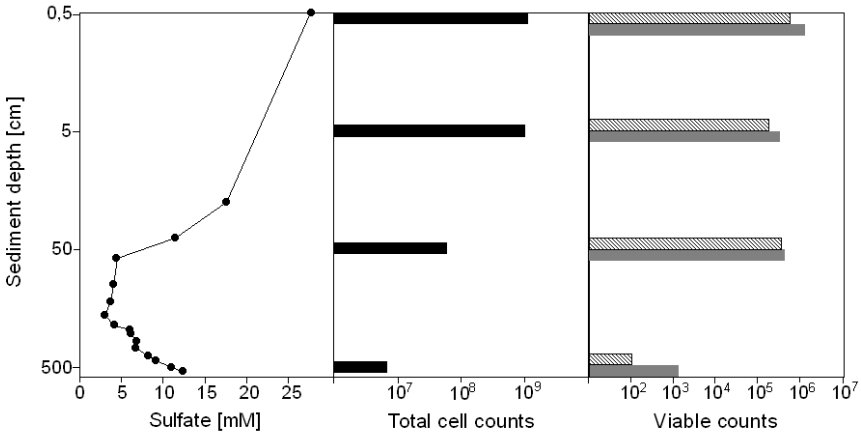

Fig. 2. Vertical sediment profiles along the top 500 cm of the Gröninger Plate core collected in June 2002. Left: sulfate concentrations, middle: total cell counts, right: viable counts obtained with monomeric substrates (grey: oxic incubation, hatched bars: anoxic incubation).

Molecular characterisation of cultivation assays

PCR-DGGE with eubacterial primers showed only minor differences in the banding pattern of the natural bacterial communities at the surface, at 5 cm and at 50 cm depth. This indicates that at these three depths the same phylogenetic groups are numerically dominating. From the PCR-DGGE analysis (Fig. 3) it can be seen that the different cultivation approaches yielded different microbial types. None of these approaches was dominated by a single band, indicating that in each sample more than one phylotype was present. Interestingly, in the cultivation assays inoculated with samples from 500 cm depth the highest bacterial diversity was found. Potential correspondence of bands found in cultivation assays with those of the natural community have to be proven by sequence analysis.

Conclusions

Even at five meters depth in intertidal sediments of the East Frisian Wadden Sea viable microbial population were detected. The microbial populations present in these deep layers show an unexpected diversity as could be demonstrated by molecular analysis of the different cultivation assays. Further investigations have to reveal which of these organisms are active *in situ* and which are present only in a dormant state.

Acknowledgements

We thank Alexander Bartholomä (Senckenberg Institute, Wilhelmshaven) for help during sampling. This work was supported by a grant of the Deutsche Forschungsgemeinschaft (DFG).

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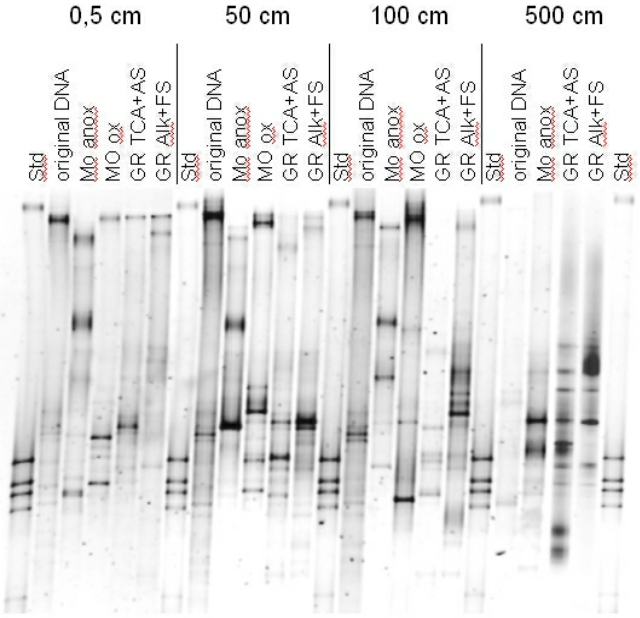

Fig. 3. Comparison of different cultivation methods using PCR/DGGE. Shown are banding patterns of MPN series with monomer media under oxic (Mo ox) and anoxic conditions (Mo anox) of gradient cultures supplemented with alcohols and fatty acids (GR Alk+FS) or with a mixture of amino acids and components of the tricarboxylic acid cycle (GR TCA+AS) as well as of the natural communities of the different depths (original DNA).

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