THE INFLUENCE OF SEASONAL CHANGES ON MICROBIAL COMMUNITIES IN THE WADDEN SEA

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Introduction

Intertidal mud flats are subjected to strong seasonal and diurnal temperature fluctuations. During low tide, the sediment surface temperatures may be up to 20°C higher than at high tide when the sediment is covered by water. Concomitantly, salinity may increase in the uppermost sediment layers due to evaporation. Seasonal temperature changes are also drastic. In winter, the temperatures may reach several degrees below 0°C, whereas in summer the water temperature may rise up to 25°C and at the surface of exposed sediments even higher temperatures may be observed. It is largely unknown if seasonal temperatures changes occur also in deeper sediment layers and if microbial communities adapt to these changing conditions.

Yet, benthic microbial communities at the sediment surface are not only affected by temperature. Nutrient supply and the quality of organic matter also show strong seasonal fluctuations. Conversely, hardly degradable organic material remains in deeper sediment layers and, if at all, only minor seasonal fluctuations of substrate supply may occur.

In the present study we tried to determine the influence of seasonal changes (temperature, possible growth substrates) on microbial sediment communities by cultivation-based methods.

Sampling

Sediment cores were taken from an intertidal mudflat (53°42.5' N, 7°42.05' E) close to the village of Neuharlingersiel (NW-Germany). Plastic cylinders (100 cm long, inner diameter 50 mm) were pushed into the sediment, sealed at the top by a rubber stopper, dug out and sealed with a rubber stopper at the bottom. Cores were immediately taken to the laboratory in upright position where they were processed within four hours. Samples were taken in September 1999, and January, March, May and August 2000.

Experimental procedures

Porewater was gained by centrifugation, filtered (0.45 µm) and immediately frozen until analysis. Determinations of chloride and sulfate concentrations, organic carbon content, grain size and total cell count were described by RÜTTERS *et al*. (2002).

Viable counts of microorganisms were determined by the most probable number (MPN) method. Samples were serially diluted in 96-well microtiter plates in an anaerobic hood. The medium used was artificial seawater, supplemented with trace elements, vitamins and buffered by the addition of sodium bicarbonate (SASS *et al*., 2001). The medium was reduced with sterile sodium sulfide. After inoculation the MPN plates were sealed with a sterile lid. The plates were incubated under oxygen exclusion in gas-tight plastic bags with a catalyst system to prevent oxygen contamination (AnaeroCult A mini, Merck).

For the determination of MPN counts at different incubation temperatures a mixture of the following substrates were used (in $mmol \cdot l^{-1}$): sodium lactate (5), sodium acetate (2.5), sodium propionate (1), sodium butyrate (1), sodium isobutyrate (0.5), alanine (1.2), arginine (0.6), cysteine (0.6), sodium benzoate (0.5) and sodium salicylate (0.5). The MPN-Plates were incubated for eight weeks (20°C, 25°C, 30°C and 40°C), three months (10°C and 15°C) or four months (4°C).

Additionally, viable counts were determined using single substrates (see Table. 3). These MPN series were incubated solely at 20°C.

Pure cultures were obtained by repeated application of the deep agar dilution method. Strains were identified by partial sequence analysis of the 16S rRNA gene (SASS *et al*. 1998). Growth was followed by turbidity measurements. Physiological activities (aerobic respiration, sulfite reduction) were analysed under defined conditions in a multielectrode chamber (volume 3.1 ml, Cypionka 1994). Whole cell fatty acid patterns were determined as described by Rütters et al. (2001).

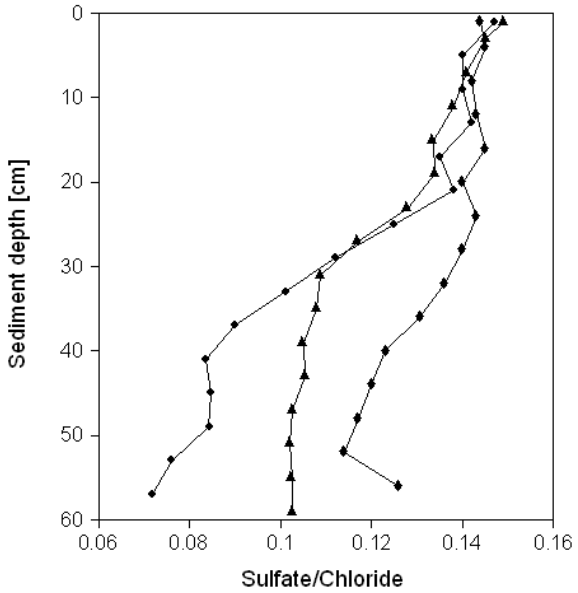

Fig. 1. Sulfate to chloride ratios along the upper 60 cm of intertidal sediments. ●: September 1999, ■: January 2000, ○: March 2000.

Chemical Gradients

At all sampling dates oxygen penetrated less than 3 mm deep into the sediment. The pH showed a nearly linear decrease from surface (around 7.5) to pH 6.7 – 6.8 at 50 cm depth. Total organic carbon ranged between 0.3 and 3% of dry weight. The highest values were observed at the sediment surface and below 50 cm depth. The highest TOC values at the sediment surface were observed in winter (3.3% of dry weight in January 2000 compared to 0.8% in September 1999), whereas in deeper layers TOC contents remained relatively constant.

Sulfate to chloride ratios were used to correct for possible effects of influx of less saline ground water. Sulfate showed a pronounced gradient in September 1999 with a 60% decrease along the upper 60 cm (Fig. 1). This decline of sulfate with depth can be assigned to dissimilatory sulfate reduction. Conversely, in January 2000 only a slight gradient was observed. Generally, the sulfate decline is stronger during summer. This finding is supported by the results obtained during the other sampling campaigns (data not shown).

In the layers directly beneath the sediment surface only a slight decrease in sulfate concentration was found, although the highest activity of sulfate-reducing bacteria is found in these layers (M. Böttcher, pers. commun.). The enhanced sulfate influx can be attributed to bioturbation.

No free porewater sulfide was found in the upper 10 cm of the sediment. Below a slight increase was observed. In 20 cm depth concentrations of free porewater sulfide were the range

Tab. 1. Sediment temperatures in the upper 50 cm of an intertidal sediment close to the village of Neuharlingersiel (NW-Germany). Measurements were performed shortly after sunrise at low tide.

Sediment depth [cm]	Sept. 1999	Jan. 2000	Mar. 2000	May 2000	Aug. 2000	Jul. 2001
-2	19.3	3.3	7.0	15.6	19.1	15.2
0	19.3	3.4	7.0	15.0	17.7	15.3
1	18.6	3.4	7.0	14.4	17.4	15.2
2	18.2	3.4	6.8	14.6	17.3	15.1
5	18.9	3.4	6.6	14.4	17.2	15.8
10	17.5	3.4	6.5	14.4	17.5	16.0
15	18.0	3.4	6.5	14.4	17.7	16.5
20	18.2	3.4	6.4	14.6	17.8	17.3
30	17.9	3.4	6.3	14.5	17.2	17.0
40	17.9	3.4	6.1	14.4	17.2	16.5
45	17.8	3.4	6.0	14.4	17.1	17.0
50	17.7	3.4	5.8	14.2	17.0	17.4

of 20 to 100 µmol·l^{-1}. Below, a constant increase occurred reaching maximum values of 1000 to 2500 µmol·l^{-1}.

Sediment temperature

Measurements of sediment temperature gradients were performed early in the morning to prevent a strong heating of the sediment surface by solar irradiation. No strong temperature gradient was found during any sampling (Table 1). Even at 50 cm depth the temperature was nearly the same as at the sediment surface.

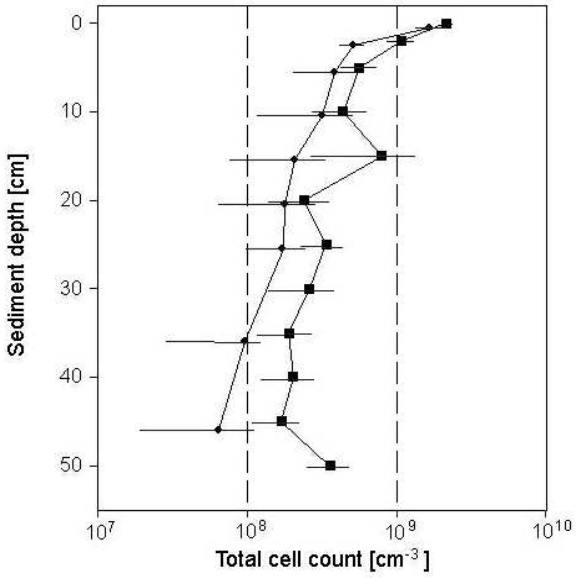

Fig. 2. Total cell counts in sediment samples from an intertidal mudflat near Neuharlingersiel (NW-Germany). Cell counts were determined by epifluorescence microscopy after staining with acridine orange.
■: March 2000, ●: January 2000.

Influence of temperature on viable counts

The highest viable counts in MPN series were obtained at 15-20°C (Fig. 3). Only weak seasonal differences in the optimum temperature were observed. In September 1999, May 2000 and August 2000 (data for the latter two not shown) the optimum temperature for the surface samples was 20°C. In September 1999 optimum temperatures for the deeper sediment layers were slightly lower, but not in May and August. In January and March the optimum temperature was around 15°C for the surface but 20°C for the deeper layers.

The drop in viable counts towards temperatures above 20°C may indicate the presence of psychrotrophic microorganisms, able to grow at temperatures below 10°C but not above 20-25°C. Similarly, the decrease in viable counts at temperatures below 15°C indicates the presence of mesophilic bacteria.

Characterization of pure cultures

Pure cultures were isolated from the highest positive dilutions in MPN series, representing the most abundant types cultured by the method used. Here, only data of sulfate-reducing bacteria are presented (Table 2).

At 40°C only spore-forming bacteria were isolated. The growth temperatures of these organisms are in the range of 30-55°C, clearly above the temperatures observed in the intertidal mudflat. These organisms did not to grow at *in situ* temperature and were most likely detected as dormant spores.

At 30°C or below, only mesophilic, but psychrotolerant bacteria (SASS *et al*., 1998b) were isolated. At 10°C all isolates were still growing. The decrease in MPN counts at this temperature may be explained by incomplete growth initiation of the microbes after inoculation. Only part of the isolates grew at 4°C. This corresponds well with the findings of the MPN series.

It is not really surprising that no psychrophilic bacteria were found. Even in the deeper sediment layers the temperatures in summer are too high for pyschrophilic microorganisms.

Temperature adaptation of isolates

The Gram-negative sulfate-reducing isolates, showed a temperature range for growth and anaerobic respiration which corresponded well with the temperatures observed *in situ*. For growth Q_{10}-values were determined which were in the range of 3.2 to 4.5 (data not shown).

A possible effect of temperature on membrane lipids was investigated by analysis of whole-cell fatty acid profiles of the isolates grown at different temperatures. Cells grown at 10°C showed a higher content of unsaturated fatty acids and shorter median chain length (Fig. 4). This result could be expected, since unsaturated fatty acids are known to increase membrane fluidity. Interestingly, the fatty acid patterns of a single strain, e. g. at 10°C and at 30°C, are more similar to each other than to those of other strains, even of the same species.

Table 2. Sulfate-reducing bacteria isolated from MPN series incubated at different temperatures and inoculated with sediment from an intertidal mudflat close to Neuharlingersiel (NW-Germany). The temperature ranges for growth are given; the characterisation of the isolates is based on Arrhenius plots for the growth data (not shown). For details see ISAKSEN & JØRGENSEN (1996).

Isolation temperature	Depth [cm]	Strain	Growth range	Classification
10℃	0.5	*Desulfovibrio acrylicus* D1	4-30℃	Mesophilic
20℃	0.5	*Desulfofrigus* sp. NA201	10-33℃	Mesophilic
	0.5	*Desulfovibrio acrylicus* NA202	10-30℃	Mesophilic
	5	*Desulfofrigus* sp. NB81	4-30℃	Mesophilic
	5	*Desulfovibrio acrylicus* NB62	4-35℃	Mesophilic
30℃	0.5	*Desulfovibrio acrylicus* NA302	4-35℃	Mesophilic
	50	*Desulfovibrio acrylicus* NC301	4-35℃	Mesophilic
40℃	0.5	*Desulfotomaculum* sp. NA401	35-50℃	Slightly thermophilic
	5	*Desulfotomaculum* sp. NB401	30-55℃	Slightly thermophilic
	50	*Desulfotomaculum* sp. NC402	30-55℃	Slightly thermophilic

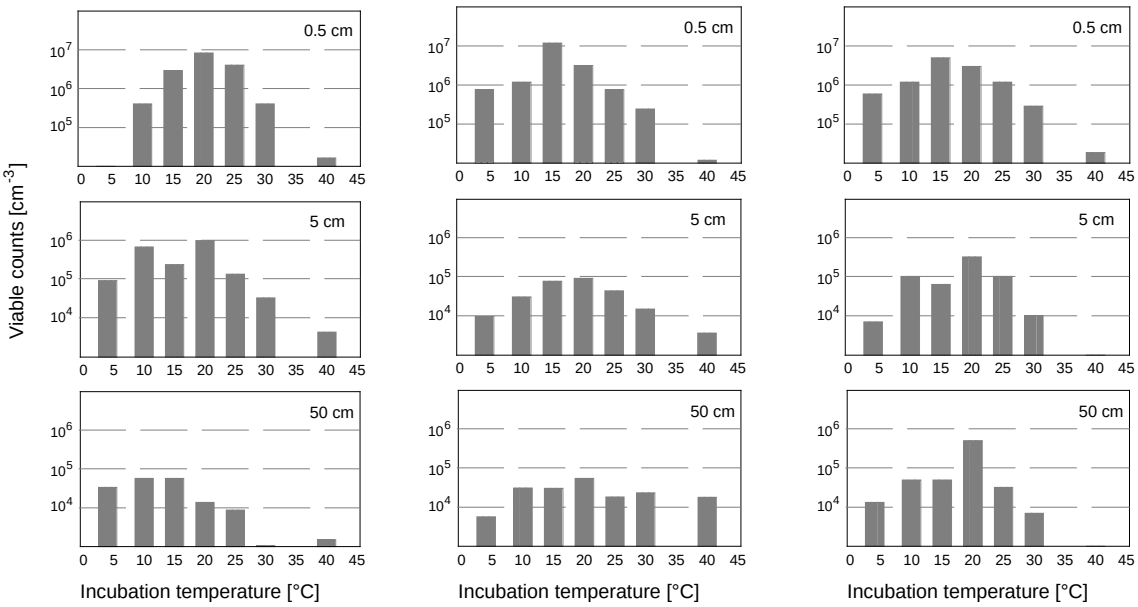

Fig. 3. Viable counts determined by the MPN method obtained at different incubation temperatures. Sediment was taken from three different depths from an intertidal mudflat close to the village of Neuharlingersiel (NW-Germany).
Left column: September 1999, mid column: January 2000, right column: March 2000.

Viable counts obtained with other substrates

Anaerobic viable counts using single substrates showed seasonal changes (Table 3) even at 50 cm depth. This indicates that seasonal changes in substrate supply may affect microbial communities even in the deeper layers.

Highest counts with amino acids were obtained in March, whereas highest values with cellulose or lactate were achieved in January. MPN counts were quite similar, irrespective of the substrate used. Even with long-chain fatty acids MPN numbers reaching 30% of those achieved with amino acids were obtained. At the sediment surface 0.1-1% of the total cell count could be grown in MPN series.

Conclusions

In intertidal sediments strong seasonal temperature changes can be found. However, we have found only little evidence that bacteria belonging to different temperature groups dominate at different seasons. Total cell counts are also relatively constant, but the presence of different metabolic groups seems to vary. Temperature fluctuations appear to have a more pronounced influence on microbial activities, as can be seen in sulfate depletion in deeper sediment layers. In the future, molecular biological analyses have to prove possible seasonal community composition changes.

Acknowledgements

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Table 3. Viable counts of anaerobic microorganisms determined by the MPN method using different substrates.

Substrate	Sediment depth [cm]	Sept. 1999	Jan. 2000	Mar. 2000	May 2000
Lactate	0.5	$2.8 \cdot 10^6$	$1.5 \cdot 10^7$	$3.9 \cdot 10^6$	$1.5 \cdot 10^6$
	5	$8.8 \cdot 10^4$	$1.5 \cdot 10^5$	$5.1 \cdot 10^5$	$1.4 \cdot 10^6$
	50	$1.4 \cdot 10^4$	$1.8 \cdot 10^4$	$1.1 \cdot 10^4$	$9.6 \cdot 10^4$
Amino acids[1)]	0.5	$1.3 \cdot 10^6$	$4.0 \cdot 10^6$	$1.9 \cdot 10^7$	$7.1 \cdot 10^4$
	5	$1.4 \cdot 10^4$	$6.2 \cdot 10^5$	$2.1 \cdot 10^6$	$2.4 \cdot 10^4$
	50	$6{,}9 \cdot 10^3$	$1.2 \cdot 10^4$	$1.0 \cdot 10^4$	$9.4 \cdot 10^4$
Short-chain fatty acids[2)]	0.5	$4.0 \cdot 10^6$	$2.0 \cdot 10^6$	$1.3 \cdot 10^6$	$3.9 \cdot 10^5$
	5	$6.9 \cdot 10^4$	$4.8 \cdot 10^5$	$3.3 \cdot 10^5$	$2.4 \cdot 10^4$
	50	$3.4 \cdot 10^4$	$3.7 \cdot 10^4$	$2.7 \cdot 10^4$	$4.0 \cdot 10^4$
Long-chain fatty acids[3)]	0.5	$1 \cdot 10^5$	$7.7 \cdot 10^3$	$1.3 \cdot 10^6$	$1.4 \cdot 10^4$
	5	$2.2 \cdot 10^4$	$6.2 \cdot 10^3$	$1.6 \cdot 10^5$	$3.4 \cdot 10^3$
	50	$8.1 \cdot 10^3$	$7.4 \cdot 10^3$	$1.0 \cdot 10^4$	$2.8 \cdot 10^3$
Cellulose	0.5	n.d.	$1.1 \cdot 10^7$	$1.4 \cdot 10^6$	$1.2 \cdot 10^6$
	5	n.d.	$2.4 \cdot 10^6$	$5.1 \cdot 10^5$	$9.1 \cdot 10^4$
	50	n.d.	$1.8 \cdot 10^5$	$3.0 \cdot 10^4$	$1.4 \cdot 10^4$

n.d. not determined [1)] Alanine, arginine, cysteine, asparagine [2)] C_2-C_6 [3)] C_{16} and C_{18}

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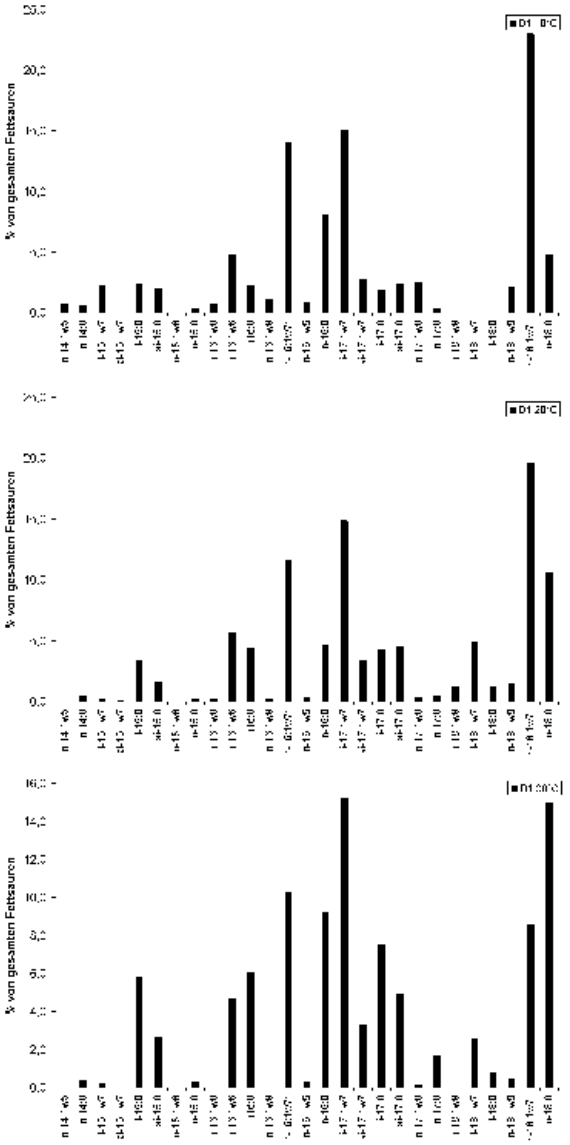

Fig. 4. Whole cell fatty acid patterns of strain *Desulfovibrio acrylicus* D1 grown at different temperatures.