MICROBIAL COMMUNITY COMPOSITION OF WADDEN SEA SEDIMENT AS REVEALED BY OPTIMIZED FLUORESCENT IN SITU HYBRIDIZATION WITH CATALYZED REPORTER DEPOSITION (CARD-FISH)

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Since the first application of fluorescence *in situ* hybridization (FISH) to Wadden Sea sediment by LLOBET-BROSSA *et al.* (1998), the method has become a much used means to directly enumerate specific microbial communities in sediments without cultivation. Depth-resolved profiles of sulfate-reducing bacteria counted with FISH showed a peak at 0.5-2 cm, corresponding with the upper local maximum of sulfate reduction rate and low concentration of organic acids such as acetate and lactate (LLOBET-BROSSA *et al.*, 2002). This suggests that FISH counting correlates well with microbial activity and chemical parameter in the sediments. On the other hand, a recent review showed that FISH enumeration of sediment samples with EUB338 (for general bacteria) has the lowest detection rate (<40%) among environments and cultivation samples (BOUVIER & DEL GIORGIO, 2003). In addition, the detection rate of EUB338 decreased with depth in sediments (LLOBET-BROSSA *et al.,* 1998, 2002). It is obvious that a substantial amount of microbes in sediments could not be detected by FISH because of their low rRNA contents.

A recent study showed that intact phospholipids extracted from Wadden Sea tidal flat samples rapidly decreased with depth but were still detectable in the deepest core sample (~50 cm), suggesting the existence of viable bacteria even in deeper parts of the sediments (RÜTTERS *et al.*, 2002). Since investigations regarding microbial communities in deeper sediment layers are scarce, enumerations of the microbial community are crucial for better understanding of the processes in this zone. More sensitive methods are needed to detect such microbes with low rRNA contents. Recent developments and applications of FISH with catalyzed reporter deposition (CARD-FISH) to marine samples showed an increase of detection rates of bacterial cells comparing to the FISH method (PERNTHALER *et al.*, 2002). It was expected that an application of CARD-FISH to sediment possibly allows improved detection of microbial communities even in deeper parts of the sediment.

Probes for CARD-FISH are labeled with horseradish peroxidase (HRP), which catalyzes deposition of fluorochrome-labeled tyramides within the target cells. Because HRP is an enzyme and a larger molecule than the fluorochromes used to label normal FISH probes, the following optimizations were necessary for the application of CARD-FISH to accurate benthic cell enumeration. First, it is unclear whether the current cell permeabilization protocol based on lysozyme works with microorganisms with unusual cell wall structure such as *Planctomyces* and Archaea (WAGNER *et al.*, 2003), which are considered to be important members of marine environments. Second, endogenous peroxidase activity in the samples, which may be abundant in the sediments and produce false signal, should be inactivated.

To examine these issues, a 40 cm deep sediment core was taken from Wadden Sea sediment close to Spiekeroog Island (Janssand) in September 2002 and sliced. Generally, the core was sandy over the whole depth interval, but some parts, especially the 18-20 cm horizon, contained black patches. The amendment of SDS into sample preparation allowed homogeneous distribution of cells on slide glass and higher DAPI counts (Fig. 1). Bright signals of *Planctomycetales* by HRP-probe Pla886 were observed without any other treatment. Achromopeptidase treatment following lysozyme treatment increased hybridization signals with Arc915-HRP (Archaea targeting) probe, although they were a minor population in the sediment communities. Methanol treatment to inactivate endogenous peroxidase activity reduced both false signal intensity and number of false-negative cells.

After these modifications, the detection rates between HRP-labeled and Cy3-labeled EUB(I-III) (bacteria targeting) per DAPI stained cells were similar in the upper layer of the sediment (92 *vs.* 82% at a depth of 2-3 cm, respectively) but more than twice in the deepest layer (63 *vs.* 26% at a depth of 35-40 cm, respectively; Fig. 2). Thus, CARD-FISH is sensitive enough to detect less active microbial communities even in the deeper sediment layers.

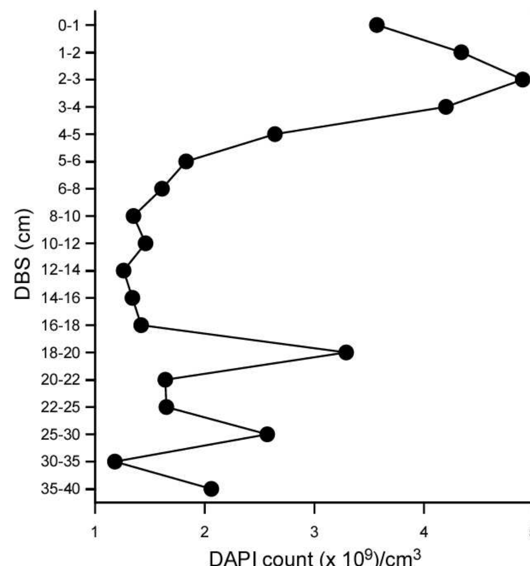

Fig. 1. The depth profile of DAPI counts. The samples were prepared with the introduction of SDS.

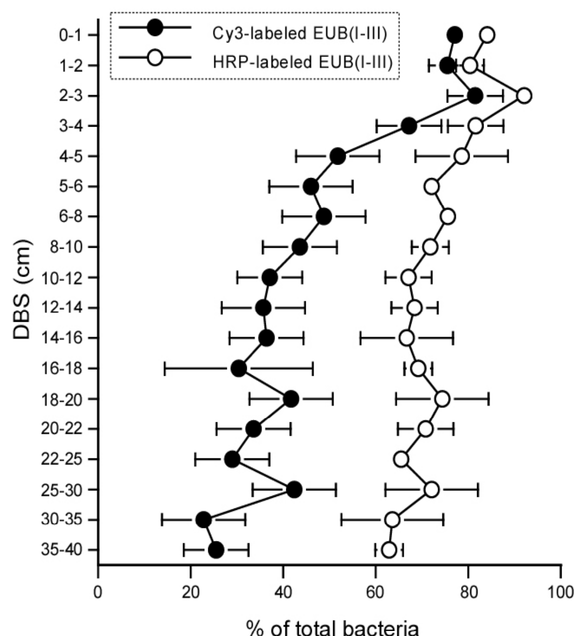

Fig. 2. Depth profiles of EUB(I-III) hybridized cell count.

Bacteria detected by HRP-probes DSR651 (*Desulforhopalus* spp.) and ALF968 (alphaproteobacteria) were found only in

the suboxic layer. On the contrary, the bacterial population evaluated by probes CF319a (*Cytophaga-Flavobacterium-Bacteroides* group), Pla886, Bet42a (betaproteobacteria) and Gam42a (gammaproteobacteria) did not show substantial change through the whole depth interval. Gam42a-hybridized cells ranged from 12 to 18% of DAPI counts, suggesting that gammaproteobacteria were the dominant heterotrophic member in the sediment. A depth profile of HRP probe DSS658 (*Desulfosarcina* spp., *Desulfofaba* sp., *Desulfococcus* spp, *Desulfofrigis* spp.) showed more than 6% of DAPI count over the depth interval with a maximum (20% of DAPI count) at a depth of 6-10 cm (Fig. 3). The lowest detection rate of this group was determind in the sample from 18-20 cm depth, which contained high amounts of black patches. On the contrary, a depth profile of sediments from Janssand taken on March 2002 showed a maximum at 23 cm depth (Fig. 4). This difference of the maxima may be explained by a difference of tidal levels, which caused different oxic layer extensions between samples (3 cm for September and 17 cm for March). In addition, the relatively smooth depth profile of this sample may be attributed to the homogeneity of the grain size distribution (low contents of black patches).

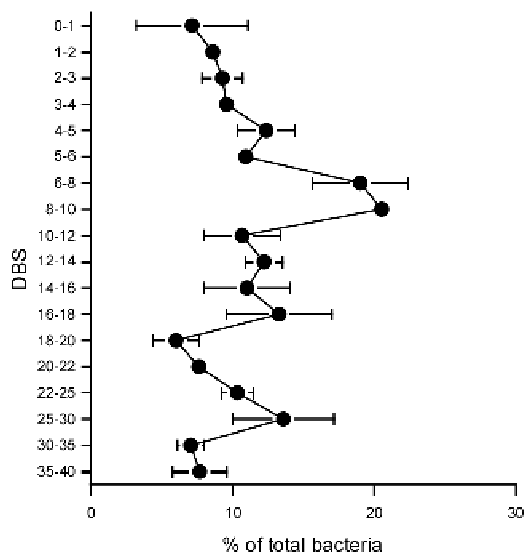

Fig. 3. A depth profile of DSS658-hybridized cell counts in September samples.

It is likely that in addition to redox potential represented by depth of oxic zones, substrate availability due to different organic sources and/or grain sizes influenced the DSS658 targeted population in the sediment. The sum of bacterial counts with these group-specific probes accounted for 50-70% of EUB(I-III) counts. Nevertheless, the activity of these bacteria detected in the deeper layers remains unknown. It may be hypothesized that they adapt to low nutrient concentrations or highly refractory organic substances. Thus, greatly improved methods enable us to further elucidate the structure of microbial communities in Wadden Sea sediments. A combination of the microbial community structure with geochemical parameters is the next interest and might improve our knowledge of tidal flat systems.

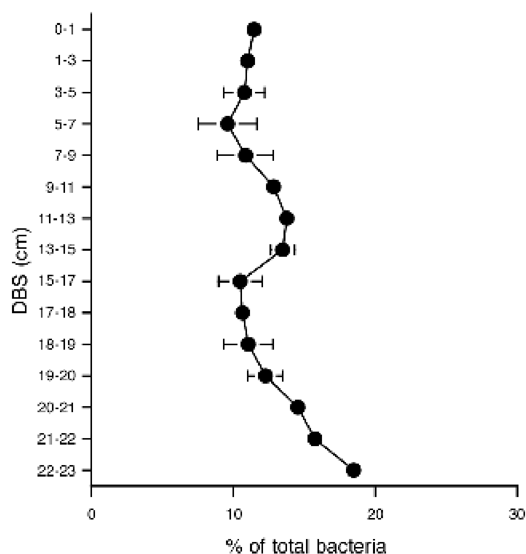

Fig. 4. A depth profile of DSS658-hybridized cell counts in March samples.

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