

# Effect of anoxia and high sulphide concentrations on heterotrophic microbial communities in reduced surface sediments (Black Spots) in sandy intertidal flats of the German Wadden Sea

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Received 24 October 2002; received in revised form 28 January 2003; accepted 30 January 2003

First published online 3 April 2003

## Abstract

Black reduced sediment surfaces (Black Spots) in sandy intertidal flats of the German Wadden Sea (southern North Sea) are characterised by elevated sulphide concentrations (up to 20 mM) and low redox potentials. It is assumed that the appearance of Black Spots is linked to elevated levels of organic matter content within the sediments. In order to establish the effect of high substrate and sulphide concentrations on the heterotrophic microbial communities in Black Spot sediments, bacterial abundances and the potential C-source utilisation patterns of microbial communities were compared in natural and artificially induced Black Spots and unaffected control sites. Bacterial numbers were estimated by direct counts and the most probable number technique for different physiological groups, while patterns of C-substrate utilisation of entire aerobic microbial communities were assessed using the Biolog<sup>®</sup> sole-carbon-source-catabolism assay. Bacterial abundances at Black Spot sites were increased, with increases in mean cell numbers, more disperse data distributions and more extreme values. Substrate utilisation patterns of aerobic microbial communities were significantly different in Black Spot sediment slurries, showing diminished richness (number of C-sources catabolised) and substrate diversity (Shannon diversity index) in comparison to unaffected sites. Principal component analysis clearly discriminated Black Spot utilisation patterns from controls and indicated that microbial communities in individual Black Spot sites are functionally diverse and differ from communities in oxidised surface sediments and reduced subsurface sediments at control sites. This work suggests that potentially negative effects on microbial communities in Black Spot sediments, through anoxia and high sulphide concentrations, are balanced by the stimulating influence of substrate availability, leading to comparable or higher bacterial numbers, but lower functional microbial diversity of aerobic microbial communities. © 2003 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

**Keywords:** Marine sediment microbial population; Biolog<sup>®</sup>; Intertidal sediment; Black Spot; Aerobic functional diversity

## 1. Introduction

In the last decades, the German Wadden Sea has been affected by large and intense phytoplankton (e.g. *Phaeocystis* sp. [1,2]) and macroalgal blooms (e.g. *Enteromorpha* sp. [3,4]). With the collapse of these blooms, organic matter is released into the ecosystem and is often subsequently

deposited in preferential sedimentation areas. Wave action or further sedimentation processes may lead to the incorporation of this organic matter into deeper sediment layers, thereby introducing microbial substrates of high trophic quality [5,6]. If the microbial oxidation of electron donors exceeds the transportation rates of oxygen into the sediments, the classical model of the vertical succession of aerobic microbial respiration to fermentation and sulphate reduction processes during the degradation of organic matter in marine sediments [7,8] becomes disturbed. In this case, sulphate reduction prevails as the dominant microbial process due to the high sulphate concentrations in

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marine habitats, resulting in the development of sulphur-eta with high concentrations of sulphide [8].

Anoxia, low redox potentials and high sulphide concentrations are characteristic of subsurface sediments in most marine habitats. However, the sudden appearance of reduced surface sediments, termed Black Spots due to the precipitation of ferrous sulphides, on intertidal sand flats of the German Wadden Sea suggests the total loss of the otherwise predominant oxic and suboxic surface and subsurface zones and indicates a major shift in the biogeochemical conditions for aerobic microbial communities.

The effects of stress and perturbation on aerobic marine microbial communities have been investigated rarely. Most studies on the impact of organic matter input into marine sediments (e.g. algal bloom deposition, sewage discharge or fish farming) focus on the significance for particular microbially controlled anaerobic processes [9,10]. Consequently, the impact of low redox potentials and high sulphide concentrations on the aerobic heterotrophic microbial communities within these sediments cannot be predicted, particularly in intertidal Wadden Sea sediments, where related studies are few [11–13]. The bacterial community has been estimated to be responsible for 70% of the degradation of organic matter in the Wadden Sea [14]. Heterotrophic microbial communities within the oxic and suboxic geochemical zones can contribute to 80% of the total bacterial turnover in marine sediments, using  $O_2$ ,  $NO_3^-$ ,  $NO_2^-$ , iron and manganese oxyhydroxides as electron acceptors [15,16] or using fermentative pathways degrading organic matter anaerobically. They play a crucial role in the initial degradation of organic matter and consequently are of major importance for the recycling of nutrients and marine ecosystem functioning [17].

Although molecular techniques, especially those based on 16S rRNA gene sequences, are now frequently used to assess microbial diversity in natural environments, they are limited in their ability to link species diversity, functional diversity and ecosystem function, and alternative molecular techniques with the potential to analyse physiological diversity within complex microbial communities are not well developed. One approach to determining the potential physiological capabilities of aerobic microbial communities is characterisation of patterns of sole carbon source utilisation, applying Biolog<sup>®</sup> assays. These assays have been developed for typing of isolates but can also be used to analyse substrate utilisation patterns of entire communities. This approach has been successful in discriminating microbial communities from different habitats and following changes in potential functional diversity resulting from management regimes or perturbations (e.g. [14,18–22]). In addition, studies based on analyses of Biolog<sup>®</sup> sole carbon source utilisation patterns have been used to construct conceptual models describing the interrelations of functional, genetic and species diversity and to assess and compare functional diversity [23]. However, few studies have been carried out on microbial communities

from marine environments [24–28] and the potential functional diversity of marine microbial sediment communities has not yet been characterised.

This study focused on the influence of shifts from oxic and suboxic to anoxic geochemical conditions in Black Spot sediments on aerobic microbial populations. The major objectives were to determine whether the occurrence of Black Spots fundamentally altered the potential functional diversity of microbial communities in surface and subsurface sediments and whether these communities retained the potential physiological properties of unaffected communities. Artificially induced Black Spots were investigated as controls with respect to hypotheses regarding the genesis and cause of natural Black Spots [29,30]. The potential functional diversity within microbial communities was analysed by comparing means and distributions of bacterial numbers and aerobic community substrate utilisation patterns.

## 2. Materials and methods

### 2.1. Study area and sampling procedures

The intertidal sand and mud flats of the German Wadden Sea extend from The Netherlands to Denmark (Fig. 1). The study area, 'Gröninger Plate', in the East Frisian subregion, is characterised by its symmetric orientation towards the tidal watershed, its uniform sediment composition (95% fine sands, particle size 125–250  $\mu m$ ), its homogeneous benthic communities (*Lanice conchilega* dominating in the upper regions and *Arenicola marina* at lower areas) and the lack of apparent anthropogenic influence. Low tides expose the sediments to the atmosphere for 5–6 h, and strong tidal currents cause permanent resuspension and sedimentation. Black Spots were present near minor and major tidal channels in the northern and north-eastern part of the sand flat, covering surface areas from a few  $dm^2$  to several  $m^2$ . Black Spot sediments were marked by reduced FeS sediments from the surface to a depth of several dm, with no cause for Black Spot development evident. Sulphide concentrations and redox potentials of Black Spots have been extensively monitored [29,30]. The sediment structure of adjacent control sites was usually consistent with the traditional model of biogeochemical zones [7,8], consisting of a 2–5-cm-thick, oxidised surface layer overlying a thin, black FeS interface layer (redox potential discontinuity (RPD) layer) above or mixed through turbation with the  $FeS_2$ -reduced blackish-grey subsurface sediment. Black Spots and adjacent control sites were sampled at random.

Artificial Black Spots were created in a field trial by burying macroalgae (20  $kg m^{-2}$  *Enteromorpha* sp. to 20 cm depth) at a site close to natural Black Spots. Total organic matter (TOM) content was determined gravimetrically after combustion at 550°C of freeze-dried samples,

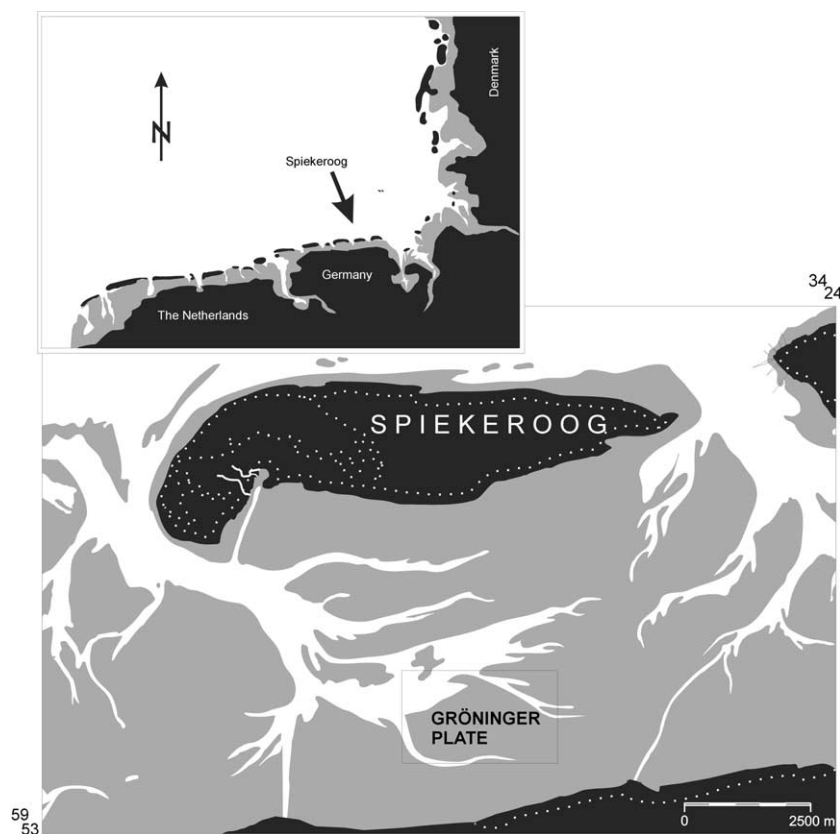


Fig. 1. Area of investigation (Gröninger Plate) in the back barrier tidal flat zone of the island of Spiekeroog.

protein concentrations were determined colourimetrically with the bicinchonic acid assay [32], the carbohydrate content was analysed with the phenol sulphuric acid assay [33], pH values were measured in wet sediments on site with an Ingold mini electrode, and porosity was defined as the water content per sample volume. TOM, protein, carbohydrate, porosity and pH values of natural Black Spots (BS<sub>N</sub>), artificial Black Spots (BS<sub>A</sub>) and controls (Cont) are presented in Table 1. Artificial Black Spots were also induced in laboratory microcosms by amending sieved and washed sediment with freeze-dried and powdered *Enteromorpha* sp. biomass (1.5 mg g<sup>-1</sup>). Microcosms consisted of 500-ml plastic beakers containing 250 g of amended sediment, submerged with filtered seawater, inoculated with Black Spot sediment and incubated at 15°C for several months. Artificial Black Spots were sampled

several weeks after development of Black Spots, which occurred within a few days, and after reoxidation and reversion to the original appearance, which occurred several months after Black Spot development. On several occasions, reoxidised natural Black Spots, which had been sampled previously, were also included. For bacterial enumeration, undisturbed sediment samples from field sites were taken from box cores with sterile syringes (1 cm in diameter with the luer end removed) at 1-cm increments up to 20 cm depth. Microcosms were sampled destructively. For Biolog<sup>®</sup> incubations, sediment volumes of approximately 50 g were retrieved from the uppermost oxidised sediment layers (0–3 cm) and the reduced sediment layers below at control sites and from equivalent depths from Black Spot sites. All sediment samples were stored at 4°C until further processing within 24 h.

Table 1

TOM, protein and carbohydrate concentrations, porosity and pH for natural Black Spots (BS<sub>N</sub>), artificial Black Spots (BS<sub>A</sub>; field trial) and controls (Cont.)

Sample group	TOM (% dw)			Protein (mg g <sup>-1</sup> )			Carbohydrates (mg g <sup>-1</sup> )			Porosity (% vol)			pH		
	BS <sub>N</sub>	BS <sub>A</sub>	Cont.	BS <sub>N</sub>	BS <sub>A</sub>	Cont.	BS <sub>N</sub>	BS <sub>A</sub>	Cont.	BS <sub>N</sub>	BS <sub>A</sub>	Cont.	BS <sub>N</sub>	BS <sub>A</sub>	Cont.
$\bar{X}$	2.1 <sub>A</sub>	1.3	1.3	5.0 <sub>A</sub>	10.6 <sub>C</sub>	4.3	1.70 <sub>A</sub>	1.06 <sub>C</sub>	0.72	20.7 <sub>AB</sub>	22.2 <sub>C</sub>	18.6	7.4 <sub>B</sub>	7.3 <sub>C</sub>	7.7
Cv%	113 <sub>AB</sub>	37	29	66 <sub>A</sub>	93 <sub>C</sub>	107	165 <sub>AB</sub>	152	64	33 <sub>B</sub>	35 <sub>C</sub>	9	10 <sub>A</sub>	14 <sub>C</sub>	3
N	182			196			105			263			183		

$\bar{X}$  = mean, Cv% = coefficient of variation. Subscripts A, B and C indicate significant differences ( $P < 0.05$ ) between sampling groups BS<sub>N</sub> and BS<sub>A</sub>, BS<sub>N</sub> and Cont. and BS<sub>A</sub> and Cont., respectively.

## 2.2. Preparation of cell suspensions for enumeration and Biolog<sup>™</sup> inoculation

Samples for total bacterial number determinations (TBN, 0.5 ml) were placed in formaldehyde in filtered seawater (4% final concentration) immediately after collection. Cell suspensions were prepared by sonication for 60 s with a Branson cell disintegrator, 100 W nominal power output. The suspensions were centrifuged ( $1000 \times g$ , 3 min), supernatants stained with 4',6-diamidino-2-phenylindole (DAPI) [34] and cells concentrated on black Nuclepore polycarbonate filters (0.2  $\mu\text{m}$  pore size). Viable cells were enumerated by dilution of samples (0.5 ml) in 4.5 ml sterile filtered seawater, containing Tween 80, final concentration 0.001%, shaken vigorously for 30 min and finally centrifuged ( $1000 \times g$ , 3 min). Serial dilutions ( $10^{-2}$ – $10^{-10}$ ) of the supernatants were prepared with sterile filtered seawater for inoculation. Inocula for Biolog<sup>™</sup> GN and GP microtitre plates were prepared by gently shaking 10 g of homogenised samples in 100 ml artificial seawater [35] without calcium components and containing Tween 80 (final concentration 0.001%) for 18 h at 10°C [28] and passage through filter paper.

## 2.3. Enumeration procedures

TBN values were estimated by epifluorescence microscopy of DAPI-stained cells, averaging counts from at least 10 randomly chosen fields. Viable counts of aerobic, heterotrophic bacteria (AB), fermentative bacteria (FB) and sulphate reducing bacteria (SRB) were determined by the most probable number (MPN) method on microtitre plates in liquid media, with five replicate inoculations from decimal dilution series. Aerobic chemoorganotrophic bacteria were cultured in Zobell's medium 2216E [36] by incubation at 20°C for 2–4 weeks. Growth was assessed as visible turbidity, with random microscopic analysis of non-turbid wells. Fermentative chemoorganotrophic bacteria were enumerated in Hugh and Leifson oxidation/fermentation medium [37], prepared using artificial seawater and containing 5 g glucose  $\text{l}^{-1}$  and incubated anaerobically at room temperature (15–17°C) for 4 weeks in a nitrogen atmosphere in gas tight plastic bags. Fermentative growth was identified by colour change of the pH indicator. SRB were enumerated following preparation of, and inoculation from dilutions dispensed separately under anoxic conditions in an anaerobic cabinet into the medium of Pfennig et al. [38] supplemented with ethanol, sodium acetate and sodium lactate (10 mM). Cultures were incubated anaerobically in an  $\text{N}_2/\text{CO}_2/\text{H}_2$  (86/10/4%) atmosphere at room temperature (15–17°C) for 10 weeks, with precipitation of black FeS indicating growth.

## 2.4. Biolog<sup>™</sup> incubations

Substrate utilisation activities and patterns of sediment

communities were estimated by inoculating Biolog<sup>™</sup> GN and GP 96-well microtitre plates containing, in total, 128 different substrates, 62 of which are duplicated and were omitted from statistical analysis [28,39]. Substrates on Biolog<sup>™</sup> microtitre plates can be grouped according to their chemical properties into the following guilds: polymers, carbohydrates, carboxylic acids, amino acids, amines/amides and others [24]. Carbon sources on the microtitre plates are incorporated into a basal medium with tetrazolium violet to indicate colourimetrically microbial utilisation of the carbon sources, with one well containing no carbon source for background readings. Biolog<sup>™</sup> plates were inoculated with 150  $\mu\text{l}$  of sediment extract in artificial seawater per well. In test trials, higher extract dilutions resulted in infrequent colour development, whereas undiluted extracts yielded results with a maximum of approximately 90% of all substrates used. Plates were wrapped in parafilm to minimise evaporation and incubated aerobically at 15°C for up to 6 days, after which readings became unreliable, as indicated by frequent colour development in control wells. Colour development  $\text{OD}_{590}$  (optical density at 590 nm) was recorded daily with a Biolog<sup>™</sup> Microplate Reader and the Biolog<sup>™</sup> Microlog 2N reader software was used for automatic subtraction of background readings. Test trials showed that the highest possible  $\text{OD}_{590}$  values for individual substrates were significantly different, biasing quantitative readings of individual substrates, and substrate utilisation was therefore expressed after the conversion of quantitative readings to ordinal scores. The Microlog 2N software discriminates between positive and negative substrate responses by calculating threshold values for each individual substrate that must be higher than 40% of the highest possible response for the particular substrate. On the basis of three replicate incubations, this threshold assignment was used for the transformation of quantitative  $\text{OD}_{590}$  values of Biolog<sup>™</sup> microplate readings into positive (1) and negative (0) scores, rendering two negative readings and one positive as negative and vice versa. For the majority of substrate scores (83%) the decision was concordant. Substrate richness was expressed as the ratio of the sum of all positive scores for each sample to the number of substrates. Accordingly, substrate activity was defined as the ratio of the sum of all positive scores for each substrate to the number of samples. Diversity of substrate usage within sampling groups was calculated according to the Shannon diversity index [23].

## 2.5. Data analysis

Data were combined in sets of sampling groups consisting of controls (Cont), natural Black Spots ( $\text{BS}_\text{N}$ ) and artificial Black Spots ( $\text{BS}_\text{A}$ ). For Biolog<sup>™</sup> analysis, control samples were discriminated between oxidised (Ox) and reduced (Red) subsurface sediments and artificial Black Spots were excluded from frequency distribution analysis

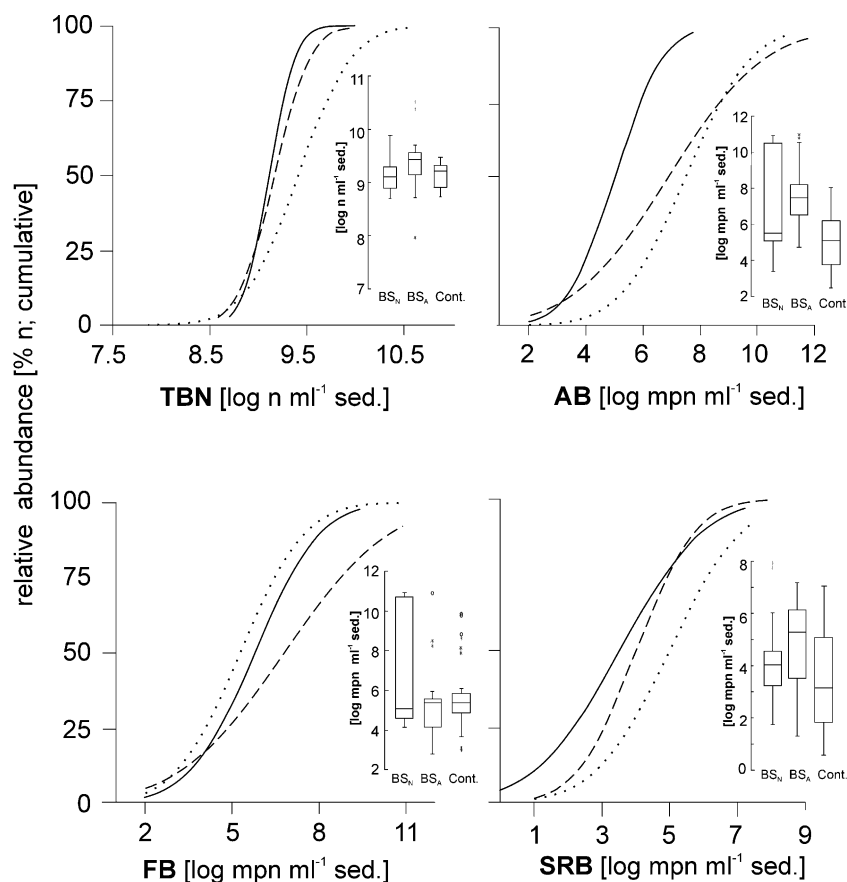


Fig. 2. Cumulative frequency distribution of bacterial numbers in natural Black Spots, artificial Black Spots (field trial only) and controls. Curves are superimposed according to normal standard distribution. Insets contain box plots showing median, 25th and 75th percentiles and extreme values. Total bacterial numbers = TBN, aerobic chemoorganotrophic bacteria = AB, fermenting bacteria = FB, sulphate reducing bacteria = SRB. Natural Black Spots (BS<sub>N</sub>) = dashed line; artificial Black Spots (BS<sub>A</sub>) = dotted line; controls (Cont.) = solid line.

because of the low number of samples. The natural heterogeneity within the control group was discriminated from the effect of the special geochemical conditions in Black Spots by independent tests of significance ( $P < 0.05$ ). The rank sums  $U$ -test by Wilcoxon Mann and Whitney was used to test for significant differences between means of sampling groups and the Kolmogoroff-Smirnoff test to test for significant differences in data distributions. Trends among microbiological and chemical data were analysed with Spearman rank correlation coefficients. Only statistically significant correlations were used to demonstrate trends between variables. Of 34 sediment cores, complete simultaneous analysis of all uni-variate parameters investigated was achieved for  $> 80\%$  of all

samples with the exception of carbohydrates for which the dataset was complete for less than 50% of all samples. Carbohydrates were therefore excluded from correlation analysis. Patterns of Biolog<sup>TM</sup> utilisation were subjected to principle component analysis (PCA) using the first and second principal components (PC1 and PC2) to demonstrate relations between sampling groups [40]. The influence of individual carbon sources within the different sampling groups was demonstrated using the loadings of principle components. Bi- and multivariate operations were performed with standardised values of quantitative data ( $z$ -scores) following log-transformation of bacterial numbers. Statistical analysis of data was achieved with the SYSTAT V. 5.2.1 software package (SPSS Inc.).

Table 2

Bacterial numbers for natural Black Spots (BS<sub>N</sub>), artificial Black Spots (BS<sub>A</sub>; field trial) and controls (Cont.)

Sample group	TBN (log $n$ ml <sup>-1</sup> )			AB (log MPN ml <sup>-1</sup> )			FB (log MPN ml <sup>-1</sup> )			SRB (log MPN ml <sup>-1</sup> )		
	BS <sub>N</sub>	BS <sub>A</sub>	Cont.	BS <sub>N</sub>	BS <sub>A</sub>	Cont.	BS <sub>N</sub>	BS <sub>A</sub>	Cont.	BS <sub>N</sub>	BS <sub>A</sub>	Cont.
$\bar{X}$	9.2 <sub>A</sub>	9.4 <sub>C</sub>	9.1	6.9 <sub>AB</sub>	7.5 <sub>C</sub>	5.0	4.0	5.0	3.5	4.0 <sub>A</sub>	5.0 <sub>C</sub>	3.5
$Cv\%$	3 <sub>A</sub>	5 <sub>C</sub>	2	39 <sub>AB</sub>	24 <sub>C</sub>	27	35	35	56	35 <sub>AB</sub>	35 <sub>C</sub>	56
$N$	182			259			222			209		

$\bar{X}$  = mean,  $Cv\%$  = coefficient of variation. Subscripts A, B and C indicate significant differences ( $P < 0.05$ ) between sampling groups BS<sub>N</sub> and BS<sub>A</sub>, BS<sub>N</sub> and Cont. and BS<sub>A</sub> and Cont., respectively.

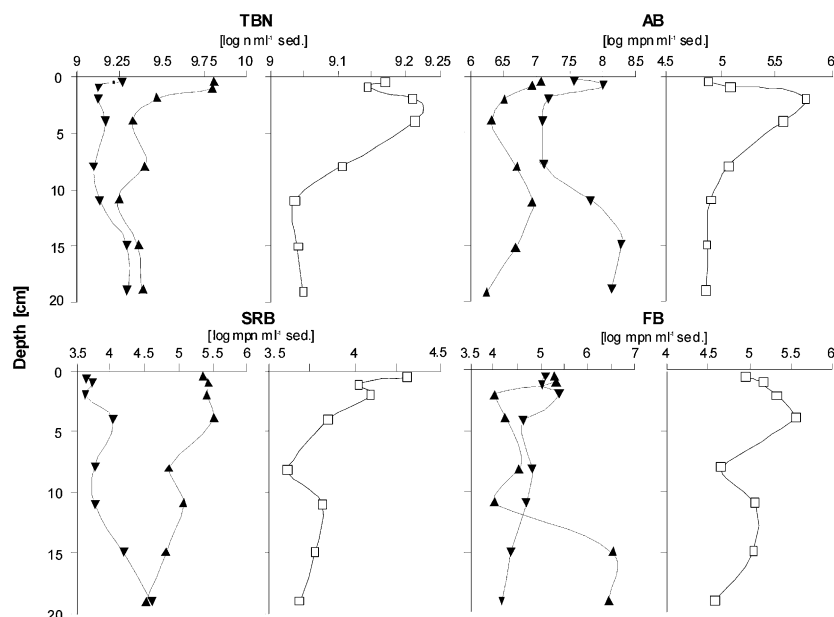


Fig. 3. Depth distribution of bacterial numbers of total bacterial numbers (TBN), aerobic chemoorganotrophic bacteria (AB), fermenting bacteria (FB), and sulphate reducing bacteria (SRB). Data points represent the mean of several enumerations. Natural Black Spots = ▲, artificial Black Spots (field) = ▼, controls = □.

### 3. Results

#### 3.1. Bacterial abundance

The majority of bacterial cell counts differed significantly between control, natural and artificial Black Spots ( $P < 0.05$ ) (Table 2, Fig. 2). Significantly higher means, more disperse data distributions and extreme values were more prominent in natural and artificial Black Spot sites than in controls. Differences associated with Black Spot formation were greater for artificial than natural sites. The significance of differences between means and data distributions of  $BS_A$  and  $BS_N$  were similar to those between  $BS_A$  and controls. Means were significantly higher and distributions of data were more scattered for viable numbers than total counts. AB counts showed the highest difference in mean and most dispersed data distribution for the BS groups. SRB numbers were also higher in BS samples, but data distributions followed the opposite trend, with significantly decreased data dispersion for  $BS_N$  sites.

A similar trend was observed for FB counts but was not statistically significant.

Integrated depth profiles of mean bacterial numbers showed similar trends for TBN, AB and FB values in control sites (Fig. 3) with highest numbers in below subsurface sediments within or above the RPD layer and decreasing numbers with increasing depth. In contrast, TBN and AB means were highest at the sediment–water interface and in deeper sediment layers from artificial and natural Black Spots. Increasing bacterial numbers with increasing depth were also observed for SRB in natural and FB in artificial Black Spot sites.

Significant correlations were found between bacterial numbers and abiotic sediment characteristics for all sampling groups (Fig. 4). The control group was characterised by similar trends in all viable bacterial number parameters, strong positive correlations of AB and FB counts with protein and porosity and correlations between TBN counts and porosity. The Black Spot groups were distinguished from the control group by the mutual negative

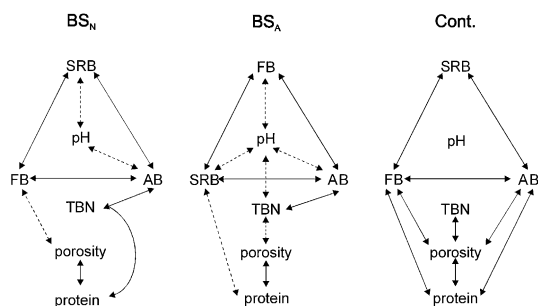


Fig. 4. Trends among biotic and abiotic variables according to significant Spearman rank correlations ( $r > 0.6$ ;  $P < 0.05$ ). For abbreviations see legend for Fig. 2. Dotted lines = negative correlations.

Table 3

Substrate richness and activity calculated on substrate utilisation scores from the 120-h incubation of Biolog GN and GP plates for natural Black Spots ( $BS_N$ ), reduced subsurface controls (Red) and oxidised controls (Ox)

Sample group	Mean richness			Mean activity		
	$BS_N$	Red	Ox	$BS_N$	Red	Ox
$\bar{X}$	0.25 <sub>AB</sub>	0.28	0.41	0.18 <sub>AB</sub>	0.24 <sub>C</sub>	0.39
$Cv\%$	30 <sub>A</sub>	27	20	25 <sub>AB</sub>	27	37 <sub>C</sub>

$\bar{X}$  = mean,  $Cv\%$  = coefficient of variation; subscripts A, B and C indicate significant differences ( $P < 0.05$ ) between sampling groups  $BS_N$  and Red,  $BS_N$  and Ox and Red and Ox, respectively.

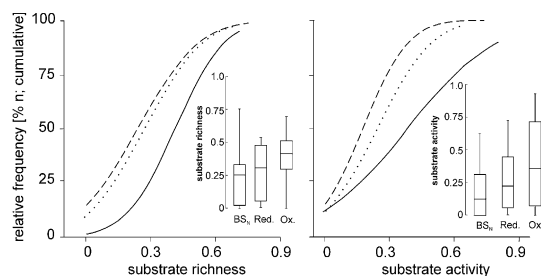


Fig. 5. Cumulative frequency distribution of data on substrate richness and substrate activity of sediment slurries in natural Black Spots, and controls. Curves are superimposed according to normal standard distribution. Insets contain box plots showing median, 25th, 75th percentiles and extreme values. Control samples were grouped according to oxidised or reduced sediment properties. Natural Black Spots = dashed line,  $n=16$ ; reduced subsurface controls = dotted line,  $n=9$ ; oxidised controls = solid line,  $n=14$ .

correlation of AB and FB numbers with pH values at Black Spot sites. A direct correlation between TBN and any of the viable number parameters was only observed in the Black Spot groups.

### 3.2. Substrate richness, activity and diversity

Statistically significant lower mean substrate richness and activities, and more dispersed data distributions were found for  $BS_N$  sediment slurries compared to oxidised and reduced control samples (Table 3, Fig. 5). An average of only 25% of all substrates were utilised in  $BS_N$  sediment slurries, whereas over 40% of substrates were utilised in oxidised control sediments. Accordingly, each substrate was only utilised in an average of 18% of all  $BS_N$  samples compared to 37% of all oxidised control samples. This tendency was reflected in the time dependent substrate utilisations (Fig. 6). In total, communities in reduced subsurface sediments from control sites showed intermediate richness, activity and time dependent utilisation compared to communities from Black Spots and oxidised controls. In contrast, identical values of the Shannon diversity index were established for oxidised and reduced control sites (4.44), with a lower value for  $BS_N$  (4.35).

### 3.3. Substrate utilisation patterns

No difference was evident between sampling groups in the substrate richness of carbon sources according to groups of chemical guilds [24]. The following ranking order of substrate richness was valid for all sampling groups: polymers > others > amino acids > carbohydrates  $\geq$  carboxylic acids > amines-amides. Among the 15 preferentially utilised substrates of the individual sampling groups, four substrates were identified that were utilised with high activities in all sampling groups: inosine, adenosine, adenosine-3'-monophosphate and L-aspartate. Pyruvic acid and bromosuccinic acid were consumed preferentially in the control group and 3-methyl-glucose in the

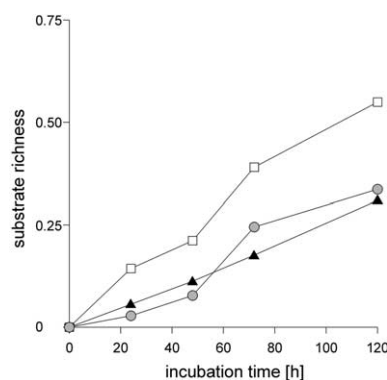


Fig. 6. Time dependent development of bacterial substrate richness. Control samples were grouped according to oxidised and reduced sediment properties. Natural Black Spots =  $\blacktriangle$ ,  $n=16$ ; reduced subsurface controls = grey circles,  $n=9$ ; oxidised controls =  $\square$ ,  $n=14$ .

reduced control sediments. L-Histidine was the only substrate that was preferentially utilised in the  $BS_N$  group.  $\beta$ -Hydroxybutyric acid, D-tagatose,  $\alpha$ -methyl-D-mannoside, xylitol and 2'-desoxyadenosine were poorly utilised in all sampling groups and D-sorbitol, amygdalin and D,L- $\alpha$ -glycerolphosphate were utilised with low intensities in the  $BS_N$  group only.

PCA of substrate utilisation patterns discriminated microbial communities in Black Spot sediment slurries from controls with 54.5% of the total variance explained by

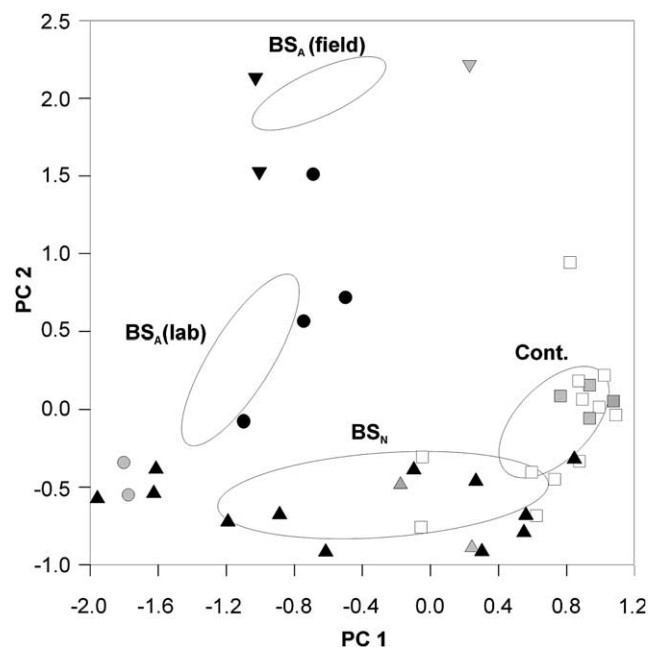


Fig. 7. Principle component scores of substrate utilisation patterns after an incubation period of 120 h. Ellipses demonstrate the 80% confidence region of the principle component values of the sampling groups. Natural Black Spots ( $BS_N$ ) =  $\blacktriangle$ , artificial (field) Black Spots ( $BS_A$ ) =  $\blacktriangledown$ , artificial (lab systems) Black Spots ( $BS_A$ ) =  $\bullet$ , reoxidised natural Black Spots ( $BS_N$ ) = grey upward triangles, reoxidised artificial (field) Black Spots ( $BS_A$ ) = grey downward triangles, reoxidised artificial (lab systems) Black Spots ( $BS_A$ ) = grey circles, controls (oxidised) (Cont.) =  $\square$ , controls (reduced) (Cont.) = grey squares.

both the first (PC1) and second (PC2) principal components (Fig. 7). The scale of principle components was dominated by the distances of the artificial and natural Black Spot samples, resulting in a cluster of control samples. Separation within identical sampling groups or from the same experimental set-up was more pronounced than separation of samples of comparable geochemical type. For example, samples from reduced sediment layers of control sites were orientated in close proximity to samples from oxidised control samples and reoxidised samples from Black Spot sediments were projected in close proximity to reduced Black Spot sediment samples. PCA trends were confirmed by corresponding results from hierarchical cluster analyses.

## 4. Discussion

### 4.1. Methodological aspects

This study employed cultivation dependent techniques to discriminate abundance and functional diversity of microbial communities from Black Spots and natural sediment and is therefore likely to exclude organisms that are unculturable, dormant or inactive under laboratory conditions. In addition, functional microbial diversity defined as the substrate richness and the type of substrate utilisation pattern used by a sample community [23] does not necessarily reflect the physiological potential of a natural microbial community in situ [41]. Nevertheless, the selection of fast growing opportunistic species that are also most likely to be accessed by culture methods is favoured by the availability of substrates of high trophic quality [42]. Hence, when assessing microbial communities in natural environments by viable counts, high numbers may indicate favourable conditions for opportunistic species, whereas the complex substrate requirements of microbial communities in later successional states may be reflected by lower viable numbers. In this sense, the use of miniaturised culture dependent techniques (MPN, Biolog<sup>®</sup>) that allow the fast screening of high numbers of samples is appropriate as a first step in characterising microbial communities and permits the use of statistical tools when different habitats are compared.

### 4.2. Effects of anoxia and high sulphide concentrations on microbial communities

The high bacterial numbers in Black Spot sediments suggest that, for the investigated physiological groups, the increased substrate availability indicated by significantly increased TOM, protein and carbohydrate values prevails over presumed negative effects of anoxia, low redox potential and high concentrations of sulphide. Additionally, the high sulphide concentrations (up to 20 mM, [29,30]) are toxic to most protozoa and metazoa [31], thus

reducing grazing of bacteria and, potentially, bacterial numbers. Predictions of effects of high sulphide concentrations on the heterotrophic aerobic microbial communities in Black Spot sediments are uncertain, as related studies are few. Hoppe et al. [43] reported adverse effects on natural heterotrophic communities in the presence of 20–30  $\mu\text{M}$  sulphide and growth rates of *Desulfovibrio desulfuricans* are reduced by 50% at lower concentrations [44], similar to those reported for Black Spot sediments. It therefore seems likely that high sulphide concentrations inhibit a variety of aerobic and anaerobic species of the indigenous sediment microflora, restricting growth to sulphide tolerant species. However, as in this study, Sundbäck et al. [45] observed no significant adverse effect of high sulphide concentrations on total bacterial numbers in an experimental marine system with high macroalgal biomass loadings. The high bacterial numbers at control sites in subsurface samples (Fig. 4) are in good agreement with comparable studies from marine environments where total bacterial numbers, numbers of dividing cells and thymidine incorporation rates were highest in surface and subsurface layers [16]. High UV-radiation, grazing pressure and shear stress through wave action and strong currents may outweigh the growth advantages for bacteria of free oxygen availability within the uppermost millimetres of the sediments. Additionally, the high activities of sulphate reducing bacteria in these layers [16,46] may result in high substrate turnover rates and lead to increased bacterial biomass of aerobe/facultative anaerobe microbial communities. Higher bacterial numbers in Black Spot surface sediments possibly reflect the microbially active zone of overlapping sulphide and oxygen gradients at the sediment–water interface and, in deeper sediment layers, the factors leading to Black Spot formation, i.e. strata containing organic matter of high substrate value, buried in deeper sediment layers.

Sample groups were discriminated on the basis of total and viable bacterial numbers and in differing patterns of significant correlations between bacterial numbers and abiotic sediment characteristics. pH changes within marine sediments are usually tightly coupled to the carbon dioxide and sulphide buffer system [47] but have in similar studies on the effect of high biomass loading also been linked to the fermentative production of organic acids [48]. The negative correlations of pH values with bacterial numbers are thus probably indicating the accumulation of fermentation products rather than unfavourable growth conditions due to the presence of sulphuric acid and carbonic acid for an indigenous microbial community with a narrow range of optimal pH. The negative correlation of the significantly decreased pH values with bacterial numbers was prominent only in Black Spot sites, a shared trend that indicates the comparable geochemical conditions in both artificial and natural Black Spot sites. Porosity is a measure of the interstitial space in sediments and strongly influences permeability that determines the advective



transport for solutes and particles [49,50]. The permeability of sandy sediments is generally assumed to be favourable for the exchange of bacterial electron acceptors and substrates [51] and the strong correlations with bacterial numbers in control sites may reflect this relation. Increased porosities in natural Black Spot sites may indicate sedimentation events of fine-grained material thus increasing the pore space but reducing the hydraulic conductivity and advective transport. Higher porosities in Black Spot sites are likely to be at least partially created by expansion of pore space through extensive methanogenesis as the ebullition of ignitable gases has frequently been observed from artificial and natural Black Spot sediments. The negative correlations or the absence of correlations of bacterial numbers with porosity in Black Spot sites implies major disturbances within the complex relations of microbial activity and geochemical conditions. The lack of correlation of bacterial numbers with the biomass indicator protein at Black Spot sites furthermore suggests that other geochemical factors (i.e. redox potential, sulphide concentrations) may be the dominant factors determining microbial growth. The strong correlation of AB and FB numbers (regression coefficient 0.9) in all sampling groups demonstrates that these physiological groups may at least be partially identical. However, only TBN and AB counts were significantly correlated at Black Spot sites, suggesting that, in these nutrient rich environments, viable numbers reflect trends in the total bacterial community.

PCA clearly discriminated the a priori defined sample groups and confirmed differences in the potential functional diversity of microbial communities in Black Spots and controls and between artificial Black Spots from field trials and microcosms. The clustering of unaffected control group samples implies aerobic microbial communities with highly similar functional diversity. The alteration of geochemical conditions in Black Spot sediments seems to have a pronounced effect on the microbial communities. In addition to high sulphide concentrations, the community composition will be affected by the type of introduced substrates and the state of succession of community development, resulting in the observed marked discrimination of individual microbial communities in Black Spot sediments. Similarly, the marked discrimination of microbial communities in reoxidised Black Spots from those in controls suggests that the reversion of the geochemical state does not lead to restoration of the microbial communities. The similarity of substrate utilisation patterns of microbial communities from oxidised and reduced controls and their dissimilarity from reduced and reoxidised Black Spots support the hypothesis that microbial communities in Black Spots are characteristic of a new, different habitat and not identical with communities from deeper layers of unaffected control sediments.

Decreased substrate richness, activity and diversity indicate significantly diminished aerobic functional diversities of Black Spot microbial communities, compared to

controls. However, substrate utilisation patterns in individual Black Spot sites suggest communities that differ significantly from each other and from communities at control sites. With respect to microbial numbers, the special geochemical conditions within Black Spot sediments seem to be beneficial for microbial communities. Consequently, numbers of specialised sulphide tolerant microbial communities in Black Spot sediments are similar to those in control sediments but may have a reduced functional aerobic diversity. It should be emphasised however, that the single carbon source utilisation patterns obtained with the Biolog<sup>™</sup> assay reflect only the potential of the investigated microbial communities for energy gain via oxic electron transport phosphorylation under aerobic incubation conditions and tetrazolium redox indicators used in the Biolog<sup>™</sup> assay do not assess fermentative versatility. It cannot be excluded, therefore, that although of diminished functional diversity with regard to oxic electron transport phosphorylation, the microbial communities in Black Spot sediments may be of equal or greater functional diversity under anaerobic conditions.

#### 4.3. General ecological implications

The development of sulphureta is not unusual in marine environments, but is mainly associated with conditions of little physical disturbance where microbial activity results in the stratification of geochemical gradients. The appearance and persistence of Black Spots and Black Spot areas of several km<sup>2</sup> within a highly dynamic and oxic environment has caused concern about the ecological state of the German Wadden Sea [29]. However, as we are not aware of accountable records of previous comparable Black Spot area events, it is not clear if Black Spots in intertidal flats of the German Wadden Sea are new phenomena, indicating a major ecological change, or natural periodic events highlighted by growing ecological awareness and monitoring. Few studies have been published on the ecological consequences of anoxia, low redox potentials and high sulphide concentrations in Black Spots but the effects on the macrofauna [31] and the chemical balance of the sediments seem to be pronounced and long lasting [52,53]. This is the first study on the microbial properties of Black Spot sediment systems but viable cell numbers and aerobic community carbon source utilisation data suggest a profound change in the functional diversity of aerobic microbial communities that may be an indication of the state of the whole ecosystem.

#### Acknowledgements

We would like to acknowledge the contribution of Dipl. Biol. C. Becker, for assistance with bacterial enumeration. This study was partly supported by the German Environmental Ministry (UBA Grant 10802085/21).

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