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ORIGINAL ARTICLE

Time- and sediment depth-related variations in bacterial diversity and community structure in subtidal sands

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Bacterial community structure and microbial activity were determined together with a large number of contextual environmental parameters over 2 years in subtidal sands of the German Wadden Sea in order to identify the main factors shaping microbial community structure and activity in this habitat. Seasonal changes in temperature were directly reflected in bacterial activities and total community respiration, but could not explain variations in the community structure. Strong sediment depth-related patterns were observed for bacterial abundances, carbon production rates and extracellular enzymatic activities. Bacterial community structure also showed a clear vertical variation with higher operational taxonomic unit (OTU) numbers at 10–15 cm depth than in the top 10 cm, probably because of the decreasing disturbance by hydrodynamic forces with sediment depth. The depth-related variations in bacterial community structure could be attributed to vertical changes in bacterial abundances, chlorophyll *a* and NO_3^-, indicating that spatial patterns of microbes are partially environmentally controlled. Time was the most important single factor affecting microbial community structure with an OTU replacement of up to 47% over 2 years and a contribution of 34% to the total variation. A large part of this variation was not related to any environmental parameters, suggesting that temporal variations in bacterial community structure are caused by yet unknown environmental drivers and/or by stochastic events in coastal sand habitats. Principal ecosystem functions such as benthic oxygen consumption and extracellular hydrolysis of organic matter were, however, at a high level at all times, indicating functional redundancy in the microbial communities.
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Introduction

The coastal seas represent large and economically valuable ecosystems directly affected by global change, including overfishing and other types of resource exploitation, eutrophication, pollution, coastal development and introduction of alien species (Essink *et al.*, 2005; DFO, 2006). Increasing scientific effort concentrates on the analysis of natural and man-made changes in biodiversity of animals and plants of coastal seas, but little is known about temporal and spatial variations of microbial communities that metabolize a major share of deposited and locally produced organic matter. Important questions remain as to the link between environmental disturbances and patterns in microbial diversity or activity, and as to their ultimate effects on ecosystem function.

Several studies have been conducted to determine the link between bacterial community structure and composition with environmental parameters in benthic habitats. Sediment depth-related patterns in bacterial community structure were, for instance, detected for a variety of benthic habitats, such as cold seep sediments (Inagaki *et al.*, 2002), the warm deep Mediterranean sea (Luna *et al.*, 2004), the western Pacific coast (Urakawa *et al.* 2000), the Antarctic continental shelves (Bowman and McCuaig, 2003), coral reef sediments (Hewson and Fuhrman, 2006), as well as for continental shelf sediments of the southern North Sea (Franco *et al.*, 2007).

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Environmental parameters such as wave impacts (Hewson and Fuhrman, 2006), organic carbon content and chlorophyll *a* in the sediment (Polymenakou *et al.*, 2005), sediment type (Franco *et al.*, 2007), enzyme (alkaline phosphatase) activities and sediment water content (Hewson *et al.*, 2007), eutrophication associated with fish farms (Vezzulli *et al.*, 2002) and inorganic nutrients enrichment (Hewson *et al.*, 2003) have been correlated to shifts in benthic bacterial community structure. All benthic studies to date have, however, included a comparably low number of environmental parameters and generally did not consider temporal dynamics of the highly complex coastal ecosystem. To our knowledge, only one recent study has treated seasonal changes in bacterial community composition in the coastal sandy sediments (Musat *et al.*, 2006). This study, however, used fluorescent *in situ* hybridization and RNA slot-blot hybridization instead of high-throughput fingerprinting techniques, and did not attempt to link changes in bacterial community composition to ecosystem dynamics.

We undertook an intensive field study over 2 years on a shallow subtidal sand flat in the German Wadden Sea, characterized by strong hydrodynamic forces of waves and tides, strong storm impacts and considerable seasonal changes in temperature and light availability. Earlier, we had investigated the temporal and vertical variations in microbial activities and carbon turnover at this field site (Böer *et al.*, 2008). Here, we determined the bacterial community structure by automated ribosomal intergenic spacer analysis (ARISA). We have analyzed these diversity data using 22 different contextual environmental parameters to answer the following questions: (1) Are there depth-related patterns in microbial activities and community structure despite the strong physical forces (for example, tidal currents and wind induced waves), and if so, are they the same for microbial activities and community structure? (2) Are the strong seasonal dynamics in temperature and productivity also reflected in microbial activities and community structure? (3) Which environmental parameters correlate best with depth-related patterns and temporal changes in microbial community structure?

Materials and methods

Study site and sample collection

The study was conducted on a shallow subtidal sand flat in the Sylt-Rømø Basin in the North Frisian Wadden Sea ('*Hausstrand*', List on Sylt) (55°00′47.7″N, 008°25′59.3″E; Supplementary Figure S1). The tidal amplitude at the site is ~2 m with a water depth ranging from 0.5–2.5 m. Sediments consist of sands with homogeneous grain sizes (mean diameter 350 μm). Ten push cores (3.6 cm inner diameter, 15–20 cm length) were collected in August 2004, October 2004, February 2005, April 2005, July 2005, November 2005, and on 1 March 2006 (March I 2006) and 27 March 2006 (March II 2006) during low tide. Pooled sediment from sectioned cores (0–5, 5–10 and 10–15 cm) was used for molecular and biogeochemical analyses. Four additional cores (8.4 cm inner diameter, 30 cm length) were collected to determine benthic oxygen consumption rates. In November 2005, these cores could not be recovered because of stormy weather and high water level. For a detailed description of the sample treatment see the supplementary information.

Water column data consisting of chlorophyll *a*, pH and temperature were obtained from the Sylt time series (for example, Martens and van Beusekom, 2008). Wind speed data were obtained from the Deutsche Wetterdienst (German Weather Bureau) and derived from the weather station in List on Sylt.

Biogeochemical analyses, bacterial cell counts and activities, benthic oxygen consumption

For nutrient analyses, pore water was blown out with nitrogen (Billerbeck *et al.*, 2006). Pore-water samples were passed through 0.45-μm nylon syringe filters and stored at 4 °C (dissolved silicate, salinity) or −20 °C (NH_4^+, PO_4^{3-}, NO_3^- and NO_2^-), respectively, before the analyses. Dissolved silicate was photometrically measured according to Grasshoff *et al.* (1983). NH_4^+, PO_4^{3-}, NO_3^- and NO_2^- were spectrophotometrically measured with a continuous-flow analyzer (Bran & Lübbe GmbH, Norderstedt, Germany) using a variant of the method of Grasshoff *et al.* (1983). Pore water salinity was measured with a hand refractometer (Reichert Scientific Instruments Co., Depew, NY, USA). Chlorophyll *a* and phaeophytin concentrations, as well as total and EDTA-extractable carbohydrates were analyzed as described in Böer *et al.* (2008). The same reference describes the procedures for measurement of bacterial abundances, bacterial carbon production rates, potential extracellular enzymatic activities and benthic oxygen consumption.

Community structure analysis by automated rRNA intergenic spacer analysis

DNA was extracted from 1 g of homogenized sediment sample using the UltraClean soil DNA isolation kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's instructions, but involving 2 × 45-s speed bead beating with a FastPrep Instrument (Q-BIO gene, Morgan Irvine, CA, USA). Changes in bacterial community structure were estimated by ARISA (Fisher and Triplett, 1999). PCR reactions were conducted in triplicates and PCR-amplified fragments were discriminated by capillary electrophoresis after purification with Sephadex G-50 Superfine (Sigma Aldrich, Munich, Germany). ARISA profiles were analyzed using the GeneMapper Software v 3.7 (Applied Biosystems,

Carlsbad, CA, USA). The total peak area per sample was normalized to one, and only fragments above a threshold of 50 fluorescence units and between 100–1000 bp length were considered to account for size calling imprecision (Supplementary Figure S2). The number of peaks >1000 bp was negligible in our samples (Supplementary Figure S3). GeneMapper output files were reformatted using custom Perl scripts and further analyzed by custom R scripts (Ramette, 2009). A 'fixed window' binning strategy with a bin size of 2 bp was applied to the ARISA generated data, and the binning frame that offered the highest pairwise similarities among samples was subjected to multivariate analyses. Detailed descriptions of the ARISA protocol, capillary electrophoresis analysis, and further peak calling and quality check are provided as supplementary information.

Statistical analyses

The consensus ARISA table (samples by operational taxonomic unit (OTUs)) was used to calculate pairwise similarities among samples based on the Bray–Curtis similarity index. The resulting matrix was examined for seasonal and spatial (depth) patterns in bacterial community structure by using non-metric multidimensional scaling as implemented in the R package *vegan*. Analysis of similarity (ANOSIM) was further carried out to test for significant differences between *a posteriori* sample groupings. Multiple linear correlations between all environmental and biological parameters were calculated by using the Pearson's correlation coefficient for the complete dataset ($n = 24$), and their significances were adjusted for multiple comparisons by the Bonferroni method (for example, Ramette, 2007). The ARISA table was first subjected to principal component analysis to extract uncorrelated axes of variation. The first four principal component analysis axes, explaining ~80% of the overall variation, were retained and *z*-transformed (Ramette, 2007) before carrying out the correlation analyses.

The relationships between environmental variables and patterns in bacterial community structure were further examined by multivariate analyses using the software packages CANOCO (v4.5; ter Braak and Šmilauer, 2002) and R, as follows. Detrended correspondence analysis (Hill and Gauch, 1980) was carried out to determine whether linear or unimodal species models better fitted the current ARISA dataset (Ramette, 2007). Canonical redundancy analysis (RDA) was used to test which environmental parameters significantly explained the variation in bacterial community structure. In RDA models, sampling dates were set as nominal variables and most of the data (except depth, pH, water temperature, wind speed and salinity) were $\log_{10}$-transformed before the analysis in order to normalize their distribution. Significant variables for the analysis were pre-selected from groups of variables (sampling time, depth, nutrients, photopigments, chemical parameters, physical parameters, water column data, enzymatic activities, bacterial abundances and growth) by forward model selection. The significance of the RDA models and of the selected variables were determined by 999 Monte Carlo permutations at $P < 0.05$ for each group. The respective effects of variables or groups of variables on the variation in bacterial community structure were further investigated by canonical variation partitioning (Legendre and Legendre, 1998; Ramette and Tiedje, 2007a).

Results

Seasonal variation in the water column and benthos at 'Hausstrand'

Daily average water temperatures showed clear seasonal patterns with the highest values in summer (21 °C) and the lowest values at the end of winter (−2 °C; Figure 1a). Daily average wind speed ranged from 1.9 m s^{-1} to 16.5 m s^{-1} and was generally higher in winter compared with summer (data not shown). The spring blooms followed the first temperature increases in March. Daily average chlorophyll *a* concentrations in the water column reached 22 μg l^{-1} during the diatom spring bloom of March 2005 (33 μg l^{-1} in March/April 2006), followed by a *Phaeocystis* bloom in May/June with concentrations of up to 21 μg l^{-1}; a common succession for the List tidal basin (Loebl *et al.*, 2007). Chlorophyll *a* concentrations during the rest of the year were on an average 4.2 μg l^{-1}.

Total integrated benthic chlorophyll *a* concentrations (Figure 1b and Supplementary Table S1) varied much less than pelagic chlorophyll *a*. Maximum values were reached during times of the phytoplankton spring bloom (6.8–7.5 mg l^{-1}). Lowest chlorophyll *a* concentrations were detected in August 2004 and on 1 March 2006 (4.5 mg l^{-1}). Benthic total carbohydrate inventory did not follow cyclical patterns but declined between August 2004 (1180 mg gluc. equiv. per l) and March 2006 (~530 mg gluc. equiv. per l). Total community respiration was closely related to temperature and varied between a high of 21–29 mmol C m^{-2} d^{-1} in summer to a low of ~5.5 mmol C m^{-2} d^{-1} in winter. Interstitial NO_3^- (Supplementary Figure S4a) was highest just before and during the pelagic spring bloom (43–57 μM on average in the top 0–5 cm) and was depleted during summer and autumn. Interstitial PO_4^{3-} was variable but showed enhanced concentrations in summer and lows during winter and spring bloom events.

Extracellular enzymatic activities and bacterial carbon production showed strong temporal variations in all three depth layers that were mostly temperature related (Figures 1c–e and Supplementary Table S2). Maximum bacterial secondary production was reached in summer (2.3 mmol C l^{-1} d^{-1}

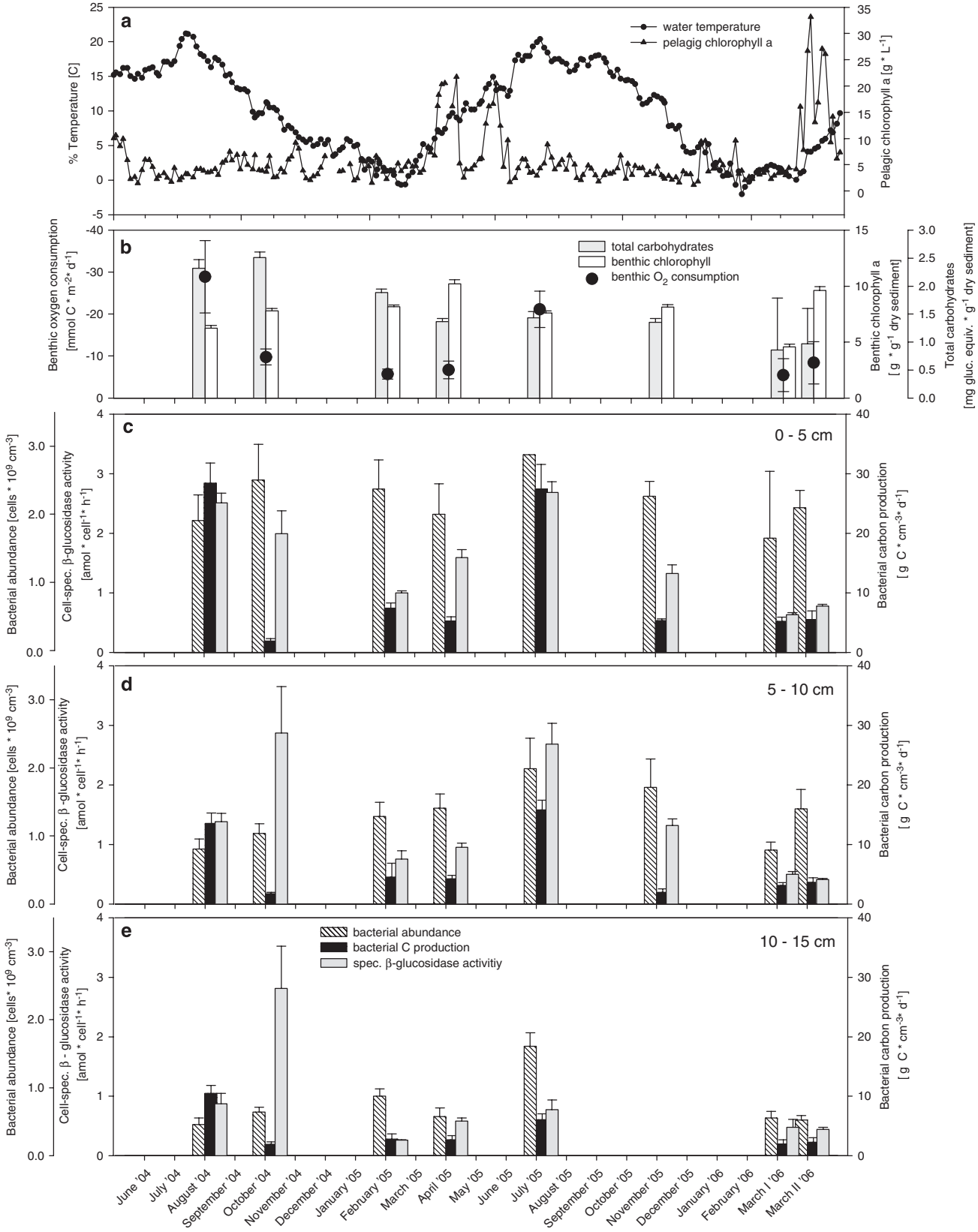

Figure 1 Seasonal patterns of major environmental variables at the sampling site. Error bars represent s.d. (**a**) Pelagic chlorophyll *a* concentrations (triangles) and water temperature (circles). (**b**) Mean benthic chlorophyll *a* concentrations integrated over the upper 5 cm of sediment (dotted bars) and benthic oxygen consumption rates (circles, $n = 4$). Bacterial abundances (black bars, $n = 6$), bacterial carbon production rates as measured by thymidine incorporation (striped bars, $n = 4$–5) and cell-specific β-glucosidase activities (gray bars, $n = 5$) for sediment horizons 0–5 cm (**c**), 5–10 cm (**d**) and 10–15 cm (**e**). Panels a and b were adapted from Böer *et al.*, 2008.

at 0–5 cm), a low was detected in October 2004 (0.17 mmol $C\,l^{-1}\,d^{-1}$), whereas in all other months rates were ~0.42–0.58 mmol $C\,l^{-1}\,d^{-1}$. Hydrolysis rates of extracellular enzymes acting on carbohydrates (α-, β-glucosidase, chitobiase) also varied three–sixfold between summer and winter months (0–5 cm). Total bacterial abundances showed only very weak temporal variations (Figures 1c–e).

Sediment depth-related variation in environmental parameters

NO_3^- concentrations were highest at 0–5 cm sediment depth and decreased rapidly below (Supplementary Figure S4a and Supplementary Table S1). PO_4^{3-} (Supplementary Figure S4b) and NO_2^- followed comparable depth patterns with highest concentrations of 13.7 μM and 4.5 μM in the upper 3–7 cm of sediment, respectively. NH_4^+ concentrations reached a peak of 38–62 μM on an average at −6 cm below sediment surface without any apparent seasonal trend.

Briefly, the sediment showed persistent vertical gradients in bacterial abundances, bacterial carbon production and extracellular enzymatic activities at all times (Figures 1c–e and Supplementary Table S2). On average, values decreased 1.4–1.8-fold from the 0–5 cm layer to the 5–10 cm layer, and by 2.3–3.8-fold compared with the 10–15 cm layer (except for lipase activity). In some cases, especially for lipase and chitobiase, highest enzymatic activities were recorded in the 5–10 cm layer.

Variation in bacterial OTU number and community structure

A total pool of 376 OTUs (corresponding to binned ARISA peaks) were detected for the entire range of sampling months and depths. The highest OTU number was obtained in August 2004 with 302 OTUs and the lowest OTU numbers occurred in fall (195 and 178 OTUs for October 2004 and November 2005, respectively; Figure 2). Ninety-seven OTUs (~26% of all OTUs) were discovered throughout the entire 2-year sampling period. Thereof, 21 OTUs (~6% of all OTUs) were common to the entire depth range. Pairwise comparison of presence/absence of OTUs showed between 53 to 95% shared OTUs between two sampling dates (Supplementary Table S3). Thereby, an average of 57% of OTUs discovered in August 2004 could be recovered in any of the other sampling months and an average of 80% of OTUs when any other sampling dates were compared. Strongest bacterial community dynamics were observed between August 2004, October 2004 and February 2005 with a loss of 47% between August and October, and a loss of 38% between October and February. All other pairs of sampling dates had at least 76% of OTUs in common (Figure 2). Approximately 25% of the entire OTU pool was exclusively detected within the first three

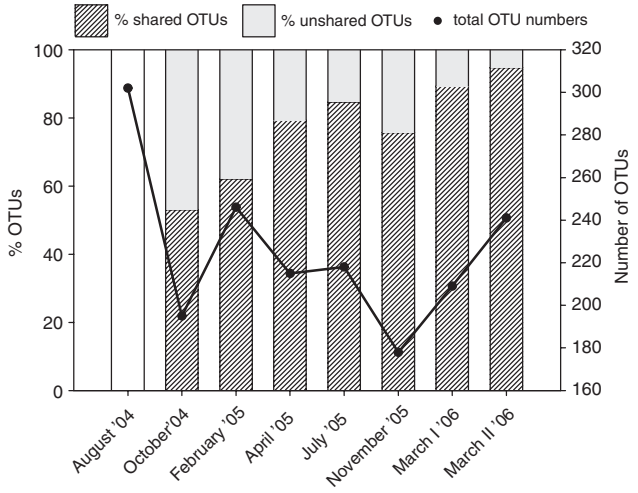

Figure 2 Operational taxonomic unit (OTU) turnover over successive sampling times. The solid line represents the total number of OTUs obtained from automated ribosomal intergenic spacer analysis (ARISA) profiles from all three sediment horizons combined. Bars show which percentages of OTUs in any of the investigated months are shared (striped) or not shared (gray) with the corresponding earlier sampling date.

sampling months, whereas community structure in general was temporally more stable between April 2005 and March II 2006. Half of the 284 OTUs that were detected between April 2005 and March II 2006 were found throughout these five sampling dates.

Operational taxonomic unit numbers varied over sampling depth, with the highest number of ARISA bands in the 10–15 cm sediment layer (178 ± 28.7) compared with the 0–5 cm (145 ± 45.7) or the 5–10 cm sediment layer (144 ± 28.8; Supplementary Figure S5). The difference in OTU numbers between the 0–5 cm layer and the 10–15 cm layer was marginally significant ($P = 0.05$; Student's *t*-test).

Main correlations between environmental parameters and bacterial diversity

Considering all samples ($n = 24$), the environmental variables comprised time, sampling depth, average wind speed on the day before sampling, benthic chlorophyll *a*, the ratio of benthic chlorophyll *a*: phaeophytin, sedimentary SiO_2, PO_4^{3-}, NO_2^-, NO_3^- and NH_4^+, salinity, water pH and temperature, bacterial cell abundance, bacterial carbon production as well as all enzymatic activities (results of correlation analyses are provided in Supplementary Table S4 and Figure 3). Briefly, amongst the environmental parameters, bacterial abundance, benthic chlorophyll *a* and the chlorophyll *a*: phaeophytin ratio exhibited a negative relationship with sediment depth (−0.85, −0.97 and −0.75, respectively) and were positively correlated to each other ($r \geq 0.71$). NH_4^+ was positively correlated to sediment depth ($r = 0.71$). Sampling time was negatively correlated with pH, salinity and PO_4^{3-}

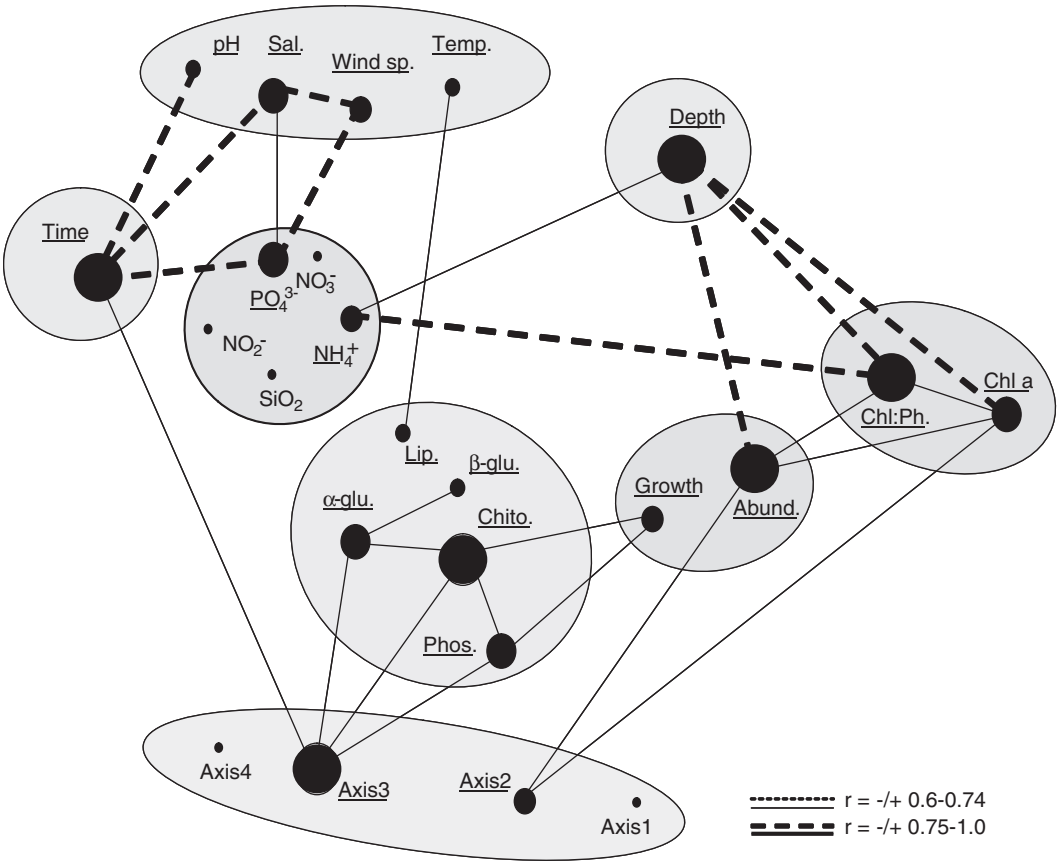

Figure 3 Correlations between temporal, spatial, environmental and microbial variables measured at all eight sampling dates between August 2004 and March 2006. Only significant Pearson's correlations at the Bonferroni-corrected level $P \leqslant 0.0001$ (0.05/465) are depicted. Thin and large connecting lines represent significant correlations with coefficients <0.75 and $\geqslant 0.75$, respectively, whereas continuous and dashed lines represent positive and negative coefficients, respectively. The size of the dots is proportional to the number of significant correlations a respective variable has with other parameters. The variables include: time (all sampling months), depth, wind speed (Wind sp.), salinity (Sal.), temperature (Temp.), pH, NO_2^-, NO_3^-, NH_4^+, PO_4^{3-}, SiO_2, lipase (Lip.), β-glucosidase (β-glu.), phosphatase (Phos.), chitobiase (Chito.), α-glucosidase (α-glu.; all enzymatic activities refer to cell-specific enzymatic activities), bacterial abundance (Abund.), bacterial carbon production (growth), benthic chlorophyll *a* (Chl a), chlorophyll *a*:phaeophytin ratio in the sediment (Chl:Ph), and the four major axes of the principal component analysis that explain variation in the bacterial community structure (Axis 1, Axis 2, Axis 3 and Axis 4).

($r = -0.77$, -0.82 and -0.85, respectively). Bacterial carbon production was significantly correlated with phosphatase and chitobiase activity ($r = 0.78$ and 0.74, respectively). The model was recalculated for samples from which total carbohydrates and EDTA-extractable carbohydrates were measured ($n = 20$). There were no significant correlations between EDTA extractable carbohydrates and total carbohydrates with any of the other variables, following Bonferroni corrections of the significance level. At a significance level of $P < 0.01$, EDTA-extractable carbohydrates and total carbohydrates showed strong correlations with sediment depth and variations in bacterial community structure (see below). In addition, EDTA-extractable carbohydrates were correlated with chlorophyll *a* and sedimentary NO_3^-, whereas total carbohydrates were correlated with bacterial abundance (data not shown).

Variation in bacterial community structure as extracted by principal component analysis consisted of four major axes explaining 38, 29, 8 and 4% of the total variation in ARISA data, respectively, which were included in correlation analyses. Variation in bacterial community structure was significantly correlated with sampling time ($r = 0.76$), bacterial abundance ($r = 0.75$), benthic chlorophyll *a* ($r = 0.78$), chitobiase activity ($r = 0.74$), α-glucosidase activity ($r = 0.77$) and phosphatase activity ($r = 0.74$).

In order to further determine the environmental variables associated with changes in bacterial community structure, an RDA was applied to those contextual parameters that significantly explained variation in the ARISA table in the forward model selection (time, depth, chlorophyll *a*, NO_3^-, wind speed and bacterial abundance; Table 1). It yielded two main axes that explained 36 and 27%, respectively, of the total variation in bacterial community structure (Figure 4), with the selected environmental variables accounting for 83% of this variance. The major part of the variance was explained by

Table 1 Conditional effects of forwardly selected environmental parameters as determined by RDA

Environmental variable[a]	*Lambda-A*[b]	F-*ratio*
August	0.17	4.35**
October	0.16	5.25**
February	0.13	4.90***
April	0.03	1.10^{NS}
November	0.03	1.00^{NS}
July	0.01	0.44^{NS}
March I	0.01	0.28^{NS}
Depth	0.23	6.60***
NO_3	0.14	3.56**
SiO_2	0.07	1.90^{NS}
NH_4	0.07	2.07^{NS}
PO_4	0.05	1.38^{NS}
NO_2	0.02	0.39^{NS}
Chlorophyll *a*	0.26	7.63***
Chl *a*/phaeophytin ratio	0.02	0.56^{NS}
Wind speed	0.1	2.57*
PH	0.07	1.66^{NS}
Salinity	0.08	2.02^{NS}
Temperature	0.04	1.23^{NS}
β-glucosidase	0.09	2.28^{NS}
Lipase	0.06	1.71^{NS}
Phosphatase	0.06	1.77^{NS}
α-glucosidase	0.1	2.32^{NS}
Chitobiase	0.04	1.04^{NS}
Bacterial abundance	0.21	5.90***
Bacterial carbon production	0.05	1.28^{NS}

Abbreviation: RDA: redundancy analysis.
[a]Horizontal lines mark groupings for forward selection.
[b]Lambda-A represents the variance each variable explains in the model. Statistical significance is indicated by ***($P<0.001$), **($P<0.01$), *($P<0.05$), and NS (not significant), as determined by 999 Monte Carlo permutations under the full multivariate models.

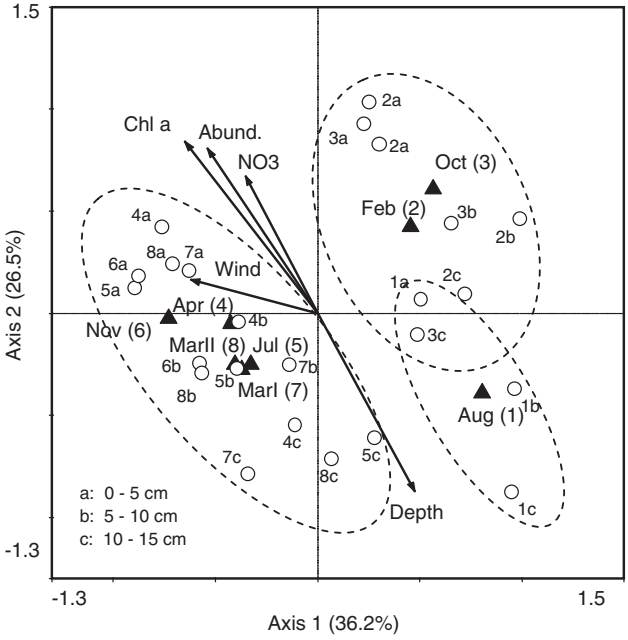

Figure 4 Redundancy analysis (RDA) biplot of bacterial diversity and contextual parameters. Open circles represent consensus automated ribosomal intergenic spacer analysis profiles for all eight sampling dates and the three sediment horizons analyzed ($n=24$). Numbers next to the circles indicate the sampling dates August 2004 (1), October 2004 (2), February 2005 (3), April 2005 (4), July 2005 (5), November 2005 (6), March I 2006 (7) and March II 2006 (8). Letters associated with the numbers correspond to sampling depth 0–5 cm (a), 5–10 cm (b) and 10–15 cm (c). Dotted lines surround groups of sampling months that differed significantly in bacterial community structure. Bacterial community structure within the three groups was not significantly different (Analysis of similarity (ANOSIM)). Sampling months were set as nominal variables in the analyses and are represented as filled triangles. Only environmental variables that significantly explained variability in microbial community structure in the forward selection procedure were fitted to the ordination (arrows).

time and wind speed on the first RDA axis. Axis 2 was mainly associated with depth-related patterns in organic carbon availability and sediment chemistry as reflected by moderate correlations with benthic chlorophyll *a*, bacterial abundance, and NO_3^-.

Samples were found to cluster as a function of depth (0–5 cm, 5–10 cm, 10–15 cm) in the RDA biplot. *A posteriori* grouping in non-metric multidimensional scaling analysis (Supplementary Figure S6a) and further non-parametric tests confirmed that these groups were indeed significantly separated (ANOSIM $R=0.373$, $P<0.001$). The RDA biplot indicated that samples in the 0–5 cm layers were associated with high chlorophyll *a* and NO_3^- concentrations as well as with high cell abundances in contrast with samples from deeper layers for which decreasing values of these environmental parameters were predicted.

Grouping of samples in the RDA plot also indicated the existence of three groups according to sampling dates (Figure 4). Group 1 contained all August 2004 samples, group 2 contained all October 2004 and February 2005 samples and group 3 comprised all remaining samples. The significance of the groupings was independently confirmed by non-metric multidimensional scaling analysis combined with ANOSIM significance testing (Supplementary Figure S6b). The groups were highly separated (ANOSIM $R=0.78$, $P<0.001$), however sampling months within group 2 and 3 turned out to be quite similar (ANOSIM $R\sim0$).

A second RDA (data not shown) was carried out on a subset of samples ($n=20$; four samples from the deepest horizon lacked measures of total and EDTA-extractable carbohydrates), for which wind speed was not retained in the stepwise model selection. Instead, total and EDTA-extractable carbohydrates were included as explanatory variables. The two main RDA axes explained 42 and 26% of the total variance in bacterial community data, respectively. All first four axes explained up to 81% with selected environmental variables in this model accounting for 92% of the total variation. The overall ordination of samples and environmental variables were mostly maintained in the new analysis with RDA axis 1 mainly correlating with sampling date and total carbohydrates and axis 2 mainly explained by

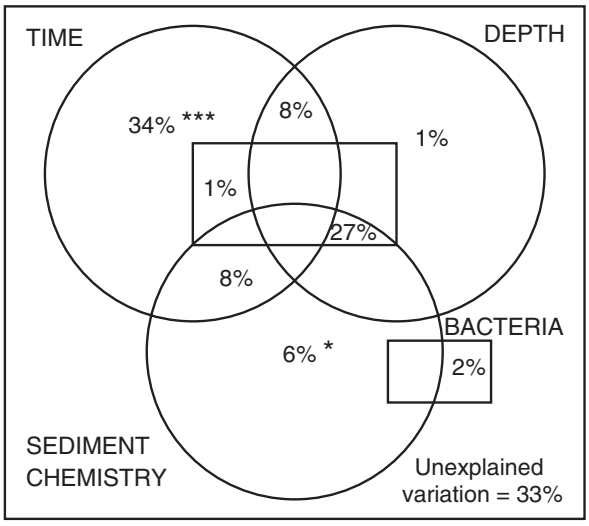

Figure 5 Partitioning of the microbial variation into the relative effects of significant contextual parameters alone or in combination. Statistically significant contributions of pure fractions to automated ribosomal intergenic spacer analysis (ARISA) variation are indicated at $P<0.001$ (***) and $P<0.05$ (*), as determined by 999 Monte Carlo permutations under the full multivariate model.

sediment depth, chlorophyll *a*, NO_3^- EDTA extractable carbohydrates and bacterial abundance.

The respective effects of each factor on the variation of community patterns were disentangled by variation partitioning analysis (Figure 5). The model on the basis of the complete dataset ($n=24$) included the following sets of explanatory variables: time (all seasons), sediment depth, sediment chemistry (chlorophyll *a*, NO_3^-) and bacterial parameters (bacterial abundance). The amount of variation explained by all pure and co-varying factors was 67%. The pure fractions (that is, the fraction explained by a factor when removing the effects of all other factors or variables in the model) contributed 34 (time, $P<0.001$), 1 (depth, non-significant), 6 (sediment chemistry, $P=0.032$) and 2% (bacterial abundance, non-significant) of the variation in bacterial community structure. The combination of sediment depth, bacterial abundance and sediment chemistry had the largest co-varying effect and accounted for 27% of the variation in the community data.

Discussion

In this study we have analyzed whether spatial and temporal variations in the physicochemical conditions of the environment may be associated to changes in bacterial diversity and community structure. A widely accepted hypothesis on the biodiversity of microorganisms is that these are everywhere, but under strong environmental selection (Baas-Becking, 1934). However, several studies have evidenced that microbial communities display temporal and spatial patterns, which are not explained by variations in contemporary environmental parameters, but rather as a result of the community history (reviewed in Martiny *et al.*, 2006; Ramette and Tiedje, 2007b). In the following sections, we discuss whether and how the strong spatial and temporal variations in environmental parameters that are typical for temperate sandy coasts may control bacterial diversity and community structure.

Despite their constant exposure to strong hydrodynamic forces, such as tidal currents and wind-induced wave surge, sandy sediments at *Hausstrand* (List, Sylt) consistently show strong vertical gradients in bacterial abundances, bacterial carbon production and extracellular enzymatic activities (Böer *et al.*, 2008). These gradients were found to be tightly coupled to the distribution of the microphytobenthos, causing a strong vertical shift in both availability and nutritional quality of degradable organic matter in the sediment, reflected in the decrease in chlorophyll *a*, total and EDTA-extractable carbohydrates with sediment depth (Böer *et al.*, 2008). Another important factor in the spatial structuring of sandy sediments is the advective pore water flow induced by currents and waves, which at least temporarily oxygenates the uppermost sediment layers, and thus influences the redox state of the sediment.

Extracellular enzymatic activities, bacterial carbon production and benthic community respiration showed strong temporal variations that were mostly related to seasonal temperature change, whereas they were barely reflected in microphytobenthic or bacterial biomass (Böer *et al.*, 2008). It is to be expected that other factors than temperature, for example substrate availability (Sander and Kalff, 1993), grazing (Epstein, 1997) and viral lysis (Fuhrman, 1999), may add to the dynamics of the microbial community in sandy sediments.

In the following sections we discuss (1) how depth-related patterns in bacterial OTU numbers and community structure may be explained by variation in specific environmental parameters; (2) how temporal changes in microbial activity and biomass may be linked to variations in bacterial OTU numbers and community structure; and (3) which environmental parameters may better explain those temporal changes in microbial community structure.

Effects on OTU numbers

It is generally assumed that energy availability, niche variation and the number of food sources are higher at the surface seafloor than in deeper sediment layers, as a result of the presence of protozoans, meio- and macrofauna and the availability of a large variety of different organic resources (labile and refractory, of floral, faunal, microbial or terrestrial origin) and electron acceptors. One would thus expect OTU numbers to be

higher in shallow sediment depths compared with deeper sediment layers. However, in the *Hausstrand* sands, OTU numbers were higher in the deeper layer (10–15 cm) as compared with the mid (5–10 cm) and upper (0–5 cm) layers (Supplementary Figure S5). Increased OTU numbers with depth have been described earlier for western Pacific, southern North Sea, as well as for Namibian coastal sediments, using various molecular techniques (for example, Urakawa *et al.*, 2000; Franco *et al.*, 2007; Julies, unpublished data).

We hypothesize that the relationship between intermediate disturbance and diversity, which has been shown for a variety of organisms (for example, Connell, 1978; Townsend *et al.*, 1997), but not yet tested on benthic microorganisms, might explain the apparent link between sediment depth and bacterial OTU numbers at our study site. Indeed, strong hydrodynamic forces at the field site lead to a constant vertical and horizontal mixing of the upper five centimeters of sediment (Hedtkamp, 2005), probably imposing a strong selective pressure on the microbial community and restricting access to this habitat for a comparably smaller range of bacteria that are able to cope with occasional resuspension, physical abrasion generated by moving sediment particles, grazing, or rapidly fluctuating concentrations of oxygen and of nutrients (Supplementary Table S1, Figure 1 and Supplementary Figure S4). The high sediment permeability observed at the field site ($1.0–3.4 \times 10^{-11}$ m^2; Hedtkamp, 2005) suggests that advection is the major transport process in the upper 10 cm of sediment (Huettel *et al.*, 2003), as reflected in the pore water nutrient profiles. In contrast, the deeper, poorly mixed sediment layers provide a relatively stable anoxic habitat of intermediate disturbance. Owing to the lack of homogenization in deeper sediment layers, potential ecological niches should also have a higher temporal stability. These layers would thus sustain a high microbial diversity because microbes in these layers have more time to actually occupy their respective niches. In contrast, the microbes in shallower sediment depths are subject to fast changing conditions that would more frequently destroy potential niches and thus reduce diversity. It can be expected that OTU numbers would decrease with further increasing sediment depth where disturbance is entirely lacking, transport processes are limited to molecular diffusion and energy becomes limited, as observed earlier by Urakawa *et al.* (2000).

Additionally or alternatively, high nutrient availability has been described to reduce bacterial diversity (Torsvik *et al.* 1998; Hewson *et al.* 2003), presumably because of an increase in competitive exclusion (Abrams, 1995). At *Hausstrand*, OTU numbers were negatively linked to microphytobenthic biomass, bacterial abundance and activity. The high availability of inorganic nutrients and of labile organic matter produced by the microphytobenthos may have favored fast growth of presumably oxygen-tolerant, microaerophilic generalists in the upper sediment layers at our study, whereas below lack of mixing may have supported a more heterogeneous microbial community of potentially slow-growing, anaerobic taxa with specific metabolic capabilities. We suggest that the vertical increase in physical and chemical stability in combination with the decrease in mortality factors and competitive exclusion (that is, a decrease of selective forces) leads to higher diversity in deeper sediment layers at *Hausstrand*.

As to the effects of depth on dynamics in community structure, it is interesting to note that about 40% of all OTUs were found to inhabit the entire depth profile. These OTUs probably represent bacterial taxa that are able to use a wide range of organic substrates and electron acceptors for active growth and may tolerate aerobic as well as anaerobic conditions. In contrast, on average per sampling time, 13, 3 and 20% of OTUs were uniquely found in the upper, medium and deepest layer, respectively. The medium and upper layer shared 7% and the medium and deepest layer shared 12% of OTUs. These OTUs most likely represented bacteria that were restricted to the specific biological, chemical and physical conditions prevailing in these sediment horizons. Integrated over the complete sampling cycle, only 5, 2 and 6% of the entire pool of OTUs were unique to the upper, medium and deepest layer, respectively. This shows a highly dynamic bacterial community over time, most likely influenced by sediment mixing and subsequent shifts in oxygen and chemical gradients.

As to the temporal variation of bacterial diversity, OTU numbers generally varied between 178 and 246 per sampling date. Only few (0–8) OTUs were unique to any of these dates (except for August 2004 with 65 unique OTUs). Compared with other ARISA studies, these numbers match well the range of shallow water benthic bacterial diversity ($\sim$236 OTUs; Hewson *et al.*, 2003) and are higher than those in coral reef surface sediments (51–148 OTUs; Hewson and Fuhrman, 2006). Out of the 376 OTUs identified over the entire 2-year sampling period, between 47–80% of OTUs were detected at individual sampling dates, showing substantial dynamics in OTU numbers in the top 15 cm of sediment (Figure 2). Only 26% of all OTUs were temporally stable and found at all sampling months.

Environmental factors associated with depth- and time-related changes in bacterial community structure

Both RDA and variation partitioning assigned the detected depth-related variations in diversity to vertical changes in organic carbon availability and redox potentials (Figures 4 and 5). Accordingly, benthic chlorophyll *a*, bacterial abundances, and NO_3^- were all strongly negatively correlated with depth. NO_3^- can be seen as an indicator of the redox

state of the habitat. Vertical pore water nutrient profiles at *Hausstrand* show that the zone of 0–5 cm depth is flushed with bottom water (Supplementary Figures 4), and hence at least temporarily oxygenated, which may select for aerobic and oxygen-tolerant species forming rigid biofilms on the sand grains, whereas the deeper layers represent relatively stable anoxic environments. Furthermore, the distribution of microphytobenthic productivity strongly affects the spatial structuring of the bacterial community in the subtidal sands. Microphytobenthic algae have been shown to channel a major fraction of their total primary production into the synthesis of extracellular polymeric substances and thus provide a significant source of oxygen and organic matter for benthic bacterial growth (for example, Underwood and Kromkamp, 1999; de Brouwer and Stal, 2001). Particularly in dynamic, sandy areas such as *Hausstrand*, benthic autotrophic and heterotrophic processes are often tightly coupled (Middelburg *et al.*, 2000). Depth-related decreases in chlorophyll *a* concentrations, bacterial abundances, growth rates and extracellular enzymatic activities indicate vertical shifts in both the quality and the nutritional quality of organic matter in the sediment (Böer *et al.*, 2008), which have been shown earlier to influence bacterial community structure (Muylaert *et al.*, 2002; Crump *et al.*, 2003). Our results suggest that depth-related variation of the bacterial community structure may be considerably controlled by environmental factors such as these shifts in organic carbon availability.

Multivariate analyses also showed that the respective effect of time alone could significantly explain up to 34% of the strong temporal variation in bacterial community structure that we observed over the 2-year period (Figure 5). No single other contextual parameter, or combinations thereof, could explain the temporal variance. The temporal changes in bacterial community structure did not exhibit cyclical patterns as could have been foreseen from the substantial seasonal changes in temperature and substrate availability at the site (Figure 1 and Supplementary Figure S4) and from the earlier investigations of bacterioplankton diversity (Fuhrman *et al.*, 2006). The variable part of the bacterial community was neither gradually renewed, nor could specific OTUs be exclusively associated with certain seasons, but OTUs tended to be above or below detection limit over time without any apparent predictable patterns. Community dynamics were found to be stronger between the first three sampling dates than towards the end of the experiment (Figure 2). It thus seems that the bacterial community underwent a development from more dynamic towards more stable structures in the course of the study. None of the contextual parameters clearly reflected this development, which suggests that yet unknown events before August 2004 may have had long lasting effects on bacterial community structure. The only unusual observation in the Sylt time series was hot summers in the period 2002–2004 with August temperatures of 1–3 °C above the long-term average (Martens and van Beusekom, 2008), however the overall effects of such events on the ecosystem are unknown. It thus remains interesting to determine whether and to which extent bacterial community dynamics in coastal sands may be influenced by yet unmeasured environmental parameters and by stochastic events.

Hypothetically, interspecific competition between different trophic members of the bacterial community may have led to changes in bacterial community structure that were independent from external drivers. However, we cannot exclude that important ecological factors were overlooked. For instance, dissolved organic matter from different algal sources may select for different bacteria in the course of seasonal phytoplankton succession, (Pinhassi *et al.*, 2004; Abell and Bowman, 2005), potentially also in the benthos. Relatively weak temporal changes in MPB and bacterial biomass as compared with strong seasonal changes in microbial activities (particularly in August 2004) suggest that grazing pressure and/or viral lysis may have controlled microbial standing stocks at *Hausstrand*, and these two factors have been shown to affect aquatic bacterial community structures as well (for example, Hahn and Höfle, 2001; Weinbauer and Rassoulzadegan, 2004). Although the strong community dynamics among the first three sampling months were linked to high MPB-related carbohydrate concentrations (data not shown), potential strong short-term variations in carbohydrates (de Brouwer and Stal, 2001) make it difficult to interpret this relationship. Apparently, unusual (and so far unresolved) physicochemical and biological conditions may lead to strong dynamics in the bacterial community structure in the subtidal sediments, here indicated by a replacement of $>25\%$ of the OTUs within >3 months, followed by a long phase of restabilization.

In conclusion, our study suggests that bacterial communities in coastal sands may be shaped by a combination of deterministic ecological factors and stochastic events. Although vertical distribution patterns were mostly environmentally controlled, a large fraction of temporal dynamics in bacterial community structure was unrelated to our measured environmental parameters and could be attributed to unknown factors, including single events followed by a subsequent stabilization phase in the community structure. It is interesting that, despite the high dynamics in bacterial diversity observed here, general ecosystem functions mediated by bacteria such as organic matter remineralization (oxygen consumption, enzymatic activity) and productivity (bacterial growth) were not affected, indicating high functional redundancy. This study shows that the ecological modeling of high-resolution fingerprinting patterns together with their environmental context is a successful approach to test hypotheses on the causes of changes in microbial diversity and

community structure. Future work is needed to determine the composition of the microbial communities in the subtidal sandy sediments and the taxonomic levels at which environmental control may be observed.

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