Microbial Characterisation of the Sea-Surface Microlayer in the Baltic Sea

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Abstract

The sea-surface microlayer (SML) is located at the air-water interface. Its inhabiting bacterial community (bacterioneuston) encounters strong opposing environmental conditions (e.g. accumulation of organic material, enhanced UV-radiation). Additionally, high spatial and temporal variability of SML properties as well as the difficulties to sample the SML cause large uncertainties in our understanding of the bacterioneuston, and contradictory results of its activity and diversity have been reported so far. In order to get further insights into general patterns of the bacterioneuston and its regulating factors, SML samples were taken throughout three years in the Baltic Sea and concentrations of organic material, bacterial abundance, productivity (^{3}H-thymidine -incorporation) as well as diversity (16S rRNA and 16S rRNA gene fingerprints) were studied and compared to the underlying bulk water (ULW).

Initially, a comparative approach to test available SML sampling techniques was performed to assure a reliable analysis. Eventually, a glass plate sampling device was established, which did not introduce any bias to the bacterial parameters of interest. Using this sampler, the SML was found to be generally enriched in organic material, which however, did not fuel bacterial activity as the proportion of highly active (CTC-positive) cells was comparable to the ULW and bacterial productivity was even reduced by 50 to 80 % in the SML. This reduction was constantly observed and not related to changing levels of meteorological forcing (wind speed, radiation), which influence physico-chemical properties of the SML.

In contrast, during low wind speed and enhanced radiation, the bacterioneuston community composition of the non-attached (NA) size fraction was increasingly different from the bacterioplankton community composition, especially in the 16S rRNA fingerprints, i.e. the active parts of the communities. Overall highly similar communities in the SML and ULW were observed in the NA size fraction. Contrarily, the similarities of the particle-attached (PA) communities in the SML and ULW were highly variable and these changes were only related to wind speed. In both size fractions, active bacterioneuston members, which were exclusively detected in the SML, showed high 16S

rRNA gene sequence similarities to environmental clones from diverse habitats, especially from water columns, and could be affiliated to *Cyanobacteria*, *Bacteriodetes* and *Proteobacteria*.

Under very calm wind conditions the formation of an extensive surface film ('slick') was observed. This slick was characterised by a high accumulation of particulate organic material, significant enrichments in bacterioneuston cell numbers, productivity and was accompanied by strong changes in bacterioneuston community structure. In order to further examine the temporal effects of minimized wind influence on bacterial assemblages in the SML, mesocosm experiments were conducted in a marina to artificially calm the sea-surface. Here, a similar pattern of changes in bacterioneuston parameters as during slick formation was observed and strong relations between particulate organic material and bacterioneuston abundance and activity were found.

These results imply that the SML is generally not a favorable habitat for bacteria, albeit the accumulation of organic material. The bacterioneuston is strongly influenced by the ULW and unlikely resembles a specifically adapted community in the Baltic Sea. However, under specific conditions (fresh organic material, low wind speed), the SML undergoes a succession and an uncoupling of the bacterioneuston community from the ULW results.

Zusammenfassung

Der Oberflächenfilm aquatischer Habitate (SML – sea-surface microlayer) befindet sich an der Luft-Wasser Grenzschicht. Die bakterielle Gemeinschaft dieses Oberflächenfilms (Bakterioneuston) ist stark gegensätzlichen Umweltfaktoren ausgesetzt, wie z.B. der Anreicherung organischen Materials und einer erhöhten UV-Strahlung. Aufgrund der immensen räumlichen und zeitlichen Variabilität in der SML sowie der schwierigen Beprobung dieses Habitats, gibt es große Unklarheiten über das allgemeine Verständnis des Bakterioneustons. In der Literatur finden sich dementsprechend widersprüchliche Aussagen zur Aktivität und Diversität bakterieller Gemeinschaften in der SML. Um weitere Einblicke in generelle Muster des Bakterioneustons und dessen regulierende Faktoren zu erhalten, wurde die SML der Ostsee beprobt und die Konzentration von organischem Material sowie die bakterielle Abundanz, Aktivität (^{3}H-Thymidin-Aufnahme) und Diversität (16S rRNA und 16S rRNA-Gen-Analysen) bestimmt und mit denen der darunter liegenden Wassersäule (ULW – underlying bulk water) verglichen.

Zu Beginn der Arbeit wurden vergleichende Studien von verschiedenen SML-Sammelmethoden durchgeführt, um eine verlässliche Analyse zu gewährleisten. Letztlich konnte ein Glassplattensammler etabliert werden, der keine selektiven Fehler während der Analyse der untersuchten bakteriellen Parameter verursachte. Die mit dieser Methode gewonnenen SML-Proben zeigten eine Anreicherung von organischem Material, was jedoch keinen positiven Einfluss auf die bakterielle Aktivität hatte, da der Anteil sehr aktiver Zellen (CTC-positiver Zellen) in der SML vergleichbar zur ULW war. Darüber hinaus war die bakterielle Aktivität in der SML um 50 bis 80 % reduziert. Diese Reduzierung wurde durchgängig in allen Proben beobachtet und war unabhängig vom Einfluss unterschiedlicher Wetterbedingungen (Windgeschwindigkeit, Sonneneinstrahlung), die sich wiederum stark auf die physiko-chemischen Bedingungen in der SML auswirken können.

Im Gegensatz dazu gingen abnehmende Windgeschwindigkeiten und steigende Strahlungen mit stärkeren Unterschieden zwischen den Gemeinschaftsstrukturen des Bakterioneustons und Bakterioplanktons in den nicht-assoziierten (NA) Größenfraktionen einher. Dies war insbesondere in den Analysen der 16S rRNA

fingerprints, d.h. der aktiven Gemeinschaften, evident. In den NA Größenfraktionen wurden generell sehr ähnliche Gemeinschaften zwischen der SML und der ULW festgestellt. In den partikel-assoziierten Größenfraktionen waren die Ähnlichkeiten zwischen der SML and der ULW im Gegensatz dazu sehr variabel und konnten nur durch veränderte Windgeschwindigkeiten erklärt werden. In beiden Größenfraktionen zeigten aktive Organismen des Bakterioneustons, die ausschließlich in der SML detektiert wurden, starke 16S rRNA Sequenzähnlichkeiten zu Umweltklonen aus verschiedenen Habitaten, insbesondere aus der Wassersäule, auf. Diese Sequenzen konnten den phylogenetischen Gruppen der Cyanobakterien, der Bakteriodetes und der Proteobakterien zugeordnet werden.

Aufgrund sehr ruhiger Windbedingungen konnte die Ausbildung eines extrem starken Oberflächenfilms („slick“) beobachtet werden. Dieser slick war durch eine starke Anreicherung von partikulären, organischen Material, gesteigerten Zellzahlen und Aktivitäten sowie drastischen Veränderungen der Gemeinschaftszusammensetzung des Bakterioneustons gekennzeichet. Um die zeitlichen Abläufe der Veränderung der Bakterioneustongemeinschaft unter minimierten Windbedingungen weitergehend zu untersuchen, wurden Mesokosmosexperimente im Yachthafen von Warnemünde durchgeführt, in denen die Wasseroberfläche künstlich beruhigt wurde. In diesen Mesokosmen konnten vergleichbare Reaktionen des Bakterioneustons wie während der slick-Ausbildung beobachtet werden. Insgesamt korrelierte die Anreicherung des partikulären, organischen Materials mit der Zunahme bakterieller Abundanz und Aktivität in der SML.

Diese Resultate implizieren, dass der Oberflächenfilm der Ostsee, trotz der Anreicherung von organischem Material, kein günstiges Habitat für bakterielle Gemeinschaften darstellt. Das Bakterioneuston wird stark durch die ULW beeinflusst und scheint dementsprechend nicht an die Bedingungen der SML adaptiert zu sein. Dennoch durchläuft die SML unter bestimmten Bedingungen (neues organisches Material, geringe Windgeschwindigkeiten) eine Sukzession, in deren Folge das Bakterioneuston sich zunehmend vom Einfluss der ULW loslöst.

Introduction

Prologue

The term 'neuston' was introduced by Einar Naumann in 1917, in order to distinguish organisms inhabiting the 'skin' of small lakes from plankton in the bulk water (Naumann 1917). He described different phylogenetic groups within this 'skin' and mentioned active movement between the two compartments. Furthermore, he proposed a strong impact of meteorology on organisms in the 'skin' and that they developed adaptive mechanisms to counteract these environmental factors. In this pioneering report, Naumann therefore posed the intriguing questions about the specificity of neustonic organisms and their controlling factors, which were continually asked by aquatic biologist thereafter. Despite the ongoing development of analytical methods and the investigations of an increasing number of aquatic habitats, even recently the question was asked, whether a 'successful' (bacterio)neuston community exists (Maki 1993, 2002).

In the following, the physico-chemical properties of the 'skin' (now being termed as the sea-surface microlayer) will be introduced to characterise this unusual habitat and how samples can be retrieved for *ex situ* analysis. An overview about microbiological research in the past will show the existing and lacking knowledge on prokaryotic communities in this habitat, in order to finally illustrate the aims of the present thesis.

The Physico-Chemical Nature of the Air-Water Interface

Structural definition of the air-water interface

The air-water interface represents the boundary between the atmosphere and the hydrosphere. Early investigations identified lipids and fatty acids as main constituents of this interface (Jarvis 1967) and a thin layer of dry and wet surfactants of 0.1 μm thickness was envisioned (Norkrans 1980). However, polysaccharides and proteins (Baier 1972) as

well as high concentrations of polymerized materials, such as humic substances, were additionally found (Hunter & Liss 1981). Therefore, Sieburth replaced this model by the one of a hydrated gel of 1 μm thickness with dominance of dissolved and colloidal material creating a matrix of intertangled molecules (Sieburth 1983). Recent studies support the idea of the SML to be a gelatinous film (Wurl & Holmes 2008, Cunliffe & Murrell 2009). In either model, the existence of several strata was proposed in which the deeper layers are colonized by bacteria and larger organisms (Maki 1993). Due to the different ideas on the structure of the air-water interface and the remaining ambiguity about its thickness, a non-uniform definition of these layers resulted and most studies described them as either 'surface film', 'surface microlayers' or 'surface microlayer'. In the following, the term 'sea-surface microlayer' (SML) will be used for convenience with most of the stated literature. In the present study, this term does not explicitly imply any of the models described above to be more valid, but rather refers to previous comments, that the SML is defined operationally by the methods used to collect the samples (Wangersky 1976, Norkrans 1980, see below).

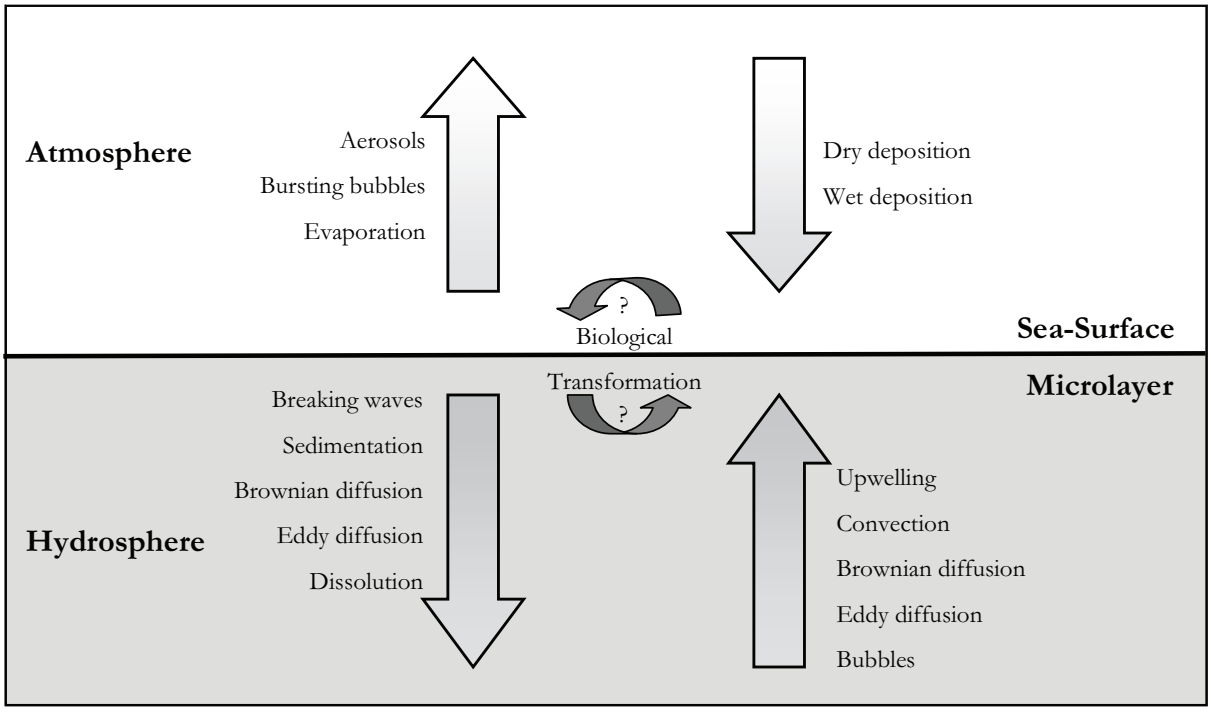

Fig. A Import and export processes influencing material concentrations and properties of the sea-surface microlayer.

Material import and export in the air-water interface

Independent of its structure, the SML is defined as the uppermost top of any water body. Buoyancy, adsorption and surface tension are physical processes, which are responsible for holding material in the interface (Sieburth 1983). However, the SML is a very dynamic system, due to the interplay of meteorological forcing on the sea surface and diverse import- and export processes into and away from the interface (Fig. A). Generally, material in the SML originates from the underlying bulk water (ULW), e.g. by upwelling, convection, diffusion or bubble transport as well as from wet or dry atmospheric deposition, e.g. by dust input or pollination events (Södergren 1987). Material will leave the SML by evaporation and aerosol formation into the atmosphere as well as by dissolution or sedimentation into the ULW. Sedimentation results from e.g. a collapse of the SML due to increasing surface pressure and convergent forces (Wheeler 1975).

Wind is one of the main factors constantly acting upon the sea surface and induces aerosol formation as well as wave breaking. Breaking waves disrupt the SML and induce a transport of material from the SML into the ULW. Furthermore, breaking waves as well as photodegradation of dissolved organic matter cause formation of bubbles in the ULW (Wangersky 1976). Bubbles, which ascend through the water column, collect surface-active compounds, e.g. produced by phytoplankton, as well as particulate material and cause a transport back into the SML (Blanchard 1974). Bursting bubbles will release part of this material into the atmosphere (Bezdek & Carlucci 1972). Therefore, after disruption events, bubbles may speed up the reestablishment of the SML which has been described to be very fast (Hardy 1982). In turn, high concentrations of surface-active compounds modify physical processes in the air-water interface, e.g. capillary wave dampening and bursting bubble phenomena (Garrett 1972). This is especially pronounced in visible surface films (slicks) which are formed during high concentrations of surface-active compounds and thus by a strong reduction of surface tension under low turbulence conditions and are frequently observed in marine systems (Romano 1996). Langmuir circulation, visible through bands with altered reflectance characteristics on the sea surface, acts as a local concentrating mechansism (Wangersky 1976) and additionally causes small scale variations in SML-properties. Taken together, the dynamic interplay of physical processes constantly disrupts and renews the constituents of the SML (Dragcevic

& Pravdic 1981) and transformation processes due to (photo)chemical and biological activity additionally cause high spatial and temporal variability in the SML (see below).

Chemical characterisation of the sea-surface microlayer

Theoretically, any chemical component which is present in the bulk phase of the upper hydrosphere or lower atmosphere may enter the air-water interface. Indeed, extensive research has shown that a broad range of substances accumulate in the SML, which will have either detrimental or stimulating effects on neustonic organisms. The SML is known to contain high concentrations of organic pollutants (Wurl & Obbard 2004), heavy metals (Hardy et al. 1985, Hardy & Cleary 1992), pesticides (Maki 1993) as well as polyaromatic hydrocarbons (Guitart et al. 2007). Inorganic nutrients were also reported to be enriched in the SML (Carlucci et al. 1986, Williams 1986). Nevertheless, most attention has been drawn to organic compounds.

Organic material in the SML derives from both living cells as well as detritus and is a complex mixture of substances (Williams 1986). Hydrocarbons, such as fatty acids (Jarvis 1967) and lipids (Kattner et al. 1985) accumulate in the SML, favoured by the hydrophobic parts of their molecules. Glycoproteins and proteoglycans (Baier 1972), carbohydrates (Williams 1986) as well as amino acids (Carlucci et al. 1991, Kuznetsova & Lee 2002, Reinthaler et al. 2008) are also enriched in the SML. It is well known that the dissolved fraction of organic material is slightly, but consistently enriched in the SML (Hunter 1997). Relative ratios for dissolved organic carbon in the SML compared to the ULW rarely exceed 1.5, i.e. a 50% increase in concentration (Momzikoff et al. 2004, Wurl & Holmes 2008). Significant enrichments of dissolved material in the SML might be inhibited by processes of polymerization (Chin et al. 1998). High concentrations of colloidal material (Bigg et al. 2004) and transparent exopolymer particles (TEP) (Wurl & Holmes 2008) support the model of the SML being a gelatinous film (Cunliffe & Murrell 2009). Generally, a strong enrichment of particulate opposed to dissolved material has often been found in the SML (Hunter 1997, Cincinelli et al. 2001). For instance, 40-fold enrichments of particulate organic carbon in the SML were reported for coastal slicks (Carlson 1983, Garabetian et al. 1993).

Strong correlations of organic material concentrations between the SML and the ULW suggest that the ULW is a major source of these compounds (Carlson 1983, Baastrup-Spohr & Staehr 2009). Dissolved and particulate materials are partly transported to the SML by buoyant particles or rising bubbles (Wallace & Duce 1978). Phytoplankton activity can support this accumulation by production of transparent exopolymer particles (TEPs) or surfactants (Gašparović et al. 2007, Wurl & Holmes 2008). The strong enrichment of particulate material might arise from aggregation in the ULW and subsequent floatation of material into the SML (Kerner et al. 2003, Azetsu-Scott & Passow 2004) or from assembly in the SML by convergent forces (Wheeler 1975). Likewise, a lack of correlation of dissolved free amino acid (DFAA) and particulate amino acid concentrations between the SML and the corresponding ULW was suggested to be due to formation of new particles in the SML or adsorption onto existing particles in the SML (Kuznetsova et al. 2004). Furthermore, autochthonous production and high extra-cellular release by phytoneuston was suggested to cause an accumulation of DFAA in the SML (Hardy & Apts 1989, Reinthaler et al. 2008), revealing the importance of neustonic activity on air-water interface properties.

Biological and chemical transformations in the SML

The SML forms the boundary for air-sea exchange of material and fluxes (Liss & Duce 1997). The limiting step of exchange, especially for gases, is the slow molecular transport across diffusive boundary layers in the liquid and gaseous phase of the air-water interface. Interfacial processes are additionally determined by chemical and biological transformations (Upstill-Goddard 2006). Photochemical reactions alter the bioavailability of organic material as well as the concentrations of byproducts such as CO (Wilson et al. 1970). Likewise, Zemmelink described high DMSO concentrations in the SML which were mainly caused by photo- rather than biological oxidation (Zemmelink et al. 2006). Transformations of SML-properties due to biological activity may include the following: (I) increased concentrations of organic material in the SML could enhance bacterial remineralisation and thus change the organic material pool as well trophic interactions near or within the SML via the micobial loop (Azam et al. 1983), (II) biological

production of surfactants changes gas-exchange rates (Frew et al. 1990), (III) concentrations of gases such as CO_2 are altered by increased production (Garabetian 1991) or consumption (Hardy 1973), and (IV) biological activity controls fluxes of trace gases across the interface (Conrad & Seiler 1988, Upstill-Goddard et al. 2003, Calleja et al. 2005).

Sampling of the Sea-Surface Microlayer

The optimal basis for studying the air-water interface is the investigation of an undisturbed SML, i.e. one, in which the native surface-to-volume ratio is maintained (Hermansson & Dahlbaeck 1983). However, since most chemical and biological characterizations depend on obtaining larger water volumes, sampling of the SML and therefore a change in its surface-to-volume ratio is unavoidable. As pointed out above, the SML is a loosely defined term, which rather refers to a sample taken by a particular sampling device than to the exact physico-chemical structure of the SML (Wangersky 1976, Norkrans 1980). Current sampling devices obtain SML layers of different thickness depending on their operating mode. The thinner a collected SML sample is, the more selective the respective sampling device is thought to be (Huehnerfuss 1981). Therefore, sampling of the SML must take the following into account (Garrett & Duce 1980, Huehnerfuss 1981): (I) as little dilution with bulk water as possible, (II) no bias due to sampler selectivity, (III) large sample volume in reasonable sampling time and (IV) good handling. Additionally, wind speed, water temperature, and wave state at the time of collection, have been reported to influence the thickness of SML samples (Carlson 1982a, Falkowska 1999a).

A common sampling technique is the employment of hydrophilic and hydrophobic sheets and membranes, which float on the sea-surface and collect very thin layers (1 - 30 μm) of the SML (Crow et al. 1975). Additionally, glass-plate (GP), rotating-drum (RD), and screen sampling devices are the most widely used in studies of the SML. Both the GP

and the RD function as solid adsorbers (Harvey 1966, Harvey & Burzell 1972). The samples collected with these devices are therefore much thinner (22–100 μm) than samples taken with the screen (150–440 μm), which traps the water by surface tension forces (Garrett 1965).

These inherent differences in layer thickness indicate the collection of distinct water samples. Laboratory experiments showed different recovery rates of artificial surface films by the GP or the screen, with the efficiency of either one dependent upon the film materials used (Hatcher & Parker 1974, Van Vleet & Williams 1980). Recently, two accompanying studies investigated extensively the possible bias of the GP and the screen for biological and chemical analysis (Agogué et al. 2004, Momzikoff et al. 2004). The authors concluded that neither the GP nor the screen influenced the characterisation of all investigated biological and most chemical parameters. However, the GP collected amino acids and fatty acids more efficiently than the screen (Momzikoff et al. 2004). This points to less dilution in GP- compared to screen samples, which is still higher than in membrane samples and possibly explains the collection of different bacterial communities using these devices (Cunliffe et al. 2009a). Membranes and sheets sample high concentrations of chemical and biological SML components (Crow et al. 1975, Kjelleberg et al. 1979), but it is still a matter of debate, whether these sampling devices are selective, especially concerning bacterial parameters (Agogué et al. 2004, Franklin et al. 2005). Taken together, these reports show that until today no sampling device is known to fulfill the several requirements for SML studies.

Generally, samples from the SML are compared to control samples from the ULW to better understand quantitative and qualitative changes within and between the compartments. Results are therefore mainly expressed as enrichment factors (EF) as defined in the following equation: $EF = [x]_{\mu}/[x]_{b}$, where [x] is the concentration of a given parameter in the SML (μ) or ULW (b) (GESAMP 1995).

Procaryotic Life at the Air-Water Interface

Naumann introduced the term 'neuston' to describe organisms found in the air-water interface (Naumann 1917), which can be further distinguished dependent upon main occurrence on the aerial side (epineuston) or aquatic side (hyponeuston) of the SML (Ruttner 1962). Diverse phylogenetic groups like fish larvae, copepods, flagellates, phytoplankton and viruses have been identified at the air-water interface (Hardy & Apts 1989, Zaitsev 1997, Joux et al. 2006), and evidence for vertical migration, specific selectivity or inhibition has been presented (Holdway & Maddock 1983, Hardy & Apts 1984, Rawlinson et al. 2005). These results suggest that strong trophic interactions can be found at the top of any water body and in the following, knowledge of the abundance, activity, and community composition of prokaryotes in the SML (bacterioneuston) will be discussed.

Bacterial abundance in the sea-surface microlayer

Due to the diverse mechanisms which transport and maintain material in the SML as well as its consistent enrichment of organic substances, it has long been suggested that bacteria are also highly concentrated in the SML. Over the past decades a large number of studies examined bacterioneuston abundances in limnic, brackish and marine habitats. SML-samples taken with different sampling-devices revealed that the bacterioneuston was enriched, yielding enrichment factors up to 10^3 – 10^4 (Table A). Only little depletion of bacterial numbers in the SML compared to the ULW was reported and in any of these studies also enriched numbers were found (Hermansson et al. 1987, Carlucci et al. 1991, Münster et al. 1998).Variations in microlayer enrichment may be caused by processes such as bubble scavenging, adsorption and diffusion (see above). Bezdek showed that bubble transport contributed strongly to bacterial accumulation in the SML (Bezdek & Carlucci 1972). Generally, import from the ULW was suggested to be the major source of enrichment of bacterial cells in coastal and open ocean SML-samples (Kuznetsova et al. 2004). A high correlation of bacterial numbers between the SML and ULW supports the

Table A Compiled literature results of relative bacterial cell numbers in the sea-surface microlayer compared to the underlying water, expressed as enrichment factor (EF). The results are categorized by the sampling device used. Different habitats as well as enumeration methods are indicated (CFU = colony forming units, EpiM = epifluorescence microscopy, FC = flow cytometry). a = mean EF, when provided in reference; b = similar results were obtained with a screen sampler

Sampler	EF	Enumeration	Habitat	Reference
Membrane / Sheet	> 10^2	CFU	marine	Crow et al. 1975
	10^2 - 10^4	CFU	brackish	Fehon & Oliver 1979
	< 10^4	CFU	marine	Kjelleberg et al. 1979
	< 79	CFU (?)	marine	Norkrans 1980
	6 - 10^2	CFU	marine	Dahlbaeck et al. 1981
	0.4 - 89	CFU	marine	Hermansson et al. 1987
	10^3 - 10^4	EpiM	marine	Sewell et al. 1981
	10^2	EpiM	marine	Dahlbaeck et al. 1982
	10^2	CFU	marine	Dahlbaeck et al. 1982
	23 - 10^4	EpiM	marine	Hardy & Apts 1984
Rotating drum / Glass plate	< 10^3	CFU	limnic	Hatcher & Parker 1974
	> 1	CFU	marine	Dietz et al. 1976
	1.7 – 3.4 [b]	EpiM	limnic	Donderski et al. 1999
	> 1	CFU	limnic	Münster et al. 1998
	0.8 – 4.8	EpiM	limnic	Münster et al. 1998
	> 1	CFU	brackish	Mudryk & Skórczewski 2000
	13 [a, b]	CFU	marine	Agogué et al. 2004
	1.2 [a, b]	FC	marine	Agogué et al. 2004
	6 [a]	EpiM	marine	Aller et al. 2005
	3.1 [a]	EpiM	marine	Matrai et al. 2008
	1 [a]	FC	marine	Reinthaler et al. 2008
	> 1	EpiM	marine	Santos et al. 2009
Screen	< 20	CFU	marine	Tsyban 1971
	0.5 - 10^3	CFU	marine	Sieburth et al. 1976
	> 1	EpiM	marine	Carlucci et al. 1985
	1.2 - 4	EpiM	marine	Carlucci et al. 1986
	0.3 – 1.4	EpiM	marine	Carlucci et al. 1991
	23 - 10^2	CFU	marine	Plusquellec et al. 1991
	1.4 – 3.2	EpiM	marine	Carlucci et al. 1992
	> 1	EpiM	marine	Kuznetsova & Lee 2001
	0.7 – 6.6	EpiM	marine	Kuznetsova & Lee 2002
	> 1	EpiM	marine	Kuznetsova et al. 2004
	1.4 - 2	EpiM	limnic	Kalwasinska & Donderski 2005
	2 – 13.3	CFU	limnic	Kalwasinska & Donderski 2005
	1.2 [a]	FC	marine	Obernosterer et al. 2005
	0.9 – 1.2	FC	marine	Joux et al. 2006
	~ 1	FC	marine	Obernosterer et al. 2008
	0.3 – 14.5	EpiM	limnic	Auguet & Casamayor 2008
	0.7	EpiM	limnic	Hervas & Casamayor 2009
	0.9 – 1.4	FC	marine	Cunliffe et al. 2009b
	~ 1	FC	marine	Cunliffe et al. 2009c

idea that abundances in the SML are strongly influenced by the bulk phase (Joux et al. 2006, Santos et al. 2009). Stability of the bacterioneuston was thought to be mainly dependent upon surface tension rather than buoancy (Sieburth 1983), and strong hydrophobicity has been reported for neustonic bacteria (Dahlbaeck et al. 1981). Thus, the SML was suggested to provide conditions comparable to solid substrata (Kjelleberg 1985). Bacteria might additionally adhere to the SML either firmly, e.g. mediated by extracellular polymeric substances (EPS), or reversible (Marshall 1985). Similar mechanisms also cause attachment of bacteria to particles and higher numbers of particle-attached bacteria have been reported in the SML compared to the ULW (Aller et al. 2005). However, only up to 3 % of bacterioneuston cells were found to be particle-attached in coastal Mediterranean samples, suggesting that the major part of the bacterioneuston remains non-attached (Obernosterer et al. 2005).

Besides the naturally induced variability, an impact of different enumeration methods on the results found in the literature is obvious (Table A). Counting of culturable bacteria (CFU = colony forming units) on solid media has generally shown strong enrichment factors. The concentrations of organic substances in the SML were speculated to be comparable to nutrient conditions in laboratory media (Sieburth et al. 1976), and thus, bacterioneuston growth on solid media might be differentially induced compared to their planktonic counterparts. The development of fluorescent dyes and their application in quantification analysis using epifluorescence microscopy (Hobbie et al. 1977) has highlighted some major drawbacks of the culture-dependent methods. Most important, the enumeration of CFU underestimates total bacterial numbers by several orders of magnitude (Staley & Konopka 1985). Flow cytometry enhanced the resolution, throughput and the reproducibility of microscopic analysis (Gasol & Del Giorgio 2000), but does not detect particle-associated bacteria, which may account for the general low enrichment factors found when this method was applied (Joux et al. 2006, Obernosterer et al. 2008, Reinthaler et al. 2008).

Bacterial activity in the sea-surface microlayer

As shown above, high CFU numbers indicate that bacteria in the SML are active. This might either reflect general high activity of bacterioneuston assemblages or their stimulation, e.g. due to reduction of stress factors under laboratory conditions compared to their natural habitat. Generally, the literature provides opposing results of bacterioneuston activity, independent from the sampling device used or the habitat investigated (Table B). Exo-enzymatic activites seem to be enhanced in the SML (Münster et al. 1998, Kuznetsova & Lee 2001, Santos et al. 2009), which may be evidence for the high concentrations of potential substrates in the SML. Bacterial cells which developed in a thick surface film showed increased formation of extracellular polymeric substances (EPS) and polyhydroxybutyrate (PHB), which might additionally indicate high organic substrate availability (Sieburth 1983). Respiring bacteria are usually enriched in the SML (Hermansson & Dahlbaeck 1983, Maki & Remsen 1989) and most of them were attached to particles (Harvey & Young 1980). Community respiration in the SML correlated to the enrichment of total organic carbon, further indicating bacterial uptake of organic material in the SML (Obernosterer et al. 2005). Likewise, a high enrichment of bacterial biomass and activity in the SML in oligotrophic but not eutrophic waters suggested that enriched substrate supply fueled bacterioneuston metabolism compared to the bacterioplankton (Carlucci et al. 1986). Furthermore, ATP concentrations increased with more stability of the SML and highest enrichment factors were reported in slicks (De Souza-Lima & Romano 1983), indicating influences of the wind speed on patterns of bacterioneuston activity.

These results show that there is living biomass in the SML. However, overall reduced incorporation of diverse substrates such as leucine, thymidine and glucose in the SML compared to the ULW has been observed (Bell & Albright 1982, Carlucci et al. 1991, Maki & Herwig 1991). Consequently, bacterial growth in the SML seems to be limited and low bacterial growth efficiencies in the SML have been measured (Reinthaler et al. 2008). It was assumed that material in the SML is too refractory for bacterial uptake (Reinthaler et al. 2008). Additionally, the physiological status of bacterial cells in the SML may be impaired by diverse stress factors acting upon the bacterioneuston community, e.g. increased exposure to UV-radiation, toxic compounds, etc. (Maki 1993). UV-radiation

possibly influenced diurnal cycles of organic matter transformations as well as changing bacterial activity in the SML (Horrigan et al. 1981, Falkowska 2001), and highest ATP levels in the SML after sunset have been observed (Freedman et al. 1982). Yet, there is contradictory evidence whether UV-radiation generally causes (diel) patterns of bacterioneuston activity (Carlucci et al. 1986, Maki & Herwig 1991, Santos et al. 2009). Sieburth reported inhibition of the bacterioneuston in slicks fom the Atlantic Ocean, which was reduced by dilution of the SML-samples with natural sea-water, indicating a negative influence of toxic compounds on bacterioneuston activity (Sieburth 1983). Natural dilution effects in the SML occur by wet atmospheric deposition and bacterioneuston activity was enhanced after rain events (Carlucci et al. 1991).

Taken together, none of the above mentioned environmental factors (e.g. UV-radiation, wind, toxic compounds, and substrate supply) fully explains patterns of bacterioneuston activity. Even when reduced activity in the SML compared to the ULW has been observed, the neuston was still active. Thus, the complexity of processes in the SML still leaves large uncertainties about the relation of bacterioneuston communities and the physical and chemical environment of the SML.

Table B Compiled literature results of relative bacterial activity in the sea-surface microlayer compared to the underlying water, expressed as enrichment factor (EF). a = mean EF, when provided in reference; b = similar results were obtained with a screen sampler

Sampler	EF	Substrate incorporation / Enzymatic activity	Habitat	Reference
Rotating drum / Glass plate	0.2 - 10	Glucose (turnover time)	marine	Dietz et al. 1976
	< 1	Glucose	marine	Bell & Albright 1982
	< 1	Thymidine	limnic	Maki & Herwig 1991
	1.8 – 13.8	Glucose	limnic	Münster et al. 1998
	0.9 – 7.5	Glucosidase	limnic	Münster et al. 1998
	0.8 [a, b]	Thymidine	marine	Agogué et al. 2004
	1.0 [a, b]	Leucine	marine	Agogué et al. 2004
	0.3 - 45	Leucine	marine	Matrai et al. 2008
	> 1	Respiration	marine	Reinthaler et al. 2008
	< 1	Acetate, Glucose	marine	Santos et al. 2009
	highly variable	Leucine	marine	Santos et al. 2009
Screen	0.5 - 1	Thymidine	marine	Carlucci et al. 1986
	0.1 – 0.5	Thymidine	marine	Carlucci et al. 1991
	0.4 – 2.2	Glutamic acid	marine	Carlucci et al. 1992
	> 1	Peptide hydrolysis	marine	Kuznetsova & Lee 2001
	< 1	Thymidine	limnic	Kalwasinska & Dond. 2005
	0.1 – 2.4	Leucine	marine	Joux et al. 2006
	0.1 – 1.2	Leucine	marine	Obernosterer et al. 2008

Bacterioneuston community composition

The unique physico-chemical properties of the SML might favour specific bacterioneuston assemblages compared to the ULW (Tsyban 1971) and the SML could therefore represent a source of new microorganisms. However, the bacterioneuston community composition is still poorly understood (Agogué et al. 2005a). Culture-dependent methods have shown similar bacterial taxa in the SML of several aquatic habitats (Table C). SML communities were dominated by *Gammaproteobacteria*, e.g. *Pseudomonas* sp. (Tsyban 1971, Fehon & Oliver 1979, Donderski et al. 1999), *Pseudomonades* sp. (Sieburth 1971), *Vibrio* sp., *Serratia* sp. or *Aeromonas* sp. (Donderski et al. 1999). Generally, these studies stressed strong differences between the bacterioneuston and bacterioplankton communties (Plusquellec et al. 1991). Similar conclusions were drawn in recent studies in which molecular-biological methods, based on phylogenetic differences of the 16S rRNA gene sequence, were applied. SML-samples which were taken with membranes revealed strong differences of the bacterial community compared to the ULW (Cunliffe et al. 2008, Cunliffe et al. 2009c). One study showed substantial lower diversity in the SML, and its inhabiting community was dominated by *Pseudoalteromonas* sp. and *Vibrio* sp. (Franklin et al. 2005). In contrast, highly comparable 16S rRNA fingerprints of screen samples from the SML and ULW were reported and show that a specific bacterioneuston community is not a common trait (Agogué et al. 2005a, Obernosterer et al. 2008).

Only few studies have determined the effects of environmental factors on the bacterial community structure in the SML. During an artificially induced phytoplankton bloom, the bacterial community was increasingly different between the SML and ULW (Cunliffe et al. 2009c). This was accompanied by an increasing accumulation of TEPs in the SML (Cunliffe et al. 2009b) and thus, a change of potential substrate supply could have influenced the bacterioneuston community structure. Studies in estuarine systems suggested that high similarities between bacterioneuston and bacterioplankton assemblages were due to strong hydrodynamics and mixing; the establishment of a distinct bacterioneuston community would occur only during calm SML-conditions (Santos et al. 2009). This is supported by results from limnic studies in which SMLs are

Table C Compilation of bacterial diversity analyses from the sea-surface microlayer (SML) and the underlying bulk water (ULW). The literature results are distinguished according to the sampling device used, the habitat investigated, and the methods applied (DGGE = denaturing gradient gel electrophoresis, T-RFLP = terminal-restriction fragment length polymorphism, SSCP = single strand conformation polymorphism, Mar-FISH = microautoradiography-fluorescence *in situ* hybridization). a = only findings from *Bacteria* are reported; exceptions (*Archaea*, functional genes) are indicated; b = all analyses based on 16S rRNA sequences, except for c = identification not based on molecular methods; d = similar results were obtained with a screen sampler

Sampler	Description [a]	Method [b]	Habitat	Reference
Membrane / Sheet	SML dominated by *Pseudomonas*	Isolates [c]	brackish	Fehon & Oliver 1979
	Less diversity in SML; SML dominated by *Pseudoalteromonas*, *Vibrio*	Clone library	marine	Franklin et al. 2005
	Bacteria, but not *Archaea* in SML different from ULW; some SML-specific functional genes	Clone library / DGGE	marine	Cunliffe et al. 2008
	Bacteria, but not *Archaea* in SML different from ULW	DGGE	marine	Cunliffe et al. 2009a
	SML different from ULW	DGGE /T-RFLP	marine	Cunliffe et al. 2009c
Glass plate	SML dominated by *Pseudomonas*, *Vibrio* sp., *Serratia*, *Aeromonas* [d]	Isolates [c]	limnic	Donderski et al. 1999
	SML similar to ULW, but some exceptions	DGGE	marine	Santos et al. 2009
Screen	Some species of *Micrococcus*, *Pseudomonas*, *Chromobacterium* only in SML	Isolates [c]	marine	Tsyban 1971
	SML dominated by *Pseudomonades*	Isolates [c]	marine	Sieburth 1971
	SML different from ULW	Isolates [c]	marine	Plusquellec et al. 1991
	SML similar to ULW; dominance of known, planktonic sequences	Isolates, SSCP	marine	Agogué et al. 2005a
	SML similar to ULW; partly different active SML community	SSCP / Mar-FISH	marine	Obernosterer et al. 2008
	Archaea in SML different from ULW	DGGE / FISH	limnic	Auguet & Casamayor 2008
	SML more similar to airborne populations than to ULW	Clone library	limnic	Hervas & Casamayor 2009
	SML similar to ULW, more specifictiy evolves during phytoplankton bloom	DGGE / T-RFLP	marine	Cunliffe et al. 2009c

less influenced by wind compared to marine systems and which are known to contain specific neustonic species, e.g. *Nevskia ramosa* (Pladdies et al. 2004) as well as archaeal assemblages (Auguet & Casamayor 2008). Finally, atmospheric deposition is another source of microorganisms in the SML (Norkrans 1980) and bacterioneuston communities in alpine lakes were more similar to airborne than to planktonic populations (Hervas & Casamayor 2009).

Despite this overall inconsistent picture of general bacterioneuston community composition, functional traits in the SML and the ULW may be distinct. Plasmid encoded efflux-systems could be used by the bacterioneuston to counteract exposure to toxic compounds like antibiotics, pesticides or heavy metals (Maki 1993). Indeed, increased frequencies of strains carrying plasmids encoding for antibiotic- and mercury-resistance have been isolated from the SML (Hermansson et al. 1987). In contrast, similar resistance patterns to antibiotics have been observed in bacterioneuston and bacterioplankton strains (Mudryk 2002). Higher numbers of pigmented strains isolated from the SML of the Swedish coast indicated that the bacterioneuston is specifically adapted to increased UV-exposure (Hermansson et al. 1987). However, no differences in pigmentation as well as UV-resistance were observed for strains from the ULW and SML of coastal Mediterranean samples (Agogué et al. 2005b). Among these, *Bacteriodetes* and *Gammaproteobacteria* contributed strongly to the highly-resistant strains in the SML (Agogué et al. 2005b). These phylogenetic groups were also dominantly involved in neustonic leucine-uptake in the Atlantic Ocean (Obernosterer et al. 2008). Taken together these results show potential differences in active members of bacterial assemblages in the SML and ULW, but there is overall lacking knowledge on how environmental factors determine the community composition in the SML.

Thesis Outline

The aim of the present thesis was to improve the understanding of the activity and phylogenetic diversity of bacterial assemblages in the SML of the Baltic Sea. Thereby, peculiar attention has been drawn on how environmental factors, especially variable concentrations of organic compounds as well as changing meteorological conditions, influence the bacterioneuston community compared to their planktonic counterparts in the ULW. In order to obtain a sufficient and reliable analysis of the SML-samples, a comparative approach to test available SML sampling techniques was unavoidable. The following issues were therefore adressed:

I) Evaluation of different sampling devices for bacterioneuston studies in the Baltic Sea
II) Influence of meteorological conditions on the activity and diversity of bacterioneuston assemblages
III) Examination of bacterioneuston dynamics under extremely low turbulence conditions

The first chapter describes the comparison of glass plate, rotating drum and metal screen sampling devices and their potential bias introduced into bacterial community analysis. After an adequate sampling technique was evaluated, SML-samples from the southern as well as central Baltic Sea were taken to examine bacterioneuston activity in these habitats.

The second chapter provides a detailed analysis about the influence of meteorological conditions on differences between the bacterioneuston and bacterioplankton community composition applying 16S rRNA fingerprint techniques. Special emphasis has been placed on the differentiation of particle- and non-attached as well as of presumably active and present communities. Furthermore, active members which were exclusively detected in the bacterioneuston fingerprints were identified by sequencing approaches in order to examine taxa inhabiting the SML.

Finally, the third chapter shows the investigation of bacterioneuston community dynamics during the formation of an extensive visible surface film (slick) in the southern Baltic Sea. Mesocosm experiments were conducted in the harbour of Warnemuende thereafter, to study the effects of artificially calmed sea surfaces on bacterial dynamics in the SML. The comparison of natural and artificial calmed sea surfaces was adressed to examine the potentials for the succession of bacterioneuston communities.

Bacterial Activity in the Sea-Surface Microlayer: *In Situ* Investigations in the Baltic Sea and the Influence of Sampling Devices [1]

(Chapter 1)

[1] Data of this chapter has been published in *Aquatic Microbial Ecology*, 2009, **58**, 67-78

1.1 Abstract

The sea-surface microlayer (SML) is considered to be an 'extreme' environment. However, it is still unclear how bacteria that inhabit the SML (bacterioneuston) react to conditions within this interface. This deficiency is partly caused by the difficulty in obtaining representative samples. Our aim was to examine different sampling devices and eventually to characterize bacterioneuston activity in the Baltic Sea. Initial *in situ* studies revealed a decreased incorporation of ^{3}H-thymidine (TdR) by up to 90 % in both glass-plate and metal-screen samples compared to the underlying bulk water. However, a series of tank experiments showed selective inhibition of bacterial productivity with either of these sampling devices, although bacterial cell counts and community composition were unaltered. The inhibition introduced by the glass plate could not be nullified by different cleaning treatments, but by the wiping technique used to scrape off the sample. Even with this modified, unbiased glass-plate technique, ^{3}H-TdR incorporation of the bacterioneuston was still reduced by 50 to 80 % compared to that in the underlying water, whereas the abundance of 5-cyano-2,3-ditolyl tetrazolium chloride (CTC)-positive cells was not affected. Our *in situ* study thus revealed that in the Baltic Sea the presence of a pronounced bacterioneuston community different from that in the underlying water is unlikely. Reduced bacterial activities within the SML support the concept of a demanding habitat. Additionally, this study emphasizes the need to carefully evaluate the sampling devices used when measuring bacterial parameters. Furthermore, it supports the view that caution is required in comparisons of results from different studies.

1.2 Introduction

The air-water interface spans 71% of the Earth's surface. This layer, which constitutes the boundary between the atmosphere and underlying water bodies, is crossed by fluxes of

mass and energy. Several models describe the organization of the interface as a more or less stratified surface film (Norkrans 1980, Sieburth 1983, Falkowska 1999b), with the top layer referred to as the sea-surface microlayer (SML). The SML is considered to be an 'extreme' environment because of its physicochemical properties relative to those of the underlying water (Maki 1993). According to this view, the bacterioneuston (i.e. bacteria within the SML) is exposed to intense UV-radiation (Maki 2002) as well as to the accumulation of pollutants (Wurl & Obbard 2004) and heavy metals (Hardy & Cleary 1992). In contrast, the accumulation of organic and inorganic substrates in the SML may be beneficial to its microbial communities (Williams 1986).

The physical, chemical, and biological properties in a variety of surface films, including those of different marine and limnic habitats, have been investigated in several studies. Earlier studies suggested that the SML is enriched in several chemical and biological compounds (Sieburth et al. 1976, Hardy 1982). However, conflicting results, especially those regarding bacterioneuston parameters, can be found in the literature. Cell counts of bacteria in the SML have been shown to be enriched in several habitats (Kuznetsova & Lee 2002, Joux et al. 2006), but similar abundances of bacterioneuston and bacterioplankton have also been reported (Hermansson et al. 1987, Reinthaler et al. 2008). The enzymatic activity of the bacterioneuston has been found to be generally enhanced compared to that of bacterioplankton (Münster et al. 1998, Kuznetsova & Lee 2001), as opposed to bacterial productivity in SML samples, which was found to be decreased or highly variable compared to bulk measurements (Obernosterer et al. 2008, Reinthaler et al. 2008). Additionally, the diversity of bacterial and archaeal communities in the SML, based on 16S rDNA and functional gene analysis, has been shown to differ from that of the underlying water (Franklin et al. 2005, Auguet & Casamayor 2008, Cunliffe et al. 2008). However, such differences have been found to be highly dynamic, implying that the presence of a different bacterial community in the SML is not a general phenomenon (Agogué et al. 2005a).

Consequently, there is no consistent view regarding the structure and function of the bacterioneuston or whether a particularly adapted bacterial community exists in the SML. This inconsistency can be explained by several reasons. (1) The SML undergoes highly dynamic spatial and temporal changes. These include longer time scales (e.g. seasonality) as well as shorter time scales (e.g. disintegration of surface slicks). Accordingly, the

bacterioneuston community structure at a single sampling site has been found to differ substantially from one day to the next (Agogué et al. 2005a). (2) The predominant problem of studying the SML is obtaining proper samples (Maki 1993). Investigation of an undisturbed SML, i.e., one in which the native surface-to-volume ratio is maintained, is preferable (Hermansson & Dahlbaeck 1983); however, since most characterizations depend on obtaining larger water volumes, a change in the SML surface-to-volume ratios is unavoidable. Ideally, SML samples should be diluted as little as possible with bulk water. Current sampling devices obtain SML layers of different thickness depending on their operating mode. The thinner a collected SML sample is, the more selective the respective sampling device is thought to be. Therefore, sampling of the SML must take the following into account (Garrett & Duce 1980, Huehnerfuss 1981): (1) thickness of the SML sample, (2) selectivity of the sampling device, (3) sampling volume and time, and (4) handling of the sampling device. Additionally, other factors, e.g., wind speed, water temperature, and wave states at the time of collection, have been reported to influence the thickness of SML samples (Carlson 1982a, Falkowska 1999a).

Glass-plate (GP), rotating-drum (RD), and metal-screen (MS) sampling devices are the most widely used in studies of the SML. Both the GP and the RD function as solid adsorbers (Harvey 1966, Harvey & Burzell 1972). The samples collected with these devices are therefore much thinner (22–100 μm) than samples taken with the MS (150–440 μm), which traps the water within its meshes by surface tension forces (Garrett 1965). These inherent differences in layer thickness imply the collection of different water samples. Indeed, laboratory experiments have shown different recovery rates of artificial surface films by GP and MS devices, with the efficiency of either one dependent upon the film materials used (Hatcher & Parker 1974, Van Vleet & Williams 1980). A recent and very extensive study investigated the possible bias of different samplers (Agogué et al. 2004). The authors concluded that neither the GP nor the nylon screen used in their study influenced the characterisation of the investigated biological parameters. However, as the authors pointed out, there is no further comparative study available, which evaluates the possible selective effects of these sampling devices on biological constituents of the SML.

In the present study, we sampled the SML using GP-, MS- and RD-type sampling devices in order to examine bacterioneuston abundance and activity in the Southern Baltic

Sea. We then conducted tank experiments to evaluate potential bias introduced by the GP and MS and to determine an appropriate sampling technique. Finally, SML samples were taken with a modified version of the GP device in the Southern and Central Baltic Sea, yielding new, unbiased measurements of bacterioneuston activity.

1.3 Materials and Methods

Sampling site

Sampling was conducted in the Southern Baltic Sea, offshore of Warnemuende, from July to September 2006 and from June to August 2007. To avoid the effects of pollutants, high nutrients loads, and the plume of the Warnow River, sampling sites opposite the wind direction were chosen. Additionally, SML samples were obtained in the central Baltic Sea (Gotland basin) in July 2007. All samples were taken under calm wind conditions (< 4 Beaufort), usually between 07:00. and 09:00 (UTC).

Sea-surface microlayer sampling

SML samples were taken with a Harvey GP and a Garrett MS from a zodiac and a RD placed on a catamaran. Sampling with the GP and the MS was usually conducted windward to avoid contamination from the zodiac. The MS had an overall area of 375 cm^2 and consisted of wire with a diameter of 0.17 mm and 1 mm^2 mesh, forming an open-space area (i.e. effective collection area) of 281 cm^2. During sampling, the MS was placed horizontally on the sea surface, lifted carefully, and then orientated vertically to allow samples to drain into sterilized glass bottles.

The GP had dimensions of 500 × 250 × 4 mm, with an effective sampling area of about 2000 cm^2 (including both sides of the plate). Samples were collected by vertically inserting

the GP into the water column and withdrawing it the same way, with a sampling velocity of about 10 cm s^{-1}. The samples were scraped off the GP with either a manual hand-wiper or framed wipers and collected in sterilized glass bottles. In both cases, the wipers consisted of 0.4 mm-thick Teflon blades. Both the MS and the GP were cleaned with Milli-Q water and ethanol (70 %) and intensively rinsed with seawater before being used.

The RD (Harvey 1966), which consists of a ceramic-coated drum with a sampling area of 5750 cm^2, was placed on a catamaran. The device is battery-driven and was operated at constant speed against the wind direction. Comparable to the GP, the samples were scraped off using a Teflon wiper and then pumped backwards for collection into sterilized glass bottles.

Bulk water was collected from a depth of 1 m with a 2 l glass collection tube and the ends were closed by a drop-weight mechanism. Results from the SML samples were compared to those from bulk-water measurements and are expressed as enrichment factors (EF) as defined in the following equation: $EF = [x]_\mu/[x]_b$, where [x] is the concentration of a given parameter in the SML (μ) or bulk water (b) (GESAMP 1995). The thickness of the SML layer was determined by the sample volume per area of the sampling device.

Tank experiments

To evaluate possible selectivity of the MS and GP sampling devices, tank experiments were carried out using 60 l of seawater filled in a plastic container. For this purpose, surface water was obtained in the morning from the pier in Warnemuende. Experiments started immediately upon the return to the institute, i.e., usually < 1 h later. To avoid formation of a sea-surface film, the water was stirred prior to each sampling. This stirring most likely does not inhibit formation of a microlayer as the reformation of organic surface films was described to be very fast (Hardy 1982). However, the water was homogenized to prevent the formation of a thickening surface film during the experiment. Reference water was obtained with a glass bottle that was opened in the middle of the tank. Three independent experiments were conducted to evaluate the MS and the GP in which the water was sampled once, twice, and 3 times in the first, second,

and third experiment, respectively (n = 6). SML thicknesses of the MS- and GP-samples were 223 ± 62 and 29 ± 5 µm, respectively. Additionally, 2 experiments were conducted to test the effect of cleaning the GP as well as a new wiping device for the GP. In each of these experiments the water was sampled 3 times (n = 3). SML thicknesses in these experiments were found to be higher (56 ± 4 and 36 ± 5 µm) than in the first experiments, but neither the cleaning procedures nor the wiping device had an influence on the thickness compared to the respective control GP. In all experiments 300 to 500 ml of water was collected with each sampling device for subsequent analysis. The water temperature in the tank did not change throughout the experiments (data not shown).

Bacterial cell counts

Bacteria were counted with a flow cytometer. Samples of 4 ml were incubated with 400 µl paraformaldehyde (1 % final concentration)/glutaraldehyde (0.05 % final concentration) in the dark for 1 h at 5°C. After fixation, the samples were frozen in liquid nitrogen and stored at -80°C. Heterotrophic bacteria were stained with SYBR Green (2.5 µM final concentration, Molecular Probes) for 30 min in the dark. Cells were counted using a Becton & Dickinson FACScalibur equipped with a laser emitting at 488 nm at a constant flow rate (35 µl min^{-1}). Yellow-green latex beads (0.5 µm, Polysciences) were used as an internal standard. Bacteria were detected by their signature in a plot of side scatter (SSC) versus green fluorescence (FL1).

Enumeration of metabolically active (CTC-positive) cells

From each sample, 900 µl was incubated with 100 µl of a 5-cyano-2,3-ditolyl tetrazolium chloride (CTC, Polysciences) solution (4 mM final concentration) in the dark at the in situ temperature for 1 to 3 h. CTC uptake was stopped by fixation of the samples with paraformaldehyde (1 % final concentration)/glutaraldehyde (0.05 % final concentration) in the dark for 10 min at 5°C. The samples were then frozen in liquid nitrogen and stored at -80°C. Cells were counted with a flow cytometer as described in 'Bacterial cell counts',

except that the beads for the internal standard were 1 μm in size and cells were detected by their signature in a plot of orange fluorescence (FL2) versus red fluorescence (FL3).

Bacterial productivity

The incorporation of 10 nM ^{3}H-methyl-thymidine (^{3}H-TdR) (60.1 Ci mmol^{-1}, Moravek Biochemicals) was measured to determine the heterotrophic bacterial productivity in 5-ml water samples. Additionally, 5-ml samples from the tank experiments were dual-labeled with 10 nM ^{3}H-TdR and 50 nM ^{14}C-leucine (261 mCi mmol^{-1}, Movarek Biochemicals) and then extracted with cold trochloroactetic acid (TCA) according to the method of Chin-Leo & Kirchman (1988). Triplicate samples were incubated for at least 1 h at the *in situ* temperature in the dark. Incorporation was stopped by fixing the cells with formaldehyde (10 % v/w) in the dark overnight at 5°C. A fourth sample, serving as a blank, was fixed for at least 10 min prior to the addition of the radioactively labeled substrates. *In situ* samples collected in 2006 were filtered on 0.22 μm nitrate-cellulose membranes. Samples from the tank experiments and those obtained *in situ* in 2007 were filtered on 0.22 μm polycarbonate filters (Millipore), as these filters yielded less background signals. Four milliliters of scintillation cocktail was added to the filters after which the incorporated substrates were counted in a scintillation counter (Packard).

DNA extraction

Water samples were filtered on 3 μm Isopore filters (Millipore), which are presumed to retain the particle-attached fraction. The flow-through (i.e., the non-attached fraction) was then filtered on 0.22 μm Isopore filters (Millipore). All filters were rapidly frozen in liquid nitrogen and then stored at -80°C. DNA from frozen filters was extracted using a phenol-chloroform-extraction method, according to Weinbauer et al. (2002). DNA in the extracts was quantified spectrophotometrically using a NanoDrop ND-1000 (NanoDrop Technologies).

Fingerprint analysis

The 16S rDNA fingerprints of the bacterial community were analyzed based on single-strand-conformation polymorphism (SSCP) or terminal restriction fragment length polymorphism (T-RFLP) analysis. For the former, DNA extracts were PCR-amplified using primers Com1 (5'CAGCAGCCGCGGTAATAC3') and Com2-Ph (5'CCGTCAATTCCTTTGAGTTT3') (Schwieger & Tebbe 1998), which amplify *Escherichia coli* 16S rDNA positions 519 to 926, and following a protocol described elsewhere (Labrenz et al. 2007). Single-stranded DNA was generated and purified and the SSCP analysis carried out as described in Schwieger & Tebbe (1998). Cluster analysis of band patterns was done with GelCompare II (Applied Maths NV). Comparisons of the samples were based on the absence or presence of individual bands (Jaccard coefficient) or on their densiometric profile (Pearson correlation).

T-RFLP fingerprints were done with PCR amplicons under the conditions described by Lehours et al. (2005), with 1 ng of template DNA and the primers 27f (5'AGAGTTTGATCCTGGCTCAG3') and 907r (5'CCGTCAATTCMTTTRAGTTT3'), according to Liu et al. (1997). Primer 27f was 5'-labeled with 6-carboxyfluorescin (FAM). PCR products were purified using a Nucleospin®Extract II kit (Macherey&Nagel). Sixty nanograms of purified amplicons were digested overnight with *Msp*I (Fermentas) following the manufacturer's instructions. The terminal restriction fragments were cleaned, separated, and analyzed as described in Hannig et al. (2006). Comparisons of the different samples followed the iterative normalization procedure as introduced by Dunbar et al. (2001).

Analysis of total organic carbon and total nitrogen

Ten milliliter water samples were sealed in HCl-precleaned and precombusted (450°C, 6 h) glass ampoules using a portable propane torch. The sealed ampoules were quickly frozen, without the addition of preservatives, and stored at -20°C until analysis in the laboratory.

Total organic carbon (TOC) and total nitrogen (TN) were quantified using a high-

temperature combustion method, carried out in the presence of a Pt-catalyst on a Shimadzu TOC-V analyzer supplemented with a Shimadzu TNM-1 nitrogen detector and a Shimadzu ASI-V autosampler. This system allows simultaneous determination of TOC and TN in the same sample. The temperature of the catalyst was kept at 680°C and carbon-free air produced by a Whatman gas generator was used as carrier gas (flow rate 150 ml min^{-1}). Acetanilide was used to calibrate (4-point calibration) TOC and TN measurements.

Prior to their analysis, the samples were acidified with 2N HCl (suprapure grade) to a final pH of 2. Concentrations of TOC and TN were calculated based on 4 injections of the sample (injection volume 75 µl). All samples were run in duplicate. The reliability of each analytical run was checked using external reference material in a seawater matrix for TOC and TN and using reference material for measurement of the blank. The reference material was obtained from the Consensus Reference Material program (Dennis Hansell, University of Miami). Measured concentrations of TOC and TN are reported as µmol C l^{-1} and µmol N l^{-1}, respectively.

Statistical analysis

The Kolmogorov-Smirnov test was applied to determine the hypothetical normal distribution of the samples, which was true for all measurements. To examine the equality of means between the SML samples and the underlying water as well between the samples from the control experiment, the t-test for independent samples with a confidence level of 95 % was chosen; $p < 0.05$ indicated that the means of the samples were significantly different. Spearman's rank correlation was applied to test the association between bacterial ^{3}H-TdR incorporation and CTC-positive cells and between these parameters and the wind speed.

1.4 Results

Sea-surface microlayer samples 2006

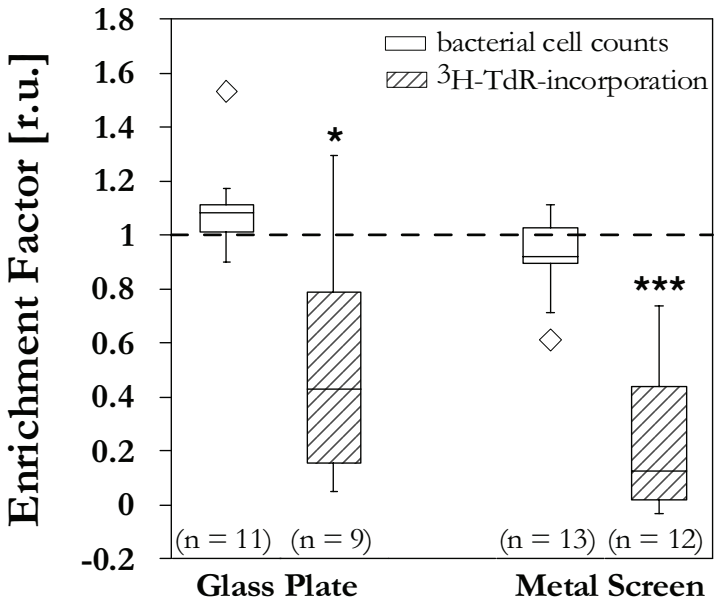

Fig. 1.1 Sea-surface microlayer samples collected in 2006 with a glass plate and a metal screen. Box-whisker plots show bacterial cell counts and ^{3}H-thymidine (^{3}H-TdR) incorporation of samples obtained with each device compared to the values in the underlying water (Enrichment factor). Dashed line indicates unity of values. Diamonds indicate box-whisker plot outliers, defined as any point falling above the following: upper quartile-(1.5 x interquartile range). r.u.: relative units Enrichment factor. *$p < 0.05$; ***$p < 0.001$

Coastal SML samples were obtained from the Southern Baltic Sea in the summer of 2006 using GP and MS sampling devices. GP samples, from 9 different stations, were 28 ± 3 µm thick compared to 267 ± 43 µm for MS samples, taken from 12 stations. The total bacterial cell counts of SML samples from the GP and MS sampling devices did not differ from those of the underlying bulk water or among each other (t-test, $p > 0.397$) (Fig. 1.1). In contrast, ^{3}H-TdR incorporation of the bacterioneuston, although highly variable, was significantly lower (20 to 90 %) than the incorporation measured in bulk water (MS: $p < 0.001$, GP: $p = 0.027$) (Fig. 1.1).

Table 1.1 Comparison of different sea-surface microlayer samples and the underlying bulk water from 12 September 2006 showing the layer thickness, bacterial cell counts, and productivity (^{3}H-thymidine incorporation; ± SD from triplicate measurements). nd: not determined

Water sample	**Layer thickness** (μm)	**Bacterial cell counts** (10^5 cells ml^{-1})	**Bacterial productivity** (pmol ^{3}H-TdR h^{-1} l^{-1})
Bulk water		9.22	19.02 ± 2.27
Glass plate	27	9.07	nd
Rotating drum	68	9.51	21.63 ± 1.92
Metal screen	264	8.42	0.33 ± 0.35

Interestingly, this decrease in ^{3}H-TdR incorporation was not measured in a 68-μm-thick SML sample collected at another time point and obtained from the RD sampler (Table 1.1). Subsequently, MS and GP samples were taken from the same station on the same day. TdR incorporation in the MS sample was reduced by an order of magnitude compared to that in either the RD sample or bulk water (Table 1.1). This difference was not due to changes in bacterial abundance or community composition. Total bacterial cell counts of SML samples taken with all 3 sampling devices were comparable among each other and with those in underlying bulk water (Table 1.1). The bacterial community composition of these samples, as revealed by 16S rDNA fingerprints from the RD and MS samples, was also similar to that in bulk water (Fig. 1.2). In both the particle-attached and non-attached size fractions, all three samples clustered together in Jaccard and Pearson comparisons. However, the similarity values of the non-attached fraction were higher (> 94.9 % in Pearson and > 86.4 % in Jaccard comparisons) than those of the particle-attached fraction (> 81.4 % in Pearson and > 50.6 % in Jaccard comparisons).

Tank experiments

A possible influence of these samplers was tested in a series of tank experiments. Homogenized seawater sampled with either the GP or the MS device indeed showed a selective reduction in ^{3}H-TdR incorporation of 70 to 80 % compared to reference water (t-test, $p = 0.011$). In contrast, the total bacterial cell counts of all samples did not differ ($p > 0.713$) (Fig. 1.3A). Additionally, T-RFLP analysis revealed a similar community composition among all samples (Fig. 1.3B), with each sample showing 30 distinct peaks.

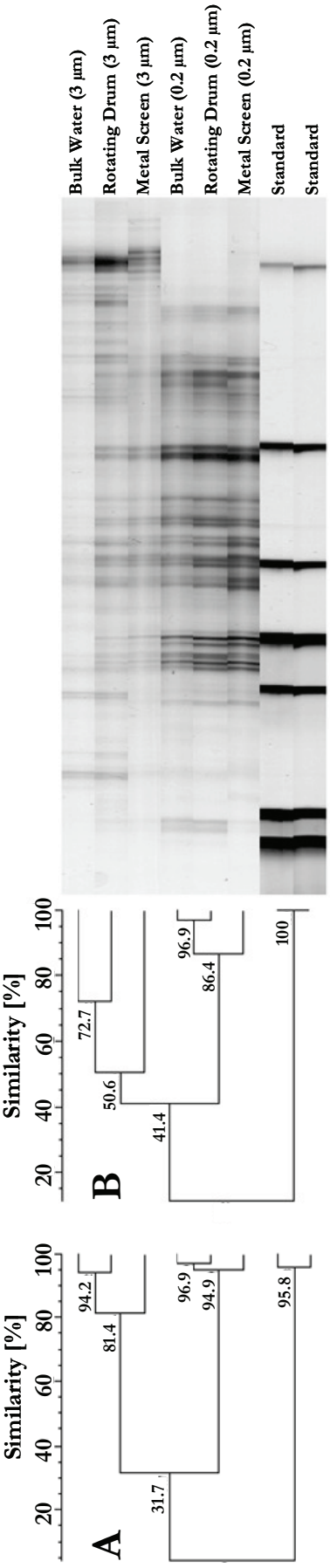

Fig. 1.2 16S rDNA fingerprints of sea-surface microlayer samples and of underlying bulk water from 12 September 2006 reveal similar bacterial community compositions in the particle attached (3 µm) and non-attached (0.2 µm) fractions. Similarity of the band patterns is shown by (A) Pearson and (B) Jaccard comparisons.

Only 5 (out of 90) peaks were solely detected in one of the samples (2 peaks in the reference water and in the GP; 1 peak in the MS). Two peaks were not detectable in the reference water and MS sample but in the other samples. Only 1 peak was not detectable in the GP sample. Taken together, all similar peaks contributed to more than 93 % of total peak height.

The effect of different GP cleaning procedures on the recovery of ^{3}H-TdR incorporation was also examined. Neither a Milli-Q water treatment nor treatment with silane or HCl showed an effect on the reduced ^{3}H-TdR incorporation (Fig. 1.3C). However, the high variability in the absolute values of ^{3}H-TdR incorporation in the control samples most probably retained statistical significance ($0.05 < p < 0.376$). Moreover, ^{14}C-leucine incorporation, used as a second parameter for measuring bacterial productivity in the same samples, was reduced to an even greater extent (60 to 80 %, $p < 0.011$).

Finally, 2 different wiping techniques were compared. The first consisted of a manual hand-wiper equipped with a Teflon blade. This device was used in all of the previously described experiments of the present study. The second technique was similar, except that the wiper blades were fixed in a PVC frame in a construction modified from the one described in Hardy et al. (1985) and by T. Reinthaler (pers. comm.). Although the layer thicknesses in this experiment did not differ between the wiping techniques, it was generally found that the fixed-blade wiper collected thicker SML samples (48 ± 6 μm) than the hand-wiper (32 ± 7 μm) in the 2007 samples. This was most likely due to more efficient retrieval of the water sample from the GP because the plate was completely dry after wiping, which was not true for the manual hand-wiper. Surprisingly, the use of this new wiping technique restored ^{3}H-TdR incorporation ($p = 0.370$) and CTC-uptake ($p = 0.705$) (Fig. 1.3D) but ^{14}C-leucine-incorporation was still reduced by about 50 % ($p = 0.009$). The fixed-blade device had no effect on total bacterial cell counts (Fig. 1.3D) or community composition (data not shown) compared to the values obtained with the reference water.

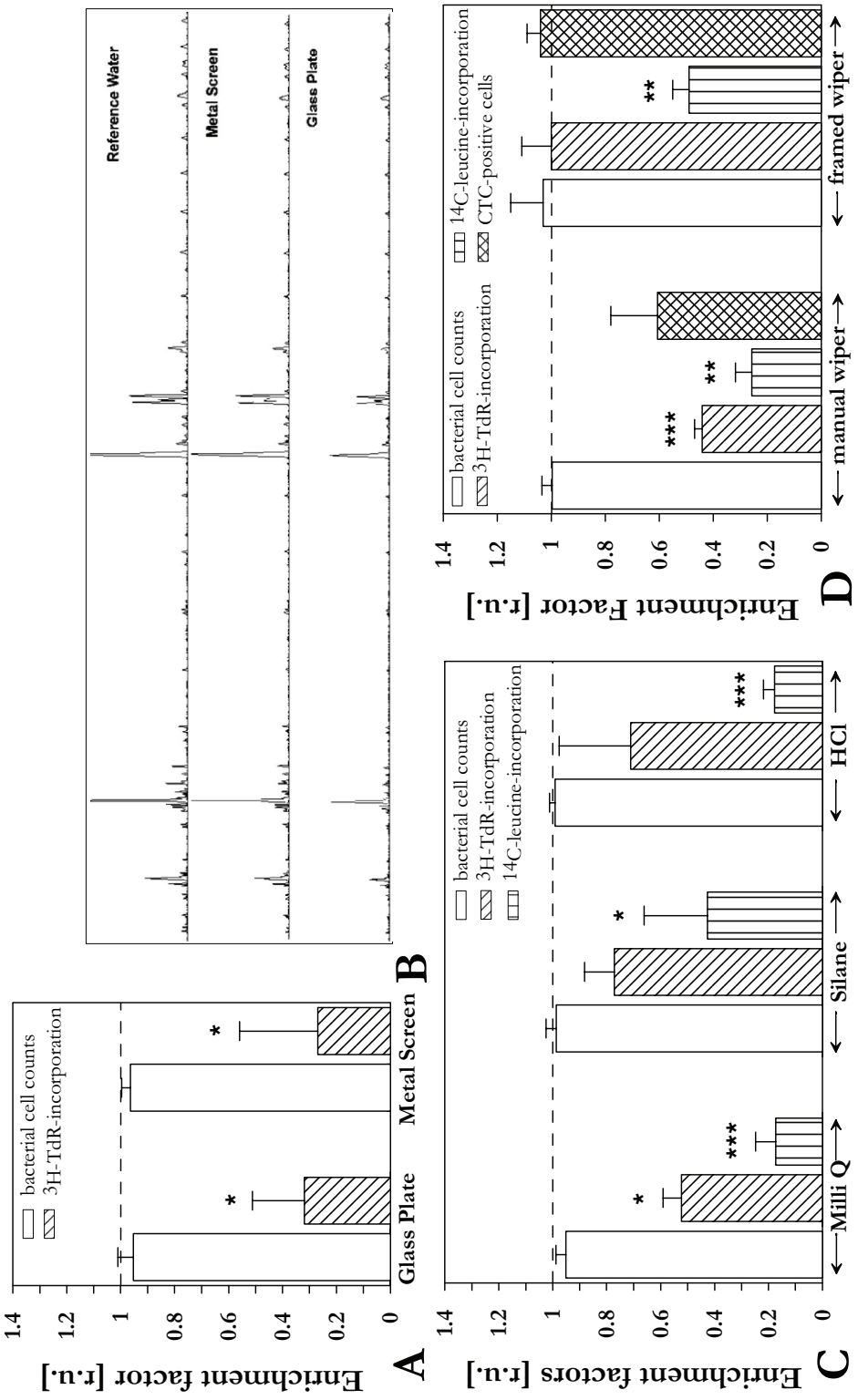

Fig. 1.3 Results from tank experiments designed to examine the potential bias of sea-surface microlayer sampling devices. (A) Bacterial cell counts and ^{3}H-thymidine (^{3}H-TdR) incorporation (n = 6) as well as (B) bacterial community composition by 16S rDNA fingerprints of glass plate (GP) and metal screen (MS) samples; (C) effects of different cleaning treatments on bacterial cell counts and ^{3}H-TdR and ^{14}C-leucine incorporation for GP samples (n = 3); (D) results of 2 GP wiping techniques, with subsequent analysis of all parameters including CTC uptake (n = 3). Results from A, C and D are expressed as enrichment factors. Dashed lines indicate unity of values between sea-surface microlayer sampling devices and reference water. r.u.: relative units. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Error bars are SD

Table 1.2 Sea-surface microlayer sampling stations in the southern (Warnemuende, WMD) and central Baltic Sea (Gotland Basin, GB). Thickness of the layer of the glass-plate samples, wind speed, and occurrence of slicks are reported for summer 2007. nd: not determined

Station	Date	Layer thickness (µm)	Location	Wind ($m\ s^{-1}$)	Visible slicks
1	18 Jun 2007	44	WMD	0.9	
2	18 Jun 2007	38	WMD	1.8	
3	19 Jun 2007	42	WMD	2.8	
4	20 Jun 2007	49	WMD	5.6	
5	10 Jul 2007	54	GB	3.5	
6	10 Jul 2007	52	GB	4	
7	11 Jul 2007	nd	GB	3	X
8	11 Jul 2007	57	GB	1.5	X
9	14 Jul 2007	nd	GB	3.9	
10	07 Aug 2007	53	WMD	2.3	X
11	14 Aug 2007	41	WMD	1.3	X

SML samples 2007

The GP combined with the fixed-blade device was found to be the sampling technique with the fewest bias for measurements of bacterial parameters. Thus it was chosen to collect a further set of SML samples from the Southern Baltic Sea (Warnemuende) and the central Baltic Sea (Gotland Basin) in summer 2007 (Table 1.2). The absolute values of the cell counts were comparable in SML and bulk samples ($p = 0.592$) (Fig. 1.4A). Interestingly, ^{3}H-TdR incorporation was still reduced, by 50 to 80 % ($p < 0.001$) compared to incorporation in the underlying bulk water (Figs. 1.4C & 1.5). This was evident throughout the sampling season and was measured in SML samples from the Southern as well as the central Baltic Sea (Fig. 1.4C). However, the enumeration of CTC-positive cells showed highly variable but overall comparable absolute and relative cell counts for bacterioplankton ($p = 0.710$) (Figs. 1.4B & 1.5).

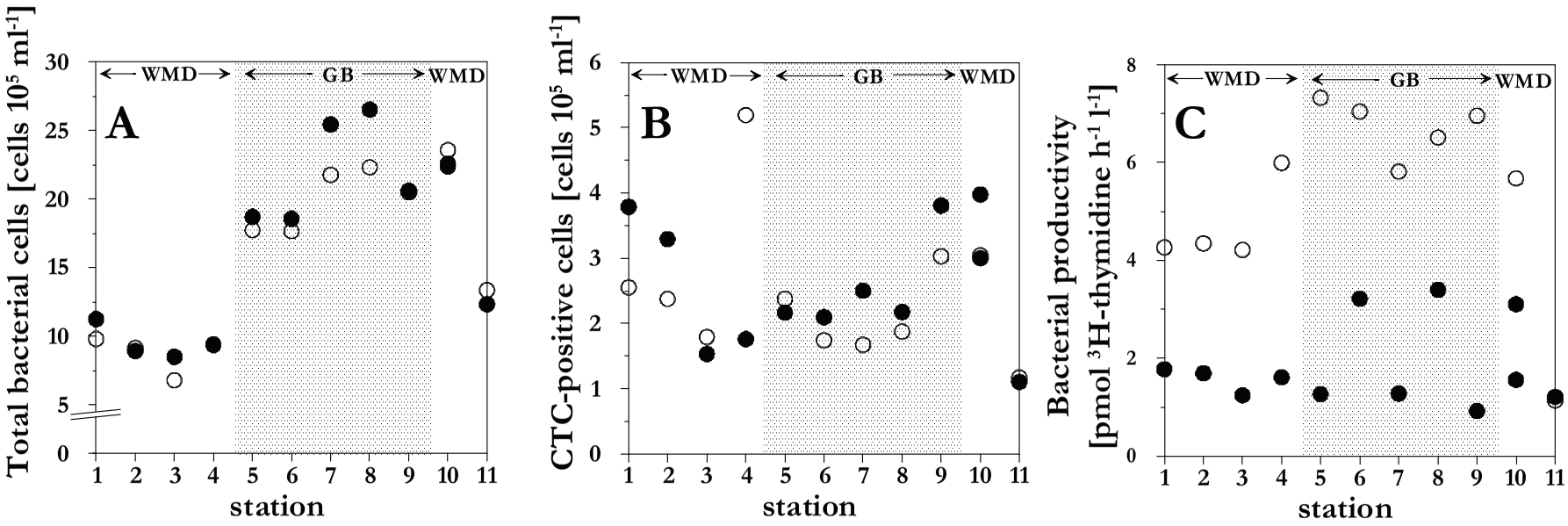

Fig. 1.4 Comparison of absolute values for (A) total bacterial cell counts**,** (B) CTC-positive cells, and (C) ^{3}H-thymidine (^{3}H-TdR) incorporation in sea-surface microlayer samples (black circles) and the underlying bulk water (open circles). Samples were collected in summer 2007 in the southern (Warnemuende, WMD) and central Baltic Sea (Gotland Basin, GB; gray shading). For detailed station information, see Table 1.2

The variability in the number of CTC-positive cells did not correlate with the rates of TdR incorporation for absolute numbers of the SML and the underlying water (data not shown) or the enrichment factors of both parameters (Spearman's rank r = 0.055; p = 0.873) (Fig. 1.6C). Additionally, the relative uptake of CTC and ^{3}H-TdR and the variations within did not correlate with the wind speed during sampling (CTC: r = -0.045, p = 0.894; TdR: r = 0.364, p = 0.272) (Fig. 1.6A,B), which is known to change the properties of the SML. Wind speed also did not correlate with either TOC or TN accumulation (data not shown); both of which were always enriched when measured. The accumulation of TN (25 to 125 %, p = 0.002) was more pronounced than the accumulation of TOC (25 to 50 %, p = 0.028) (Fig. 1.5).

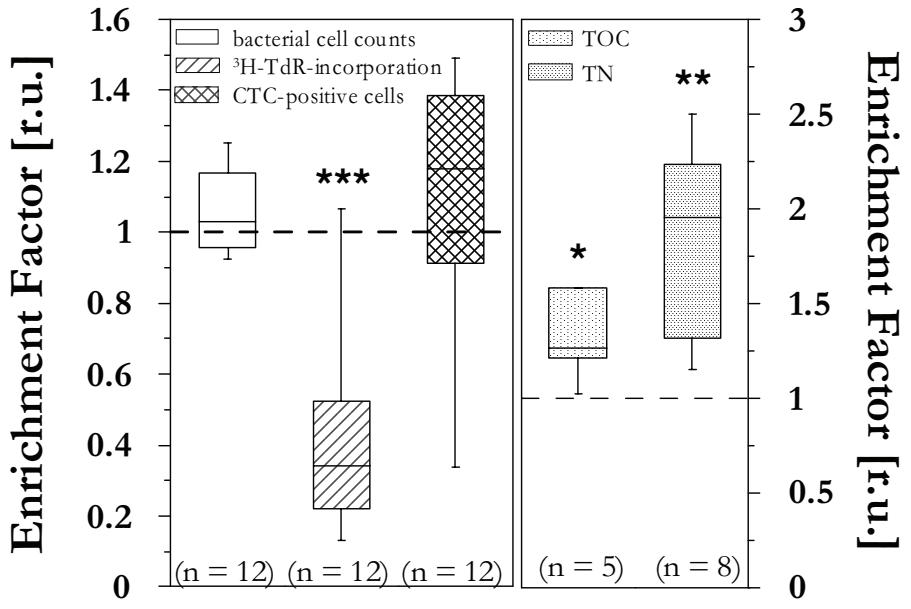

Fig. 1.5 Relative ratios (enrichment factor) of sea-surface microlayer samples compared to the underlying bulk water, both collected in summer 2007. Total bacterial cell counts, ^{3}H-thymidine (^{3}H-TdR) incorporation, CTC-positive cells, total organic carbon (TOC), and total nitrogen (TN) are shown. See Fig. 1.1 for explanation of figure

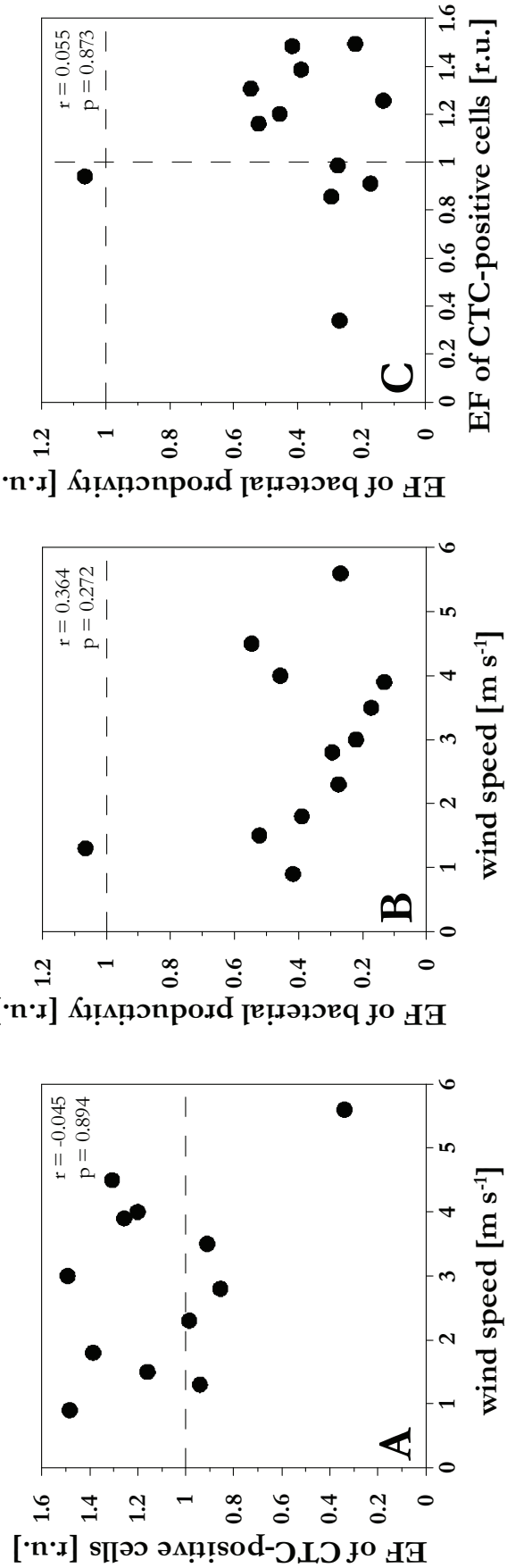

Fig. 1.6 Relationship between the relative ratios (enrichment factor, EF) of (A) CTC-positive cells and (B) ^{3}H-thymidine incorporation and wind speed during sampling. (C) Relationship between the relative ratios (EF) of both activity parameters. Dashed lines indicate unity of values between the sea-surface microlayer and the underlying water, i.e. EF = 1. r: Spearman's rank. r.u.: relative units

1.5 Discussion

The SML is known to influence exchange processes between the atmosphere and water bodies by physicochemical processes, e.g., the dampening of capillary waves induced by surface-active substances (Frew 1997). It has long been suggested that bacteria within the SML (bacterioneuston) play a pivotal role in these exchange processes. However, determining whether the bacterioneuston is 'successful' (Maki 1993) remains controversial, with previous studies reporting conflicting results regarding bacterioneuston activity, abundance, and diversity compared to bacterioplankton.

Critical evaluation of SML sampling techniques

The application of SML sampling techniques must take into account the thickness of the sampled SML and the mode of operation of the chosen sampling device, especially its degree of selectivity. Astonishingly, and as pointed out by others, only a few studies have examined the potential bias of sampling devices with respect to biological parameters (Hatcher & Parker 1974, Agogué et al. 2004, Franklin et al. 2005). In one such study, which extensively examined the possible bias of MS- and GP-type samplers, no selective effects were found for most of the bacterial parameters studied (Agogué et al. 2004).

In light of those observations, ^{3}H- TdR incorporation in different SML samples of the Southern Baltic Sea was measured. Variable but overall reduced ^{3}H-TdR incorporation was measured in GP and MS samples compared to incorporation in the underlying water; this was in contrast to the unchanged incorporation measured on one occasion in a RD sample, where MS and RD samples were taken simultaneously at the same station. Assuming that the SMLs of the MS and RD samples were identical except for the thickness of the collected layer, these results are surprising since inhibitory factors should have had a more pronounced effect on the RD samples because of less dilution with bulk water. This discrepancy was examined in tank experiments with the GP and MS samples; the results showed a strong reduction of ^{3}H-TdR incorporation in samples obtained with either device compared to the incorporation measured in reference water. A selective

reduction of bacterial productivity has not been reported before, although productivity measurements in another tank experiment involving GP and MS samplers were quite variable (Agogué et al. 2004).

Sampling devices differ in their mode of operation and in the material of the sampler itself (Huehnerfuss 1981). Based on the results of the present study, we conclude that the modes of operation of GP and MS devices are most likely not selective, because bacterial abundance and community composition, based on 16S rDNA fingerprints, were comparable to the reference water in all experiments. Adsorption of bacteria to the GP might stress the cells, reflected in a reduction of bacterial productivity, but a similarly pronounced reduction occurred in samples collected with the MS, in which the water is simply trapped within the mesh of the screen.

Another reason for the observed reduction in bacterial productivity might have been the presence of inhibitory substances, originating from the sampling devices themselves. Most authors of previous research either did not mention how or if their sampling devices were treated, or reported treatment of the GP with Milli-Q water and ethanol (Agogué et al. 2004), HCl (Reinthaler et al. 2008), or silane (Gever et al. 1996). We tested these different treatments, none of which had an effect on the reduced ^{3}H-TdR incorporation. Furthermore, in all treatments the rate of ^{14}C-leucine incorporation was reduced.

Ultimately we developed a sampling technique using a different wiping mechanism, that restored ^{3}H-TdR incorporation and CTC uptake. One possible, but highly speculative, reason for the success of this method is that it increased the speed of sampling, which was twice as fast as that of the framed-wiper technique and thus reduced the time between sampling and the start of the incubations. However, ^{14}C-leucine incorporation in our experiments was still reduced, which might have been caused by isotope dilution due to higher leucine concentrations in the GP samples. In our experiments, we did not measure amino acid concentrations, but they have been shown to be enriched in the SML (Kuznetsova et al. 2004) and considered as external dilution factors in productivity rates of the bacterioneuston (Reinthaler et al. 2008). Although the water was homogenized prior to sampling, the rapid accumulation especially of hydrophobic amino acids might have occurred. While there is no satisfactory explanation for the persistent decrease in ^{14}C-leucine incorporation, it suggests that bacterial activity parameters are unequally

affected by SML sampling devices. Furthermore, ^{3}H-TdR incorporation was found to be the suitable method to measure bacterioneuston productivity with our sampling device.

Interpretation of SML samples 2007

During our studies in the Baltic Sea, bacterial cell counts did not differ between SML samples and the underlying bulk water. Absolute cell numbers were consistent with those previously reported for the surface waters of the southern and central Baltic Sea (Heinänen 1991, Schumann et al. 2003). An enrichment of colony-forming units in some SML samples from the Western Swedish coast has also been reported (Hermansson et al. 1987). However, to the best of our knowledge, cultivation-independent bacterial cell numbers in SML samples from the Baltic Sea have, until now, not been published.

The abundance of CTC-positive cells in our SML samples was highly variable but overall similar to that of the underlying water. This was evident for absolute cell counts and relative values (8 to 37 %) compared to total bacterial cells. Additionally, flow cytometric analysis of samples of the SML and the underlying water showed a similar abundance of bacterial cells with a high nucleic acid content (data not shown), which are thought to represent the active part of the bacterial community (Gasol et al. 1999).

These results imply that an active bacterioneuston community exists. The SML is thought to reflect conditions favourable to heterotrophic bacteria (Sieburth et al. 1976) due to the accumulation of organic and inorganic substrates (Hunter 1997). Enriched concentrations of TOC have been found to correlate with increased community respiration in the SML compared to the underlying bulk water (Obernosterer et al. 2005). Elevated substrate supply might also explain enhanced enzymatic activities of the bacterioneuston in different aquatic systems (Münster et al. 1998, Kuznetsova & Lee 2001). However, several authors have reported highly variable or no significant changes of bacterial productivity in the SML (Joux et al. 2006, Reinthaler et al. 2008), or even a decrease compared to the underlying bulk water (Obernosterer et al. 2008) although in all studies organic matter was found to be enriched in the SML.

In the present study, we also detected an accumulation of TOC and TN in the SML of the Baltic Sea. The enrichment of TN was more pronounced, in agreement with a recent

report in which dissolved organic nitrogen (DON) was more enriched than dissolved organic carbon (DOC) (Reinthaler et al. 2008). Due to sample volume limitations, we were only able to determine total organic material (TOM) and therefore could not differentiate between the dissolved and the particulate fraction. A recent study found a constant accumulation of particulate organic carbon (POC) but not DOC in nylon screen samples (Obernosterer et al. 2008), a result consistent with previous work which concluded that enrichments for dissolved matter are not as pronounced as those for the respective particulate material (Hunter 1997). However, this is not a common feature, as the TOC in SML samples obtained with a GP as well as the underlying water, has been found to contain similar high ratios of DOC (Reinthaler et al. 2008).

The enrichment of TOM in our samples apparently did not fuel bacterial activity. Instead, there was an overall reduction in the incorporation of ^{3}H-TdR in SML samples collected from different stations during the summer of 2007, suggesting that DNA synthesis and thus cytokinetic activities were generally impaired. However, there is only one previous study to use ^{3}H-TdR as a measurement for bacterial production in SML samples obtained from a GP showed that incorporation was highly variable but overall not significantly changed compared to incorporation in the underlying water (Agogué et al. 2004). Diverse factors seem to influence the relationship between substrate supply and bacterial productivity in the SML. Variable ^{3}H-TdR incorporation has been discussed to be affected by diel cycles, with increasing uptake during the night, although overall incorporation was still consistently found to be lower compared to the underlying water (Carlucci et al. 1986). This might result from the inability of the bacterioneuston to take up the rich pool of nutrients during high UV exposure as reported for bacterioplankton (Herndl et al. 1993), perhaps due to direct (DNA damage) or indirect (photochemical alteration of substrate) effects. Yet these diel patterns do not always explain the variable productivity of the bacterioneuston, e.g. when possibly being masked by wind influences (Reinthaler et al. 2008).

Wind speed influences the properties of the SML. A recent study found that decreasing leucine incorporation by the bacterioneuston was correlated to low wind speeds (Obernosterer et al. 2008). In our experiments, however, the observed activity patterns did not correlate with the actual wind speed during sampling or with the wind history, defined as the mean wind speed up to 6 h prior to sampling (data not shown).

Furthermore, we found no correlation between CTC uptake and ^{3}H-TdR incorporation in the SML samples. Therefore, it seems that the bacterioneuston community remains metabolically active but does not proliferate as intensely as the bacterioplankton. This activity pattern was found in SML samples from coastal and open water samples as well as those in slick and non-slick samples.

Taken together, our data show that, despite the accumulation of TOM in the SML, bacterioneuston productivity is not enhanced. Rather, although the abundance of active cells is comparable to that in the underlying water, certain cellular activities, e.g., cytokinesis, are impaired.

1.6 Conclusions

There is an essential need to compare data from different studies in order to better characterize the bacterioneuston and to gain detailed insights into its structure and function. However, reliable comparisons of such data were thought to be impossible due to the different modes of operation of the various sampling devices (Carlson 1982a). Some other authors have suggested that only SML samples taken with the same type of sampling device can be compared (Van Vleet & Williams 1980). However, the present study shows that comparisons should be made carefully, and the results underline previous recommendations concerning the need to evaluate the sampling devices (Agogué et al. 2004).

Our *in situ* results support the view of the SML as a demanding, even stressful habitat (Dietz et al. 1976). Furthermore, the presence of a specifically adapted bacterial community in the SML is unlikely. Future studies should be aimed at specifying the factors influencing bacterioneuston activity. For this purpose, characterizations of the effects of different physicochemical parameters at the community and single-cell levels are essential.

Meteorological Conditions and Planktonic Members Shape the Coastal Bacterioneuston Community Structure in the Baltic Sea

(Chapter 2)

2.1 Abstract

The bacterioneuston resembles the bacterial community in the sea-surface microlayer (SML) and is exposed to unique physico-chemical properties and stronger meteorological influences compared to the underlying water (ULW). Despite extensive research, the structuring factors of the bacterioneuston remain enigmatic. The aim of this study was to examine the effect of meteorological conditions on differences between the bacterioneuston and bacterioplankton community structure and to identify dominant, active bacterioneuston members. We compared 19, randomly taken, bacterial assemblages in the SML and ULW of the southern Baltic Sea. The analysis was based on single-strand conformation polymorphism (SSCP) fingerprints, where, for the first time reported for the SML, present (16S rRNA gene) and active (16S rRNA) as well as non-attached (NA) and particle-attached (PA) members were distinguished. The NA communities revealed overall high similarities (> 47%) between the SML and ULW, which was more pronounced in the 16S rRNA gene fingerprints. However, during low wind speed and high radiation levels the active bacterioneuston was increasingly different from the bacterioplankton. In contrast, the similarities of the PA communities ranged from 8% to 98% between the two compartments. This strong variability was only related to wind speed in the 16S rRNA fingerprints. We were able to identify 66% of all SML-specific SSCP bands based on their relative band intensity. The active bacterioneuston in the NA and PA size fractions consisted of members from the *Cyanobacteria*, *Bacteriodetes* and *Proteobacteria* from diverse habitats, especially from water columns. Our results suggest that bacterioneuston communities are strongly influenced by the ULW, but that specific meteorological conditions (low wind, high radiation) favour distinctive populations in the SML.

2.2 Introduction

The sea-surface microlayer (SML) is located within the air-sea interface and is characterised by specific physico-chemical properties (Liss & Duce 1997). Its inhabiting bacterial community (bacterioneuston) is exposed to substantially different environmental conditions compared to the bacterioplankton in the underlying water (ULW). One characteristic is the accumulation of diverse organic and inorganic compounds in the SML (Hunter 1997). Consequently, enhanced exo-enzymatic activities in the SML have been found in different habitats (Münster et al. 1998, Kuznetsova & Lee 2001). Nevertheless, bacterial growth in the SML seems to be limited despite high concentrations of potential substrate supply (Reinthaler et al. 2008), and reduced bacterioneuston productivity suggests that the SML is a stressfull habitat (Obernosterer et al. 2008, Chapter 1). Inhibitory conditions for the bacterioneuston might result from increased exposure to organic pollutants, heavy metals and UV-radiation (Maki 1993, Wurl & Obbard 2004). Thus, the question arises, whether these environmental factors thrive a specific bacterioneuston assemblage in this unusual habitat.

Previous studies provide contradictory evidence on differences between bacterioneuston and bacterioplankton community compositions. 16S rRNA gene clone libraries from the North Sea revealed a substantially lower diversity in the SML compared to the ULW (Franklin et al. 2005). Furthermore, bacterial communities in the SML of alpine lakes were more similar to airborne than to planktonic populations (Hervas & Casamayor 2009). In contrast, 16S rRNA gene fingerprints from the Mediterranean Sea and the Atlantic Ocean showed no consistent differences between the SML and the ULW (Agogué et al. 2005a, Obernosterer et al. 2008). Additionally, nearly all bacterial strains, isolated from the Mediterranean SML, were closely related to environmental clones from diverse marine habitats (Agogué et al. 2005a). These isolates did not show different resistance patterns to UV-exposure compared to their planktonic counterparts, indicating that adaptation to environmental stress factors is not a common trait among neustonic bacteria (Agogué et al. 2005a). However, different members of bacterioneuston and bacterioplankton communities were presumably involved in leucine-uptake and trace gas metabolism (Cunliffe et al. 2008, Obernosterer et al. 2008), indicating that specific

processes include different community members of bacterial assemblages in the SML and the ULW.

At least two factors limit general comparisons between neustonic and planktonic communities: the high variability in the SML as well as the employment of different sampling devices. One characteristic of the SML is its high spatial and temporal variability. Thereby, the bacterioneuston community is much more influenced by meteorological conditions than the bacterioplankton. For instance, high wind speed causes increasing turbulence and breaking waves disturb the SML. In turn, decreasing wind speed reduces surface mixing, and under highly calmed conditions, visible surface films (slicks) are formed, which can cover large areas of coastal waters (Romano 1996). Therefore, the variability of SML-properties due to changing meteorological conditions might at least partially explain the opposing results of bacterioneuston diversity reported so far. SML-sampling techniques differ in their operating mode and consequently collect SML-samples of different layer thickness (Huehnerfuss 1981). Recent studies have adressed this problem, showing that comparison of different SML studies have always to be done with caution (Agogué et al. 2004, Cunliffe et al. 2009a, Chapter 1).

The aim of this study was to get further insights into patterns of bacterioneuston community structure and composition under varying meteorological conditions. We analysed 16S rRNA as well as 16S rRNA gene fingerprints from SML- and ULW-samples of the southern Baltic Sea offshore Warnemuende using single-strand conformation polymorphism (SSCP). Thereby, non-attached and particle-attached members of the bacterial communities were distinguished. Furthermore, SCCP-bands from the 16S rRNA fingerprints, which were solely detectable in the SML, were identified by retrieving partial 16S rRNA gene sequences. Our results show that non-attached communities in the SML and the ULW were highly similar, but that the particle-attached communities were very different. In both size fractions, decreasing wind speed was related to increasing differences between the communities, especially in the 16S rRNA fingerprints. The latter revealed that active bacterioneuston members were closely related to environmental clones from diverse habitats.

2.3 Materials and Methods

Study site and sea-surface microlayer sampling

Samples from the sea-surface microlayer (SML) were taken in the southern Baltic Sea offshore Warnemuende (Fig. 2.1) between July and September 2006, June and August 2007 as well as February and May 2008 (Table 2.1). Sampling was performed between 7 to 11 local time using the glass-plate technique (Harvey & Burzell 1972). Control samples from the underlying water (ULW) were taken from 1 meter depth with a 2 l glass collection tube which ends were closed by a drop-weight mechanism. Samples were returned to the laboratory and processed for subsequent analysis usually within 1 to 2 hours after sampling. Meteorological data were recorded and provided by the meteorological station in Rostock-Warnemuende, located at the shore in proximity to the study site. Data for total organic carbon (TOC) in the SML and the ULW were taken from Chapters 1 and 3.

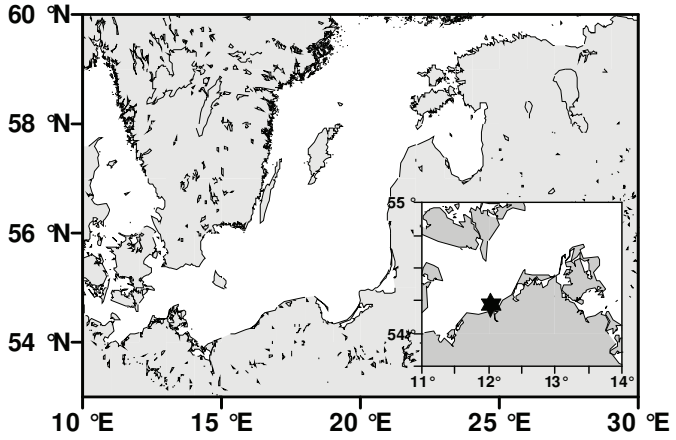

Fig. 2.1 Sampling site in the southern Baltic Sea. Samples were taken between July 2006 and May 2008 (see Table 2.1) in proximity to position N 54° 11'; E 12° 04' (asterisk).

Extraction of nucleic acids and fingerprint analysis

Water samples were filtered on 3-µm Isopore filters (Millipore), which are presumed to retain the particle-attached fraction. The filtrate (i.e. the non-attached fraction) was then filtered on 0.22-µm Isopore filters (Millipore). All filters were rapidly frozen in liquid nitrogen and stored at -80°C. DNA and RNA from frozen filters were extracted using a phenol-chloroform-extraction method according to Weinbauer et al. (2002). After the first steps of cell lysis, the samples were separated and a pH-dependent procedure was applied to extract DNA (phenol-chloroform pH = 7.5 to 8) or RNA (phenol-chloroform pH = 4.5 to 5). All extracts were washed with ethanol and quantified spectrophotometrically using a NanoDrop ND-1000 (NanoDrop Technologies).

For the analysis of the presumably active community, the RNA was reverse transcribed into complementary DNA (cDNA). Therefore, co-precipitated DNA in 10 µl aliquots (accounting for 40 ng to 1 µg total nucleic acid concentrations) was removed using DNAseI (DNA-free kit, Ambion), following the manufacturers instructions. Synthesis of cDNA from 10 to 20 ng RNA was performed in a Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR) using the universal primer 1492R (5'-GGTTACCTTGTTACGACTT-3') (Lane 1991) and the iScript cDNA synthesis kit (Bio-Rad). Complete removal of DNA was tested with an additional RT-PCR without enzyme. Successful synthesis of cDNA was checked in a following PCR, as described below.

The 16S rRNA and 16S rRNA-gene fingerprints of the bacterial communities were analyzed using single-strand conformation polymorphism (SSCP). DNA extracts as well as cDNA amplicons were PCR-amplified using primers Com1f (5'CAGCAGCCGCGGTAATAC3') and Com2r-Ph (5'CCGTCAATTCCTTTGAGT TT3') (Schwieger & Tebbe 1998), which amplify *Escherichia coli* 16S rRNA-gene positions 519 to 926, following the protocol from Labrenz et al. (2007), with an annealing-temperature of 50°C. Single-stranded DNA was generated and purified and the SSCP analysis carried out as described in Schwieger & Tebbe (1998). Pairwise comparisons of band patterns from the SML and ULW were done in the digitized images with GelCompare II (Applied Maths NV) based on their densiometric profile (Pearson correlation) after background substraction, least square filtering and optimization according to the manufacturers' instructions. The results of the comparisons are

expressed as pairwise dissimilarities, i.e. the percentage difference between the bacterioneuston and bacterioplankton community composition.

Sequence analysis

Individual bands from the SSCP gels, which were exclusively observed in the 16S rRNA fingerprints of the SML samples were excised and re-amplified according to Labrenz et al. (2007). PCR products were purified using the NucleoSpinII Extraction Kit (Macherey&Nagel) as described by the manufacturer, and were sequenced by QIAGEN using primers Com1f and Com2r. Forward and reverse sequences of all bands were checked for accuracy using the SeqMan software (DNASTAR). Phylogenetic affiliations of the partial 16S rRNA sequences were estimated using the basic local alignment search tool (BLAST) (Altschul et al. 1997). Alignments were checked and the sequences taxonomically grouped employing the ARB software package (Ludwig et al. 2004).

Statistical analysis

The Wilcoxon-test (significance level $p < 0.05$) was applied to test differences of the results of pairwise comparisons between the non-attached and particle-attached size fraction as well as between 16S rRNA and 16S rRNA gene fingerprints. Spearman's rank correlation (significance level $p < 0.05$) was applied to test the association between pairwise comparisons and meteorological conditions as well as TOC. Additionally, non-linear regression was applied for further analysis of this association.

2.4 Results

Bacterioneuston and bacterioplankton community structure

A total of 19 samples from the sea-surface microlayer (SML) in the southern Baltic Sea were taken (Fig. 2.1, Table 2.1). Its inhabiting bacterial community (bacterioneuston) was compared to the bacterioplankton community of the underlying water (ULW). Values for the pairwise dissimilarities of the non-attached (NA) communities ranged between 3.1 % and 53.3 % in the 16S rRNA fingerprints (presumably active community members) and between 1.4 % and 33.7 % in the 16S rRNA gene fingerprints (present community members) (Fig. 2.2A). The pairwise dissimilarities of the particle-attached PA communities revealed values ranging between 2.2 % and 74.6 % in the 16S rRNA fingerprints and between 3.4 % and 92.2 % in the 16S rRNA gene fingerprints (Fig. 2.2A). Mean values for the pairwise dissimilarities of the 16S rRNA and 16S rRNA gene

Table 2.1 Sea-surface microlayer samples from the southern Baltic Sea (see Fig. 2.1). Samples were taken between July 2006 and May 2008. When two samples were taken on the same day (e.g. samples 4 and 5), sampling sites were about 1 km apart.

Station	Date
1	19 Jul 2006
2	19 Jul 2006
3	03 Aug 2006
4	18 Aug 2006
5	18 Aug 2006
6	07 Sep 2006
7	19 Sep 2006
8	19 Sep 2006
9	18 Jun 2007
10	18 Jun 2007
11	19 Jun 2007
12	20 Jun 2007
13	14 Aug 2007
14	20 Feb 2008
15	27 Mar 2008
16	06 May 2008
17	07 May 2008
18	08 May 2008
19	09 May 2008

fingerprints were 15.3 % and 8.2 %, respectively, in the NA size fraction and 37.2 % and 39.2 %, respectively, in the PA size fraction. Overall, these comparisons showed significant differences between the two size fractions in both, the 16S rRNA as well as 16S rRNA gene fingerprints (Wilcoxon-test, $p < 0.001$, $n = 19$; Figure 2.2B). In the NA size fraction, values of the pairwise dissimilarities from the 16S rRNA fingerprints were consistently different from values of the 16S rRNA gene fingerprints (Wilcoxon-test, $p = 0.009$, $n = 19$; Figure 2.2B). In contrast, there were no significant differences of these values between the 16S rRNA and 16S rRNA gene fingerpints in the PA size fraction.

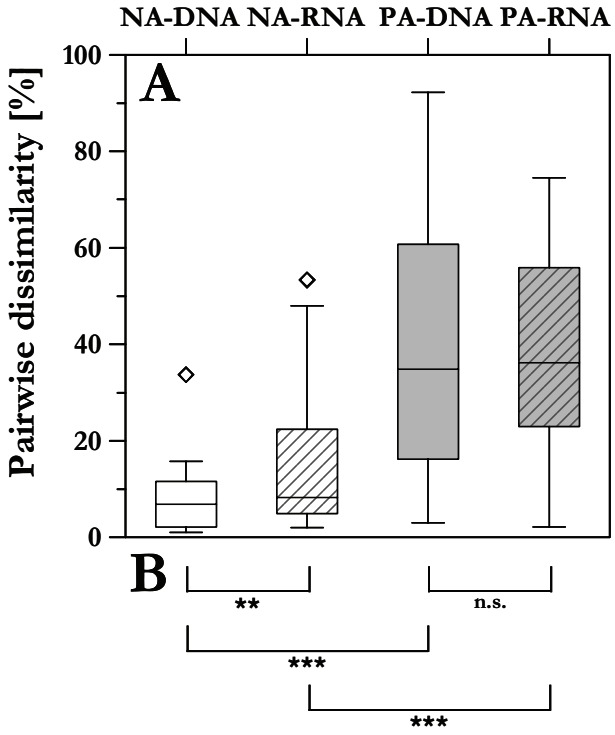

Fig. 2.2 (A) Mean values for pairwise dissimilarities between bacterioplankton and bacterioneuston communities of 16S rRNA (RNA) and 16S rRNA gene (DNA) fingerprints of the non-attached (NA) and particle-attached (PA) communities (n = 19). (B) The statistical analysis to test the differences between the four groups was based on the percentage dissimilarity between bacterial community structure in the sea-surface microlayer and the underlying water (Wilcoxon-test). **p < 0.01, ***p < 0.001, n.s. = not significant.

In order to examine possible effects of meteorological conditions on the differences between the bacterioneuston and bacterioplankton community composition, values for the pairwise dissimilarities were plotted against the wind speed, global solar and UV-A

radiation during sampling as well as against mean wind speed and mean global solar radiation 6 h prior sampling. Spearman's rank correlation revealed no relation between any of these parameters and the differences among the bacterial communities (data not shown), except between the mean wind speed 6 hour prior sampling and the 16S rRNA fingerprints of the PA community (Spearman rank correlation coefficient $r_s = 0.542$, $p = 0.016$, $n = 19$; Figure 2.3). Additionally, the relation between differences in the fingerprints and the accumulation of total organic carbon (TOC) was tested and showed only significant values related to the 16S rRNA gene fingerprints in the PA size fraction ($r_s = 0.733$, $p = 0.016$, $n = 10$). Despite this overall lack of correlation, obviously, the differences among the NA communities in the 16S rRNA fingerprints tended to increase at lowest wind speeds as well as highest radiation levels (Fig. 2.3). In order to describe these effects, non-linear regression was applied and showed that the NA 16S rRNA fingerprints were associated to mean wind speed 6 h prior sampling ($R^2 = 0.783$), mean global solar radiation 6 h prior sampling ($R^2 = 0.473$) and global solar ($R^2 = 0.467$) and UV-A radiation ($R^2 = 0.418$) during sampling (Fig. 2.3). Differences in the 16S rRNA gene fingerprints of the NA communities were only associated to the mean wind speed 6 h prior sampling ($R^2 = 0.659$). In contrast, non-linear regression did not explain any influence of meteorological conditions on the patterns within the PA size fraction (data not shown). Taken together, wind speed history was related to all pairwise dissimilarities, except in the PA 16S rRNA gene fingerprints. Moreover, all meteorological parameters, except the wind speed during sampling, were related to differences in the NA 16S rRNA fingerprints.

Identification of bacterioneuston community members

The aim of this study was not to identify and compare all community members in the SML and ULW, but to determine specific, active bacterioneuston members and thus, only SSCP-bands, which were solely detectable in the 16S rRNA fingerprints of the SML were analysed. In 13 out of the 19 stations, 133 SSCP bands were exclusively found in the SML fingerprints, from which 52 bands belonged to the non-attached and 81 bands belonged to the particle-attached size fraction. 39 % of all sequences (n = 52) yielded sufficient

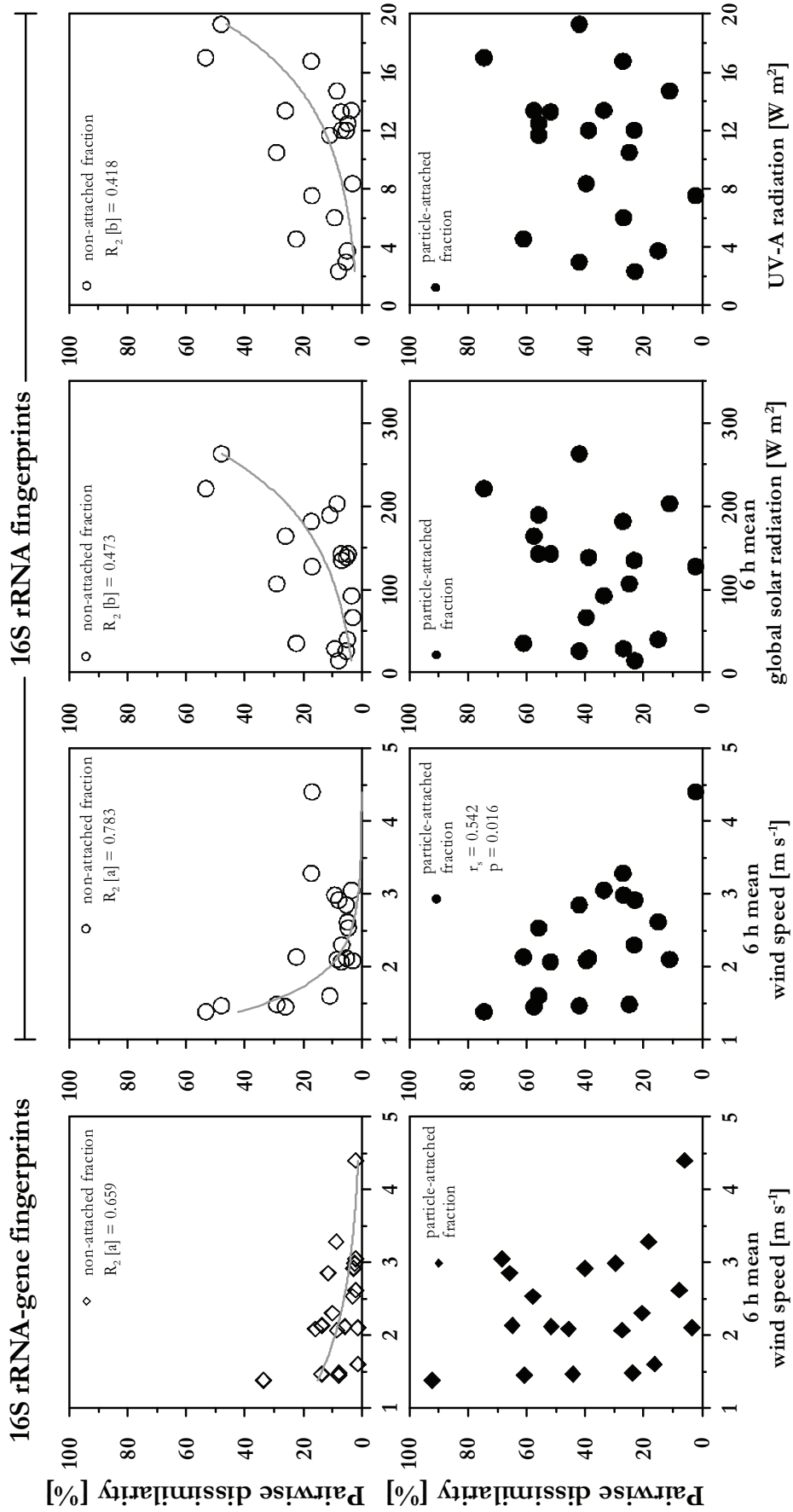

Fig. 2.3 Relationship of meteorological conditions and the pairwise comparison between bacterioneuston and bacterioplankton community structure as revealed by 16S rRNA (gene) fingerprints. Significant correlations (r_s = Spearman correlation coefficient) are given. Additionally, non-linear regression using exponential decline (R_2 [a]) and exponential growth (R_2 [b]) is indicated (grey line).

sequencing results, accounting for 66 % (± 28%, n = 13) of all SML-specific sequences, based on their relative band intensity within each sample. Sequences with bad quality (n = 81) were rejected. These sequences were mostly derived from SSCP bands which were very faint, showing < 1% of relative band intensity (data not shown). Generally, neither the number of total SML-specific bands nor the number of sequences with good quality was related to the pairwise dissimilarities between the SML and ULW or any of the environmental factors (data not shown). Notably, the highest number of sequences (n = 16) per single sample was retrieved from station 17 (Table 2.2), where a dense surface film (slick) was observed (Chapter 3).

Only 3 out of the 52 sequences showed less than 97% 16S rRNA sequence similarity to environmental clones in the NCBI database (Table 2.2). Sequences, which revealed ≥ 97% sequence similarity to the identical environmental clone, were grouped into one operational taxanomic unit (OTU). A total of 32 OTUs were identified and their closest environmental clones were from diverse habitats (Table 2.2). Relative band intensities of the OTUs were highly variable, ranging from 0.3 % to 13.8 % (Table 2.2). We identified members of the phyla *Cyanobacteria* and *Bacteriodetes* as well as of the Alpha-, Beta-, and Gamma-subgroups of the phylum *Proteobacteria* to be active members of the SML. Most OTUs belonged to the *Gammaproteobacteria* (n = 12), the *Cyanobacteria* (n = 7) and the *Bacteriodetes* (n = 5) (Table 2.2). Overall, within each phylogenetic group, several orders and families were classified, except for the *Betaproteobacteria*, where only members of the *Burkholderiales* were identified (Table 2.2). Generally, all groups showed a similar

Table 2.2 16S rRNA gene similarities (OTUs) of SSCP bands which were exclusively identified in the sea-surface microlayer. The origin of the SSCP bands is indicated by the sample number (see Table 2.1) as well as by the size fraction (N = non-attached, P = particle-attached). Furthermore, the relative band intensity of each OTU in the sample is shown. The number of sequences (n) which were grouped into one OTU and their similarity to the closest environmental clone as well as their habitat (NCBI database) is given. Moreover, the phylogenetic affiliation of the OTUs was classified according to their alignment in the ARB database. Their closest type strain as well as the distance based on neighbourhood joining distance matrix is shown.

	OTU	Sample	Fraction	Band intensity [%]	n	16S rRNA sequence similaritiy [%]	Accession	Habitat / Strain	Phylogenetic Affiliation	Type strain	Distance matrix
						BLAST (NCBI)			ARB		
Alpha	BaSML1	6	N	3.9	1	98	EU799901	marine harbour	*Rhodospirillales*	*Thalassobaculum sp. P24*	0.1107
	BaSML2	9	N/P	5.5	2	98	EF471665	coastal water	*Rhodobacteraceae*	*Marivita litorea*	0.0331
	BaSML3	11	P	0.9	1	99	AY922222	marine microbial mat	*Rhodobacteraceae*	*Maricaulis salignorans*	0.0057
	BaSML4	4	N	3.3	2	100	EF414048	sponge-associated	*Sphingomonadaceae*	*Erythrobacter seohaensis*	0.0179
Beta	BaSML5	17	N	3.5	1	98	DQ791365	geothermal-heated soil	*Burkholderiales*	*Leptothrix mobilis*	0.0240
	BaSML6	15	P	0.5	1	99	EF125953	biofilm reactor	*Burkholderiales*	*Leptothrix discophora*	0.0094
	BaSML7	7, 8	N	3.5	2	100	GU088518	tropical lagoon water	*Burkholderiales*	*Hydrogenophilus thermoluteolus*	0.0658
Gamma	BaSML8	4	N	3.6	2	100	FJ527418	forest soil	*Chromatiaceae*	*Rheinheimera aquamaris*	0.0077
	BaSML9	9	N/P	2.8	2	99	AM110966	deep sea sediment	*Chromatiaceae*	*Rheinheimera baltica*	0.0000
	BaSML10	4	P	3.4	1	99	FJ516460	enrichment culture	*Chromatiaceae*	*Rheinheimera baltica*	0.0150
	BaSML11	19	P	7.8	1	100	DQ128248	drilling fluid	*Chromatiaceae*	*Rheinheimera perlucida*	0.0168
	BaSML12	1	P	7.7	1	99	CP001161	endosymbiont (insect)	*Enterobacteriaceae*	*Pantoea ananatis*	0.1067
	BaSML13	2	P	6.3	3	99	M63248	endosymbiont (insect)	*Enterobacteriaceae*	*Pantoea ananatis*	0.1139
	BaSML14	5	N	0.8	1	98	FJ353210	lake water	*Halothiobacillaceae*	*Halothiobacillus kellyi*	0.1176
	BaSML15	1	P	1.8	1	97	AF286124	associated to insects	*Coxiellaceae*	*Legionella drozanskii*	0.1358
	BaSML16	1, 17	P	3.2	2	98	AM905315	beach sediment	*Moraxellaceae*	*Alkanindiges illinoisensis*	0.0526
	BaSML17	17	N	0.3	1	98	GU001720	sediment of gas plant	*Shewanellaceae*	*Shewnella frigidimarina*	0.0193
	BaSML18	17	N	0.7	2	95	Y13699	*S. frigidimarina*	*Shewanellaceae*	*Shewnella frigidimarina*	0.0330
	BaSML19	17	N	12.8	4	99	GQ327990	intestine of gastropoda	*Shewanellaceae*	*Shewnella decolorationis*	0.0035
Bacteriodetes	BaSML20	3	P	0.4	1	95	FJ352992	lake water	*Cryomorphaceae*	*Owenweeksia hongkongensis*	0.1296
	BaSML21	3	P	2.8	2	97	AY580718	coastal water	*Cryomorphaceae*	*Owenweeksia hongkongensis*	0.1195
	BaSML22	4	N	1.3	1	98	EF573073	marine water	*Flavobacteriaceae*	*Flavobacterium cucumis*	0.0228
	BaSML23	17	N	3.4	1	100	EU878141	Baltic Sea mesocosms	*Flavobacteriaceae*	*Polaribacter filamentus*	0.0381
	BaSML24	11	P	0.4	1	100	EU703445	lake water	*Saprospiraceae*	*Haliscomenobacter hydrossis*	0.1126
Cyanobacteria	BaSML25	12	N	9.8	2	99	EF568895	Baltic Sea	*Halospirulina*	*Halospirulina tapeticola*	0.1254
	BaSML26	14, 17, 18	N/P	13.8	5	99	FM212461	Macrophyte-associated	*Halospirulina*	*Halospirulina tapeticola*	0.1333
	BaSML27	17	P	3.8	1	99	EF568894	Baltic Sea	*Halospirulina*	*Halospirulina tapeticola*	0.1331
	BaSML28	17	P	7.4	1	97	EF638722	coastal water	*Halospirulina*	*Halospirulina tapeticola*	0.1033
	BaSML29	9, 10	P	6.0	2	99	AF268009	*Nodularia spumigena*	*Halospirulina*	*Halospirulina tapeticola*	0.1036
	BaSML30	17	P	1.9	1	99	FJ355041	lake water	*Oscillatoria*	*Planktothricoides raciborskii*	0.1824
	BaSML31	13	P	1.6	2	99	AB469950	cow manure compost	*Oscillatoria*	*Planktothricoides raciborskii*	0.1667
	BaSML32	2	N	2.4	1	94	AY537289		unclassified		

distribution in the non-attached and the particle-attached size fraction with dominance of *Gammaproteobacteria* and *Cyanobacteria* (Fig. 2.4). While several phylogenetic affiliations, e.g. *Chromatiaceae*, were found in both size fractions, some were exclusively detected in the non-attached (e.g. *Shewanellaceae*) and particle attached (e.g. *Enterobacteriaceae*) size fraction (Table 2.2). However, only 3 OTUs were detected in both size fractions, whereas the remaining OTUs were specifically found in either the NA or PA fingerprints.

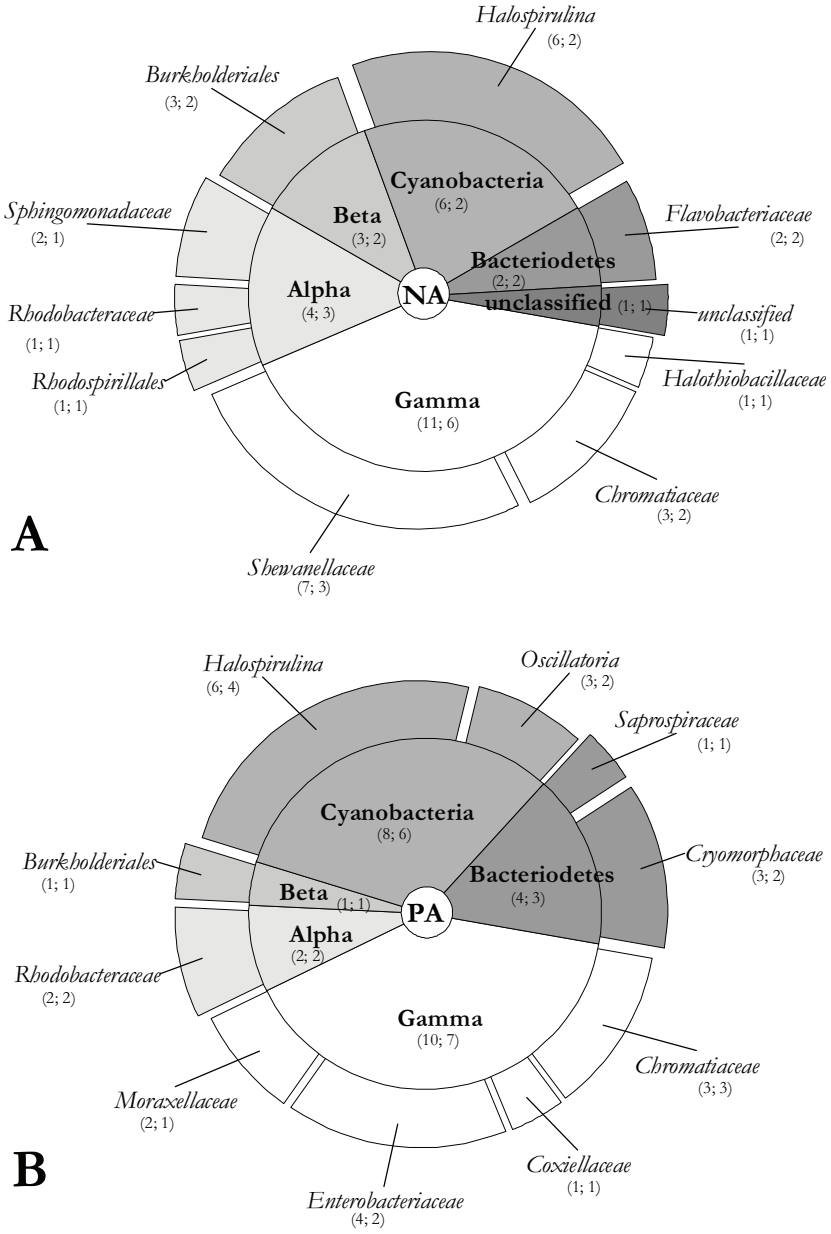

Fig. 2.4 16S rRNA sequence similarities of SSCP bands which were exclusively found in the sea-surface microlayer. The classification was based on their phylogenetic affiliation using the ARB database as well as BLAST results of the NCBI database (summary of Table 2.2). Results are differentiated between (A) the non-attached (NA) and (B) the particle-attached (PA) fraction. Values in brackets indicate (number of sequences; number of OTUs).

2.5 Discussion

The present study aimed to compare bacterial community compositions of the sea-surface microlayer (SML) and the underlying water (ULW) under varying meteorological conditions. Differences between the bacterioneuston and bacterioplankton 16S rRNA fingerprints increased with decreasing mean wind speed 6 h prior sampling. Global solar and UV-radiation were solely related to differences between the 16S rRNA fingerprints of the SML and ULW of the non-attached fraction, implying that wind history had the strongest structuring effect on bacterioneuston assemblages. Active members of the bacterioneuston, which were exclusively detected in the SML, belonged to *Cyanobacteria*, *Bacteriodetes* and the Alpha-, Beta- and Gamma-subgroups of *Proteobacteria* and were similarily distributed in the non-attached and particle-attached size fractions. A strong influence of the ULW on bacterial communties in the SML is suggested, as 16S rRNA gene sequences of these specific bacterioneuston members revealed high similarities to environmental clones mainly known from soils and different water columns.

Comparison of SML and ULW community structure

The comparison of bacterioneuston and bacterioplankton fingerprints was based on distinctions between the non-attached (NA) and the particle-attached (PA) size fraction as well as between the present (16S rRNA gene, DNA) and active (16S rRNA, RNA) community, which, to the best of our knowledge, has not been reported for bacterioneuston assemblages before. The comparisons between the SML and the ULW (i.e. pairwise dissimilarities) revealed substantial differences between the two size fractions. The pairwise dissimilarities were much more variable but generally higher within the PA compared to the NA fingerprints. The NA fingerprints revealed overall very similar community compositions in the SML and the ULW. This strong similarity in the NA size fraction was more pronounced in the DNA fingerprints compared to the RNA fingerprint. In contrast, DNA and RNA fingerprints were not significantly different in the

PA size fraction. Generally, our results point to the importance of these distinctions in order to receive higher resolutions of bacterioneuston diversity analysis.

Previous studies reported opposing results of how distinguishable bacterial communities in the SML and the ULW are. 16S rRNA gene-based community profiling showed distinctive diversity patterns of the bacterioneuston compared to bacterial assemblages in the ULW (Franklin et al. 2005, Cunliffe et al. 2008). In contrast, fingerprint techniques using the same molecular marker indicated very similar community structures between the SML and ULW (Agogué et al. 2005a, Obernosterer et al. 2008). This discrepancy is partly also caused by the different sampling techniques used (Agogué et al. 2004, Cunliffe et al. 2009a). The most important factors for high quality SML samples are: (I) less dilution with bulk water as possible, (II) no bias due to sampler selectivity, (III) large sample volume in reasonable sampling time and (IV) good handling (Garrett & Duce 1980, Huehnerfuss 1981). We choose the glass-plate sampling device for our study as (I) layer thickness was in the range of recommended 50 µm (data not shown) (Zhang et al. 2003, Cunliffe & Murrell 2009), (II) larger amounts of sample water were needed for accompanying analysis and (III) this sampling device showed no selectivity for the analysis of bacterial community composition (Chapter 1).

The influence of meteorological conditions

The present study shows that differences between bacterioneuston and bacterioplankton community compositions in both size fractions, except in the PA-DNA fingerprints, increased with decreasing wind speed. Thereby, only the wind history, expressed as the mean wind speed 6 h prior sampling, and not the actual wind speed during sampling, was related to differences in the fingerprints. Wind speed influences the general status of the SML and upon less wind-induced surface mixing the sea-surfaces calms. This in turn might promote the establishment of a distinct bacterioneuston community. Similar suggestions have been made for estuarine bacterioneuston assemblages, which are exposed to strong hydrodynamics (Santos et al. 2009) and might explain previous observations of highly distinctive bacterioneuston assemblages in mesocosm experiments as well as during slick formation (Cunliffe et al. 2009c, Chapter 3). Further evidence

comes from studies in alpine lakes, which are exposed to less wind stress than marine systems and show unique archaeal assemblages in the SML and ULW (Auguet & Casamayor 2008). Moreover, specific neustonic bacteria are known from limnic habitats, e.g. *Nevskia ramose* (Pladdies et al. 2004).

However, an association between wind speed and differences of bacterioneuston and bacterioplankton was not found in coastal Mediterranean waters (Agogué et al. 2005a), indicating that high spatial and temporal variability in the SML is also caused by other factors. Dynamic patterns in the PA size fraction of the present study were related to the accumulation of total organic carbon in the SML. The SML is generally characterised by a strong enrichment of organic and inorganic substances, especially of the particulate fraction (Hunter 1997). Under very calm conditions, e.g. in visible surface films, the particlulate fraction increasingly contributes to the total organic matter pool (Chapter 3). This suggests that the changing enrichment of particulate organic matter will describe differences in community compositions to a greater extent as was shown for bacterial abundances and productivity (Chapter 3). Further evidence of high differences of community composition between the SML and the ULW upon increased organic matter comes from mesocosm experiments, where autotrophic production caused high concentrations of transparent exopolymeric particles (TEP) in the SML (Cunliffe et al. 2009b, Cunliffe et al. 2009c).

Global solar and UV-A radiation showed only small effects on the differences between the bacterioneuston and bacterioplankton communities, implying low structuring effects on bacterioneuston assemblages. Similarily, near-surface bacterioplankton composition of the North Sea was only little influenced by changing levels of UV-radiation (Winter et al. 2001). However, biggest differences in the present study were observed under highest radiation levels in the 16S rRNA fingerprints of the NA size fraction, suggesting that increasing exposure to UV-stress changes the community composition of non-attached, active bacterioneuston members. In contrast, differences in the PA fingerprints were not related to changing levels of radiation, which might be due to shelter from UV-exposure upon association to particles (Wu et al. 2005, Hess-Erga et al. 2008).

Identification of active bacteria in the SML

In order to identify active members of the Baltic Sea bacterioneuston, SSCP-bands from 16S rRNA fingerprints of the PA and NA size fraction were sequenced. We were able to sequence most abundant members as revealed by their relative band intensities. Active members of the bacterioneuston were closely related to 16S rRNA sequences in the NCBI database which originated from diverse habitats such as marine, brackish and limnic waters as well as soils and associated to metazoa. Only 3 OTUs showed similarities < 97%, supporting previous findings that unknown taxa are not widespread among bacterioneuston populations (Agogué et al. 2005a, Hervas & Casamayor 2009). The number of OTUs which were retreived from any single station showed no relation to the environmental factors. This implies that the differences observed in the fingerprints were primarily due to relative changes in the abundance of same members in the SML and ULW and not to an increasing abundance of SML-specific taxa. In exception, 10 out of 32 OTUs were specifically detected in a slick-sample (station 17, Chapter 3), supporting the conclusions from the fingerprint comparisons, that highly calmed conditions promote distinctive bacterioneuston populations.

Most OTUs within the *Cyanobacteria* were detected in the PA size fraction and were dominantly related to sequences from planktonic habitats, e.g. *Nodularia spumigena*, which is highly abundant in Baltic Sea surface blooms (Ploug 2008). Several OTUs from the *Rhodobacteriaceae*, *Burkholderiales*, *Chromatiaceae*, *Moraxellaceae* and *Shewanellaceae* of the phylum *Proteobacteria* were allocated to sediments and microbial mats and most of these OTUs were found in the PA size fraction. Organisms from the sediment-water interface and particle-attached organisms might have specific requisites to inhabit the air-water interface, which has been considered to provide conditions comparable to solid substrata (Kjelleberg 1985). However, water depth at the sampling site in the southern Baltic Sea is shallow (~ 10 m). Therefore, sediment turbulence and passive transport of these OTUs into the SML could also have occurred, rather than specific colonization within the SML.

A large proportion of the OTUs in the NA and PA size fraction showed highest similarities to planktonic members from limnic, brackish and marine waters. Among these, all *Bacteriodetes* were related to bacterioplankton sequences. Interestingly, *Flavobacteriaceae* were only detected in the NA size fraction and among these, one OTU

was closely related to *Polaribacter filamentus*. This organism is known to contain gas vacuoles (Gosink et al. 1998) and indicates that members of the bacterioneuston may actively regulate their duration of stay in the SML. Generally, *Bacteriodetes* are widepread among bacterioneuston populations (Agogué et al. 2005a, Cunliffe et al. 2008, Obernosterer et al. 2008), which has been speculated to result from their high potential of degrading complex polymeric substances (Kirchman 2002). The dominance of the *Gammaproteobacteria* in the present study confirms previous findings from marine bacterioneuston assemblages using culture-independent (Franklin et al. 2005, Obernosterer et al. 2008) and culture-dependent (Tsyban 1971, Fehon & Oliver 1979, Donderski et al. 1999) methods. It was discussed to be due to their ability in quickly responding to nutrient enrichments (Pinhassi & Berman 2003) and additional evidence comes from experiments, where artificial dust input fueled *Gammaproteobacteria* (Reche et al. 2009). Isolated members of the *Gammaproteobacteria* and *Bacteriodetes* from the Mediterraneaen bacterioneuston had a strong contribution to highly UV-resistant strains (Agogué et al. 2005b). Furthermore, leucine-uptake in the SML was dominated by *Gammaproteobacteria* and *Bacteriodetes*, further indicating their strong contribution to bacterioneuston assemblages (Obernosterer et al. 2008). Notably, within the *Gammaproteobacteria* of the present study, *Chromatiaceae* showed a high contribution and were identified in the PA and NA size fractions. These sequences clustered closely together with *Rheinheimera baltica* and *Rheinheimera perlucida*, which were isolated from Baltic Sea surface waters and described to be aerobic and chemoheterotrophic (Brettar et al. 2002, Brettar et al. 2006). The high contribution of *Rheinheimera* sp. related organisms further suggests that the dominant active bacterioneuston members in the Baltic Sea do not specifically inhabit the SML.

2.6 Conclusions

Taken together, the identification of active bacterioneuston members and the high similarity between the NA communities in the Baltic Sea supports previous findings that the SML is biologically strongly influenced by the ULW (Joux et al. 2006). Future studies have to elucidate how atmospheric deposition contributes to SML-populations in marine systems, as it significantly influences limnic bacterioneuston assemblages (Hervas & Casamayor 2009). The high variability in the PA size fraction is only weakly linked to wind speed and probably reflects the importance of particles in this unusual habitat. Differences in NA community structure between the SML and the ULW are influenced by meteorological conditions. Under these conditions, certain processes, e.g. gas exchange, might be specifically influenced by the bacterioneuston (Cunliffe et al. 2008).

Succession of the Sea-Surface Microlayer in the Baltic Sea Under Natural and Experimentally Induced Low Wind Conditions [1]

(Chapter 3)

[1] Data of this chapter has been published in *Biogeosciences*, 2010, **7**, 2975-2988

3.1 Abstract

The sea-surface microlayer (SML) is situated within the boundary between the atmosphere and hydrosphere. High spatial and temporal variability of SML-properties, however, cause large uncertainties in our understanding of interactions between biotic and abiotic parameters within or across the air-sea interface. Obviously, wind speed is a major factor of changing the physical and chemical environment of the SML. In order to examine the temporal effects of minimized wind influence, SML-samples from the southern Baltic Sea as well as from mesocosm experiments in a marina were taken to study naturally and articifcially calmed sea surfaces. We analysed organic matter as well as abundance, ^{3}H-thymidine incorporation and community composition of bacteria in the SML (bacterioneuston) compared to the underlying bulk water (ULW). In all SML-samples dissolved organic carbon and nitrogen were only slightly enriched and showed low temporal variability, whereas particulate organic carbon and nitrogen were generally strongly enriched and highly variable. This was especially pronounced in a dense surface film (slick) which developed during calm weather conditions as well as in the artifically calmed mesocosms. Overall, bacterioneuston abundance and productivity correlated well with changing concentrations of particulate organic matter. Moreover, changes in the community composition in the field study were stronger in the particle-attached compared to the non-attached bacterioneuston. This implies that decreasing wind enhances the importance of particle-attached assemblages and finally induces a succession of the bacterial community in the SML. Eventually, an uncoupling of the bacterioneuston from the ULW, upon very calm meteorological conditions, results.

3.2 Introduction

The air-sea interface constitutes the boundary between the atmosphere and water bodies, which cover about 70 % of Earth's surface. The sea-surface microlayer (SML) is defined as the uppermost top of the water column and is located within this interface. Despite its small layer thickness (< 1 mm), the SML influences exchange processes due to its unique physico-chemical properties compared to the underlying water (ULW) (Liss & Duce 1997). However, the functional importance of bacteria in the SML (bacterioneuston) remains unclear. Despite their potential role in influencing gas exchange (Cunliffe et al. 2008), contradictory results of organic matter enrichment in the SML and resulting responses of the bacterioneuston are puzzling.

For decades, the SML has been shown to be enriched in diverse organic and inorganic substances compared to the ULW (Hardy 1982, Williams 1986, Liss & Duce 1997). Material in the SML originates from atmospheric deposition, e.g. by dust input or pollination events (Södergren 1987) or from production in the SML (Reinthaler et al. 2008). High correlations of organic and inorganic matter between the SML and the ULW suggest that the ULW is a major source of these compounds (Carlson 1983, Joux et al. 2006, Baastrup-Spohr & Staehr 2009). High concentrations of colloidal material (Bigg et al. 2004) and transparent exopolymer particles (TEP) (Wurl & Holmes 2008) support the model of the SML being a gelatinous film (Sieburth 1983, Cunliffe & Murrell 2009). A strong enrichment of particulate opposed to dissolved matter has often been found in the SML (Hunter 1997). Nevertheless, elevated concentrations of dissolved substances, like amino acids and carbohydrates, might support bacterial growth in the SML (Williams 1986, Kuznetsova & Lee 2002).

Indeed, enhanced enzymatic activities in the SML suggest that bacterioneuston metabolism is fueled (Münster et al. 1998, Kuznetsova & Lee 2001) and that organic matter in the SML is not only highly concentrated but also utilisable for microbial uptake. However, low bacterial growth efficiencies (BGE) in the SML indicate that the bacterioneuston is rather maintaining cellular biomass and does not grow strongly (Reinthaler et al. 2008). Other studies reported increased bacterial respiration rates as well

as decreased bacterioneuston productivity in the SML and support this observation (Obernosterer et al. 2005, Chapter 1).

High spatial and temporal variability in the SML might be a reason for this overall inconsistent picture. Variations of SML-properties are due to import- and export processes (atmospheric deposition, turbulent mixing), productivity of the habitat (seasonality, trophic state) as well as accumulation of inhibitory substances (organic pollutants, heavy metals). Moreover, meteorological conditions cause variable patterns in the SML. For instance, UV-radiation was suggested to influence diurnal cycles of organic matter transformations as well as changing bacterial productivity in the SML (Carlucci et al. 1986, Falkowska 2001). Furthermore, concentrations of particulate organic carbon and bacterial productivity in the SML were related to wind speed (Obernosterer et al. 2008). However, these factors do not always simply explain patterns of bacterioneuston activities (Reinthaler et al. 2008, Santos et al. 2009). Therefore, the complexity of processes in the SML still leaves large uncertainties about the relation of bacterioneuston communities and the physical and chemical environment of the SML.

The aim of this study was to elucidate the dynamics of organic matter accumulation as well as bacterial abundance, activity and community composition in the SML during minimized wind-induced surface mixing. Wind influences general physico-chemical properties of the SML and upon decreasing wind speed the stability of the SML is expected to increase. Therefore, SML-samples were taken in the southern Baltic Sea on four consecutive days under constant low wind conditions, following the formation and disintegration of a visible surface film (slick). Additionally, mesocosms were installed in a Baltic-Sea marina, in order to compare naturally influenced and artificially calmed sea surfaces.

3.3 Material and Methods

Field study site and sampling

Samples from the sea-surface microlayer (SML) were taken in the southern Baltic Sea offshore Warnemuende from May 6th to 9th 2008. Sampling was performed between 9 and 11 local time from a zodiac against wind direction to avoid contamination of the samples. SML samples were obtained using the glass-plate technique (Harvey & Burzell 1972). There is evidence that glass-plate samples underestimate concentrations of parameters in the SML due to dilution with bulk water (Cunliffe et al. 2009a). However, this sampling device was choosen to obtain sufficient quantities of the SML. Furthermore it was shown to introduce no bias on measurements of many bacterial parameters (Agogué et al. 2004, Chapter 1). Control samples from the underling water (ULW) were taken from 1 meter depth with a 2 l glass collection tube which ends were closed by a drop-weight mechanism. Samples were returned to the laboratory and processed for subsequent analysis usually within 1 to 2 hours after sampling. SML measurements were compared to results from the ULW and are expressed as enrichment factors (EF) according to following definition: $EF = [x]_{\mu}/[x]_{b}$, where [x] is the concentration of a given parameter in the SML (μ) and the ULW (b) (GESAMP 1995). Wind speed was recorded and the data provided by the meteorological station in Rostock-Warnemuende, located at the shore in proximity to the study site.

Mesocosm experiments

Mesocosm experiments were performed in a marina within the estuary mouth of the river Warnow using circular floatation barriers. Each mesocosm had a surface area of 10.5 m^2 and was open to the bottom. Three independent experiments were conducted in November and December 2008 and each experiment was performed for four consecutive days. Initially, three mesocosms were employed in each experiment. However, in each

experiment only one mesocosm stayed intact during the four-day period. All samples were taken between 8 to 9 local time each day. About 50 ml of the SML was sampled, which was achieved by 7 to 10 glass-plate samplings. Given the layer thickness of 27 μm to 38 μm and a sampling area of 2000 cm^2 of the glass-plate, 13% to 19% of the total area inside the mesocosms was sampled. The small volume was chosen to avoid sampling of the complete surface inside the mesocosms. The SML outside the mesocosms was sampled in triplicates. Samples of the ULW were taken from 30 cm depth inside and outside the mesocosms.

SML samples from the first day inside the mesocosms were not included in the analysis in order to reject potential bias introduced by the mesocosms. We observed different enrichments in the SML between in- and outside the mesocosms upon installation of the mesocosms, which was most likely caused by mixing of the sea-surface as well as by material adhering to the mesocosms in preceeding experiments, although the mesocosms were intensively rinsed prior each experiment. However, after 24 hours this artificial shift was balanced and comparable to values of the SML outside the mesocosms.

Bacterial abundance and activity

For the analysis of total bacterial cell numbers, samples were fixed with paraformaldehyde (1 % final concentration)/glutaraldehyde (0.05 % final concentration) in the dark for 1 h at 5°C, then frozen in liquid nitrogen and stored at -80°C until analysis by flow cytometry. Heterotrophic bacteria were stained with SYBR Green (2.5 μM final concentration, Molecular Probes) for 30 min in the dark. Cells were counted using a Becton & Dickinson FACScalibur flow cytometer equipped with a laser emitting at 488 nm at a constant flow rate (35 μl min^{-1}). Yellow-green latex beads (0.5 μm, Polysciences) were used as an internal standard. Bacteria were detected by their signature in a plot of side scatter (SSC) versus green fluorescence (FL1).

For assessing the abundance of highly active bacteria in the meocosm experiments, 900 μl of a sample were incubated with 100 μl of a 5-cyano-2,3-ditolyl tetrazolium chloride (CTC, Polysciences) solution (4 mM final concentration) in the dark at the in situ temperature for no longer than 1 h (Gasol & Arístegui 2007). CTC-uptake was stopped

by fixation of the samples with paraformaldehyde (1 % final concentration)/glutaraldehyde (0.05 % final concentration) in the dark for 10 min at 5°C. The samples were then frozen in liquid nitrogen and stored at -80°C. Cells were counted with a flow cytometer as described above, except that the beads for the internal standard were 1 µm in size and cells were detected by their signature in a plot of orange fluorescence (FL2) versus red fluorescence (FL3).

The incorporation of ^{3}H-methyl-thymidine (^{3}H-TdR) (60.1 Ci mmol^{-1}, 10 nM final concentration, Moravek Biochemicals) was measured to determine heterotrophic bacterial productivity in 5-ml water samples from the field study according to the method of Chin-Leo & Kirchman (1988). Triplicate samples were incubated for 1 h at the *in situ* temperature in the dark. Due to sample volume limitation, only 2.5 ml of the samples were incubated in the mesocosm experiments. This, however, had no effect on the observed bacterial productivity compared to 5 ml samples (data not shown). Incorporation was stopped by fixing the cells with formaldehyde (10 % v/w) in the dark overnight at 5°C. A fourth sample, serving as a blank, was fixed for at least 10 min prior to the addition of ^{3}H-TdR. All samples were filtered on 0.22-µm polycarbonate filters (Millipore). 4 ml of scintillation cocktail were added to the filters after which the incorporated substrates were counted in a scintillation counter (Packard).

Extraction of nucleic acids and fingerprint analysis

Water samples from the field study were filtered on 3-µm Isopore filters (Millipore), which were presumed to retain the particle-attached fraction. The filtrate (i.e. the non-attached fraction) was then filtered on 0.22-µm Isopore filters (Millipore). Water samples from the mesocosm experiments were completely filtered on 0.22-µm Isopore filters. All filters were rapidly frozen in liquid nitrogen and stored at -80°C. DNA and RNA from frozen filters was extracted using a phenol-chloroform-extraction method, according to Weinbauer et al. (2002). After the first steps of cell lysis, the samples were separated and a pH-dependent procedure was applied to extract DNA (phenol-chloroform pH = 7.5 to 8) or RNA (phenol-chloroform pH = 4.5 to 5). Extracts were washed and quantified

spectrophotometrically using a NanoDrop ND-1000 Photometer (NanoDrop Technologies).

For the analysis of the presumably active community, the RNA was reverse transcribed into complementary DNA (cDNA). Therefore, co-precipitated DNA in 10 µl aliquots (accounting for 40 ng to 1 µg) was removed using DNAseI (DNA-free kit, Ambion), following the manufacturers instructions. Synthesis of cDNA from 10 to 20 ng RNA was performed in a Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR) using the universal primer 1492R (5'-GGTTACCTTGTTACGACTT-3') (Lane 1991) and the iScript cDNA synthesis kit (Bio-Rad). Complete removal of DNA was tested with an additional RT-PCR without enzyme. Successful synthesis of cDNA was checked in a following PCR, as described below.

The 16S rRNA and 16S rRNA gene fingerprints of the bacterial communities were analyzed using single-strand-conformation polymorphism (SSCP). DNA extracts as well as cDNA amplicons were PCR-amplified using primers Com1 (5'CAGCAGCCGCGGTAATAC3') and Com2-Ph (5'CCGTCAATTCCTTTGAG TTT3') (Schwieger & Tebbe 1998), which amplify *Escherichia coli* 16S rRNA gene positions 519 to 926, following the protocol from Labrenz et al. (2007), with an annealing-temperature of 50°C. Single-stranded DNA was generated and purified and the SSCP analysis carried out as described in Schwieger & Tebbe (1998). Cluster analysis and pairwise comparisons of band patterns were done with GelCompare II (Applied Maths NV) based on their densiometric profile (Pearson correlation) after background substraction, least square filtering and optimization according to the manufacturers' instructions. The pairwise comparison of the bacterioneuston and bacterioplankton community is expressed as percentage dissimilarity.

Analysis of organic carbon and nitrogen

Ten-ml water samples were sealed in HCl-precleaned and precombusted (450°C, 6 h) glass ampoules using a portable propane torch. The sealed ampoules were quickly frozen without the addition of preservatives, and stored at -20°C until analysis in the laboratory. Total organic carbon (TOC) and total nitrogen (TN) were quantified using a high-

temperature combustion method, carried out in the presence of a Pt-catalyst on a Shimadzu TOC-V analyzer supplemented with a Shimadzu TNM-1 nitrogen detector and a Shimadzu ASI-V autosampler (Shimadzu Europe Ltd.). This system allows simultaneous determination of TOC and TN in the same sample. The temperature of the catalyst was kept at 680°C and carbon-free air produced by a Whatman gas generator was used as carrier gas (flow rate 150 ml min^{-1}). Acetanilide was used for a 4-point calibration. Prior to analysis, the samples were acidified with 2N HCl (suprapure grade) to a final pH < 2. Calculations of TOC and TN concentrations were based on four injections of the sample (injection volume 75 µl). All samples were run in duplicates. The reliability of each analytical run was checked using external reference material in a seawater matrix for TOC and TN and using reference material for blank measurement. The reference material was obtained from Dennis Hansell's CRM program (Dennis Hansell, University of Miami).

For the analysis of particulate organic carbon (POC) and nitrogen (PN), between 50 ml and 500 ml (field study) as well as 95 ml and 250 ml (mesocosm experiments) of a sample were filtered through Whatman GF/F glass fibre filters (pre-combusted at 450°C for 6h). After filtration, the filters were transferred into 10 ml rolled rim vials and stored at -20°C until analysis. Prior analysis, particulate inorganic carbon (PIC) was dispersed by acidifying the thawed filter with 100 µl of 2N HCl. After an incubation of approximately 30 min the filters were dried at 60°C for 4h. For final measurements, the dry filters were transferred into small tin boats and analysed in an Elementar vario MICRO cube elemental analyser (Elementar Analysensysteme GmbH, Germany) using the standard protocol recommended by the manufacturer. Acetanilide was used for calibration. The filtrate was considered as the dissolved fraction and analysed as described for TOC and TN (see above).

Statistical analysis

Statistical analysis of results from the mesocosm experiments were based on non-parametric tests, as normal distribution was either rejected using the Kolmogorov-Smirnoff-test or sample numbers were to small for proper testing. To examine the equality of means between the SML samples and the underlying water as well as among the SML samples, the Mann-Whitney-U-test and the Kruskal-Wallis-H-test, respectively,

were chosen with a significance level $p < 0.05$, indicating that the means of the samples were significantly different. Spearman's rank correlation was applied to test the association between parameters in the SML and the ULW. Again, $p < 0.05$ was determined to indicate significant correlations.

3.4 Results

Field study

The sea-surface microlayer (SML) in the Southern Baltic Sea was sampled on four consecutive days from May 6th to 9th 2008 (day 1 to 4) using the glass-plate technique. SML layer thickness on day 2 was 45.9 μm and in the range of previously reported values (Chapter 1). All sampling days were characterised by wind speed < 3.8 m s^{-1} during sampling as well as within the last six hours prior sampling (Table 3.1). On day 2, the sea-surface was highly calmed, resulting in the formation of a visible surface film ("slick") with considerably altered visible reflectance characteristics. The following day, this slick began to disintegrate and disappeared completely by day 4 parallel to increasing wind speed up to 2.6 m s^{-1}.

Table 3.1 Wind speed in May 2008 during sea-surface microlayer sampling as well as mean values for a 6 h period prior to sampling. Samples were taken in the southern Baltic Sea offshore Warnemuende in proximity to position N 54° 11'; E 12° 04'. Asterisk indicates visible slick formation.

Date	Wind speed [m s^{-1}]	Wind speed [m s^{-1}] 6 h mean
06 May 2008	3.80	2.30
07 May 2008 *	1.90	1.08
08 May 2008	1.50	2.12
09 May 2008	2.60	2.53

Organic carbon and nitrogen

The pool of organic carbon and nitrogen in the SML underwent strong changes throughout the sampling period with strong accumulations during slick formation (day 2, Fig. 3.1A). Concentrations of particulate organic carbon (POC) and particulate nitrogen (PN) in the slick were 791.3 μmol l^{-1} and 76.4 μmol l^{-1}, respectively. This was much higher compared to 29.5 μmol POC l^{-1} and 3.9 μmol PN l^{-1} in the underlying water (ULW) as well as compared to 25.2 μmol POC l^{-1} and 5.9 μmol PN l^{-1} in the SML the day before. This resulted in high enrichment factors for POC (26.8) and PN (19.5) on day 2. The disintegration of the slick (day 3 and 4) was accompanied by a subsequent decrease in absolute and relative values of POC and PN in the SML, although there was still an overall strong enrichment compared to the ULW (Fig. 3.1A). In the slick, concentrations of POC and PN contributed to 70% and 79%, respectively, of the total organic matter pool, compared to only 11% POC and 14% PN in pre-slick conditions as well as to 6% to 9% POC and 14% to 18% PN in the ULW of all sampling days.

In the ULW, highest concentrations for dissolved organic carbon (DOC; 355.7 μmol l^{-1}) and dissolved nitrogen (DN; 34.4 μmol l^{-1}) were measured on day 1. Over the whole sampling period, concentrations of DOC and DN in the ULW were relatively stable, ranging between 310.6 to 314.5 μmol l^{-1} and 15.7 to 17.4 μmol l^{-1}, respectively. DOC and DN showed only slight accumulations in the SML (Fig. 3.1A) with highest enrichment factors on day 3 for both DOC (1.12) and DN (1.27).

Bacterial abundance, activity, and community structure

Enrichment factors for total bacterial cell numbers (3.01) and ^{3}H-TdR incorporation (2.27) were highest in the slick (Fig. 3.1B, C). Furthermore, 4.6 10^{6} total bacterial cells ml^{-1} were measured in the slick, which was nearly twice as much as compared to "pre-slick" conditions and decreased again during slick disintegration (Fig. 3.1B). A similar pattern was observed for bacterioneuston productivity which showed 7.78 pmol ^{3}H-TdR h^{-1} l^{-1} in the slick and subsequently decreased the following days (Fig. 3.1C). Interestingly, total bacterial cell numbers and ^{3}H-TdR incorporation in the ULW decreased slightly on the day of slick formation and increased during slick disintegration. Therefore, the

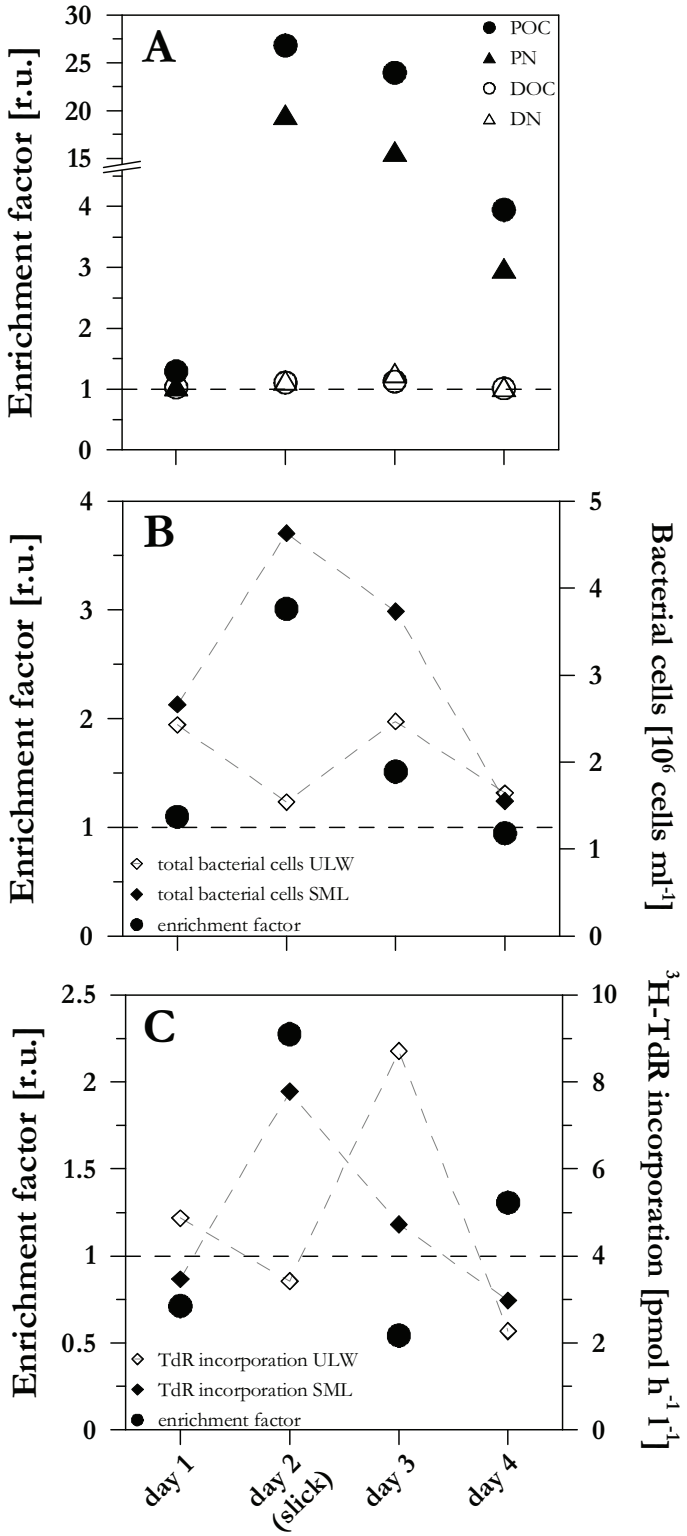

Fig. 3.1 Changes of (A) organic carbon and nitrogen, (B) bacterial abundance and (C) bacterial incorporation of ^{3}H-thymidine throughout the formation and disintegration of a visible surface film (slick) in the southern Baltic Sea. Values are given as enrichment factors (EF), i.e. relative ratios between the sea-surface microlayer and the underlying water. Dashed lines indicate EF = 1, where values in both samples are equal. Additionally, absolute values for the bacterial parameters are shown.

enrichment factor for ^{3}H-TdR incorporation on day 3 was smaller (0.54) compared to day 2 (2.27) and day 4 (1.31).

The comparison of bacterioplankton and bacterioneuston community composition, using 16S rRNA and rRNA gene fingerprints, revealed strong changes throughout the sampling period. The differences in community composition of non-attached bacteria

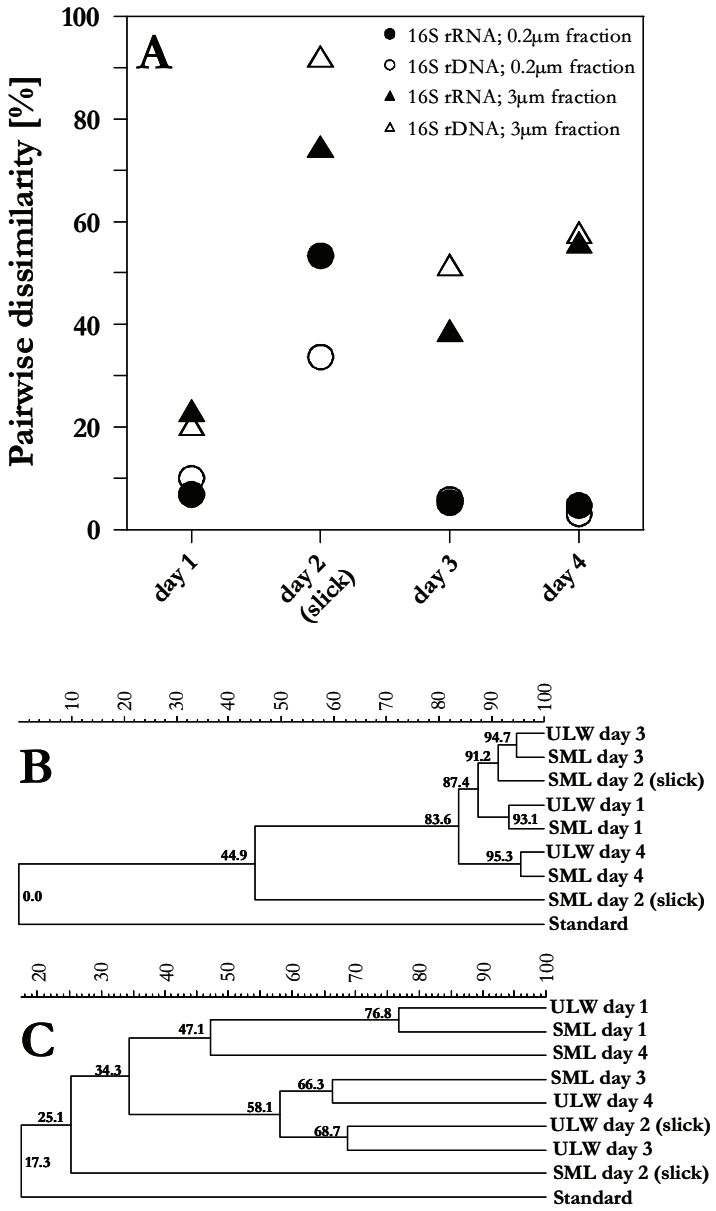

Fig. 3.2 Analysis of the bacterial community composition in the Baltic Sea field study based on 16S rRNA and 16S rRNA gene fingerprints. (A) Pairwise dissimilarity between bacterioneuston (SML) and bacterioplankton (ULW) community profiles, using Pearson correlation coefficient, for the particle-attached and non-attached size fraction. Cluster analysis of 16S rRNA fingerprints of (B) the non-attached and (C) the particle-attached fraction show a strong change of the bacterioneuston community during slick formation.

between the SML and ULW were initially < 10.1% on both DNA and RNA level (Fig. 3.2A). However, after slick formation, this dissimilarity increased to 33.7% (DNA) and 53.3% (RNA). This was due to major changes within the bacterioneuston community composition on day 2, illustrated by a cluster analysis, where all ULW-samples and the remaining SML-samples grouped strongly together (Fig. 3.2B).

An overall similar pattern was observed in the comparison of 16S rRNA and rRNA gene fingerprints of the particle-attached bacterial communities (Fig. 3.2A). However, there were also some differences: (1) the 16S rRNA gene fingerprints showed higher dissimilarities compared to the corresponding 16S rRNA fingerprints, except on day 1, (2) the dissimilarities between the bacterioneuston and bacterioplankton community were generally higher in the particle-attached than in the non-attached fraction, and (3) during slick disintegration, the dissimilarities in the particle-attached fraction did not decrease as strong as they did in the non-attached fraction. Nonetheless, in the cluster analysis of the 16S rRNA fingerprints, the particle-attached bacterioneuston community in the slick showed lowest similarity compared to all other samples (Fig. 3.2C).

In summary, slick formation in the southern Baltic Sea enhanced bacterial abundance and productivity in the SML and induced strong changes in the bacterioneuston community composition. Moreover, slick formation was characterised by a strong accumulation of particulate matter.

Mesocosm experiments

The mesocosm experiments were conducted to study temporal changes of the SML under artificially calmed conditions compared to naturally wind-affected sea-surfaces. The mesocosm study site was heavily influenced by opposing water inflow of brackish (Baltic Sea) and limnic (river Warnow) origin. Despite this fact, measurements of several biotic and abiotic parameters revealed highly comparable conditions in all ULW samples in – and outside the mesocosms (data not shown).

Dynamics of organic carbon and nitrogen

Inside and outside the mesocosms, total organic carbon (TOC) and total nitrogen (TN) in the SML showed very similar characteristics. Concentrations were highly variable but overall enriched compared to the ULW (Mann-Whitney-U-test; $p < 0.001$, $n \geq 20$; Table 3.2). Enrichment factors for TN displayed higher values than TOC (Wilcoxon-test; $p \leq 0.008$, $n \geq 9$), but both enrichments were significantly correlated (Spearman $r_s \geq 0.767$, $p \leq 0.016$, $n \geq 9$). Absolute concentrations of these parameters in the SML did not correlate to the ULW inside the mesocosms and only weakly outside the mesocosms (Table 3.2).

Outside the mesocosms, particulate and dissolved organic carbon (POC, DOC) as well as particulate and dissolved nitrogen (PN, DN) were measured on the first and the last day of each experiment. Enrichment factors for POC and PN ranged between 2.16 and 16.38 and about doubled throughout the first and third experiment. However,

Table 3.2 Comparison between samples from the sea-surface microlayer (SML) in – and outside the mesocosms with the respective underlying water (ULW). For each parameter it was tested, whether absolute values were different between the SML and ULW (Mann-Whitney-test) and if there was a correlation between these values (Spearman-Rho correlation coefficient). Moreover, it was tested, if enrichment factors were significantly different among all SML-samples in- and outside the mesocosms (Kruskal-Wallis-test). Bold values indicate significantly different (Mann -Whitney-test and Kruskal-Wallis-test) or significantly correlated (Spearman-Rho) comparison. r_s = Spearman-Rho correlation coefficient; level of significance is indicated as follows: ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$; n.s. = not significant. Kruskal-Wallis χ^2 = Chi-square; df = degrees of freedom

	Inside mesocosms SML vs ULW		**Outside mesocosms SML vs ULW**		**Enrichment factors all SMLs**
	Mann-Whitney	Spearman [r_s]	Mann-Whitney	Spearman [r_s]	Kruskal-Wallis
Total organic carbon	**p < 0.001** n = 20	0.262 n.s. ; n = 8	**p < 0.001** n = 71	0.076 n.s. ; n = 35	p = 0.099 n = 44; χ^2 = 6.272; df = 3
Total nitrogen	**p < 0.001** n = 20	-0.310 n.s. ; n = 8	**p < 0.001** n = 71	**0.384** * ; n = 35	p = 0.199 n = 44; χ^2 = 4.651; df = 3
Total bacterial cells	p = 0.058 n = 21	0.583 n.s. ; n = 9	**p = 0.026** n = 71	**0.685** *** ; n = 35	p = 0.118 n = 44; χ^2 = 5.879; df = 3
CTC-positive cells	**p = 0.001** n = 21	0.333 n.s. ; n = 9	**p < 0.001** n = 72	0.197 n.s. ; n = 36	**p = 0.032** n = 45; χ^2 = 8.824; df = 3
^{3}H-TdR incorporation	p = 0.382 n = 21	0.217 n.s. ; n = 9	p = 0.551 n = 72	**0.947** *** ; n = 36	p = 0.135 n = 45; χ^2 = 5.568; df = 3

enrichments on the last day were not as pronounced as inside the mesocosms (Table 3.3). Here, POC, DOC, PN and DN were analysed only on the last day of each experiment due to sample volume limitations. Enrichment factors for POC inside the mesocosms ranged between 9.5 and 22.6 (Table 3.3). 27% to 60% of TOC consisted of POC in the SML inside the mesocosms (Table 3.3), compared to 4% to 8% in the ULW (data not shown) or to 14% to 47% in the SML outside the mesocosms (Table 3.3). A comparable pattern was found for the enrichment of PN (Table 3.3). DOC and DN were only slightly but similarly enriched in all SML-samples (EF (DOC) = 1.05 to 1.30, EF (DN) = 1.02 to 1.69).

Table 3.3 Enrichment factors (EF) of particulate organic carbon (POC) and particulate nitrogen (PN) in- and outside the mesocosms. Outside the mesocosms samples were taken on the first and the last day of each experiment, inside the mesocosms samples were only taken on the last day of each experiment.
* mean ± SD (n = 3; values without SD n = 1); values in parenthesis indicate contribution of the particulate matter to the total organic matter pool [%]. nd = not determined

Experiment	Day of experiment	EF (POC)		EF (PN)	
		Inside mesocosms	Outside mesocosms*	Inside mesocosms	Outside mesocosms*
1	1	nd	2.16 ± 0.96 (14.1)	nd	2.17 ± 1.02 (13.2)
	4	12.76 (48.8)	6.13 ± 3.19 (30.0)	9.62 (43.0)	4.97 ± 2.86 (27.1)
2	1	nd	6.98 ± 2.38 (24.5)	nd	7.17 ± 1.99 (18.0)
	4	9.48 (27.4)	6.44 ± 1.57 (23.2)	7.74 (29.8)	4.88 ± 1.98 (20.4)
3	1	nd	5.21 ± 2.32 (26.3)	nd	4.64 ± 3.21 (31.0)
	4	22.64 (60.0)	16.38 (46.8)	30.40 (70.8)	15.34 (50.72)

Bacterial parameters

Generally, concentrations of bacterial abundance and productivity in the SML were highly variable (Fig. 3.3 C-E). CTC-positive cells were overall enriched in all SML-samples (Mann-Whitney-U-test; $p \leq 0.001$, $n \geq 21$; Table 3.2, Fig. 3.3D), but absolute values did not correlate between the SML and ULW (Table 3.2). Outside the mesocosms, total

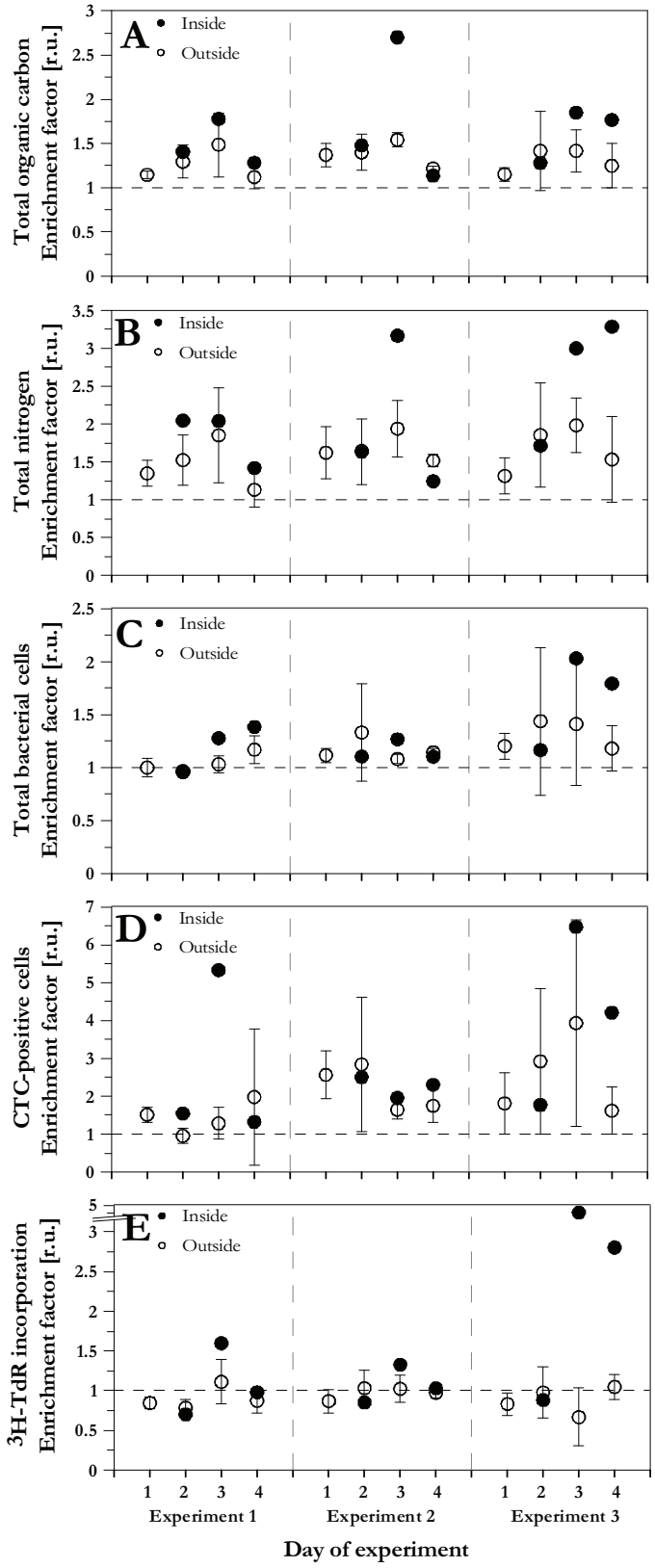

Fig. 3.3 Enrichment factors of total organic carbon (A), total nitrogen (B), abundance of total bacterial cells (C) and CTC-positive cells (D) as well as the incorporation of ^{3}H-thymidine (E) are given for the sea-surface microlayer inside the mesocosms (black circles) and outside the mesocosms (white circles). Results of three experiments are shown. Error bars indicate SD of three independent samples.

bacterial cell numbers were significantly enriched in the SML compared to the ULW (Mann-Whitney- U-test; p = 0.026; n = 71) which was not found for ^{3}H-TdR incorporation (Table 3.2, Fig. 3.3C, E). However, absolute values for both parameters were correlated to the ULW (Table 3.2). Although total bacterial cells were slightly enriched inside the mesocosms (Fig. 3.3C), absolute cell numbers as well as ^{3}H-TdR incorporation were not significantly different from the ULW (Mann-Whitney-U-test; $p \geq 0.058$; n = 21) and were not correlated to concentrations in the ULW (Table 3.2). To test whether enrichments inside the mesocosms were different from outside the mesocosms, enrichment factors among all samples were compared. This comparison revealed that only the enrichment of CTC-positive cells was significantly different between inside and outside the mesocosms (Kruskal-Wallis-H-test; p = 0.032; n = 45; χ^2 = 8.824; df = 3; Table 3.2).

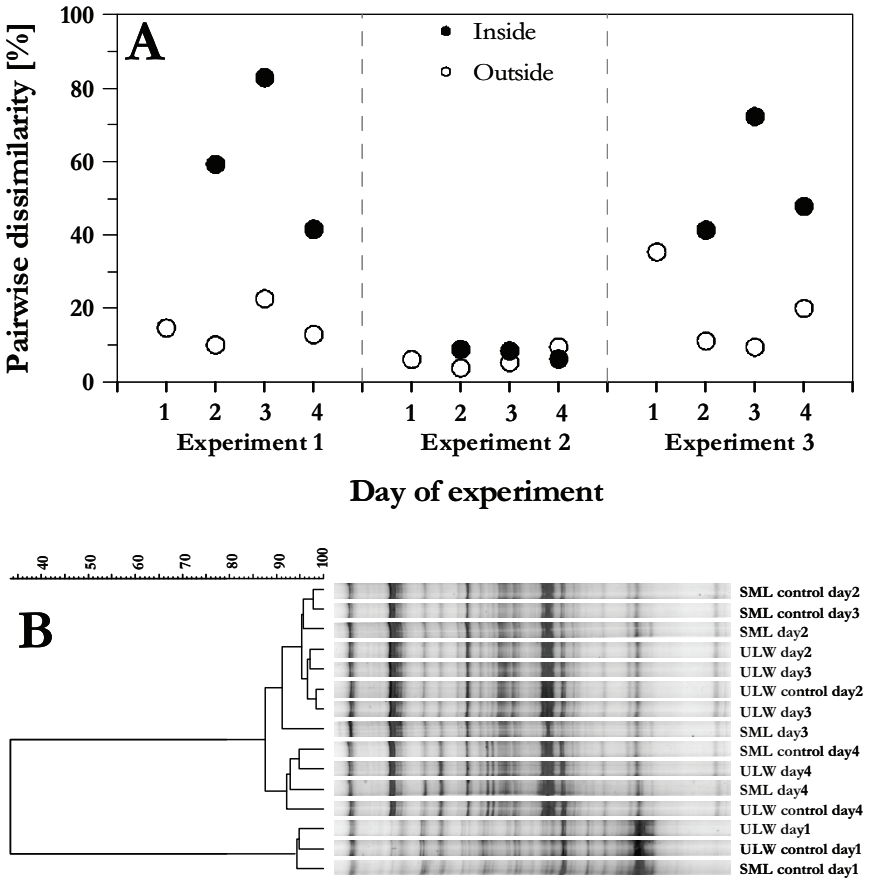

Fig. 3.4 (A) Pairwise comparison of the total bacterioneuston community inside the mesocosms (black circles) as well as outside the mesocosms (white circles) with the respective bacterioplankton community based on 16S rRNA fingerprints. (B) Cluster analysis of all communities over the course of four days during the second experiment.

The total bacterial community composition was analysed by 16S rRNA fingerpints. During the first and the third experiment, differences between the bacterioneuston and bacterioplankton communities were highly dynamic (Fig. 3.4A). In both experiments, highest dissimilarities between SML and ULW inside the mesocosms were observed on the third day (82.9 % and 72.3 % in experiment 1 and 3, respectively). Contrarily, throughout the second experiment, the dissimilarities between the SML and the ULW were constantly < 9 %. During this experiment, there was a drastic change in community composition of the bacterioplankton between the first and the second day, most likely due to an inflow of brackish water into the limnic harbour (data not shown). These changes were also observed in the SML in- and outside the mesocosms (Fig. 3.4B). Outside the mesocosms, the similarity in community composition between SML (bacterioneuston) and ULW (bacterioplankton) was always higher than inside the mesocosms (Fig. 3.4A).

Taken together, all parameters in the SML outside the mesocosms were as dynamic and variable as inside the mesocosms. Similarily, each parameter underwent changing enrichments within each experiment (Fig. 3.3). Although differences among all SML samples were not significant, except for the enrichment of CTC-positive cells (Table 3.2), the enrichment factors inside the mesocosms, especially for bacterial parameters, tended to be higher than the mean enrichment outside the mesocosms (Fig. 3.3C-E).

Table 3.4 Correlations between the enrichment of bacterial parameters and total and particulate matter. Correlations with total organic carbon and total nitrogen are based on SML samples from the southern Baltic Sea taken in 2007 (Chapter 1) and in May 2008 as well as during the mesocosm experiments. Correlations with particulate organic carbon and particulate nitrogen are based on samples from May 2008 as well as several mesocosm samples (see Table 3.3). r_s = Spearman rang correlation coefficient; p = level of significance; n = number of samples: n.s. = not significant

	Total organic carbon			**Particulate organic carbon**		
	r_s	**p**	**n**	**r_s**	**p**	**n**
Total bacterial cells	0.522	< 0.001	57	0.794	< 0.001	23
CTC-positive cells	0.555	< 0.001	58	0.583	0.003	24
^{3}H-TdR incorporation	0.380	0.003	58	0.418	0.042	24
	Total nitrogen			**Particulate nitrogen**		
	r_s	**p**	**n**	**r_s**	**p**	**n**
Total bacterial cells	0.406	0.002	57	0.835	< 0.001	23
CTC-positive cells	0.408	0.001	58	0.739	< 0.001	24
^{3}H-TdR incorporation	0.187	n.s	58	0.384	n.s	24

3.5 Discussion

Strong enrichments of bacterial abundance, productivity and particulate organic matter were observed in the sea-surface microlayer (SML) during slick formation in the Baltic Sea. This was accompanied by strong changes in the composition of the bacterioneuston community. Similar effects were observed in artificially calmed sea-surfaces and indicate that mimized wind-induced surface mixing results in a succession of the SML.

Organic matter in the sea-surface microlayer

Organic matter in the SML derives from both living cells as well as detritus and is a complex mixture of substances, to which carbohydrates, proteins and lipids have major contributions (Kattner et al. 1985, Williams 1986). It is well known that its dissolved fraction is slightly, but consistently enriched in the SML (Hunter 1997). Also SML-samples from the southern Baltic Sea in this study showed only a slight enrichment of dissolved organic carbon (DOC) and nitrogen (DN). Enrichment factors ranged between 1.0 and 1.3, which are comparable to previously reported values from different aquatic habitats (Momzikoff et al. 2004, Reinthaler et al. 2008, Wurl & Holmes 2008). Moreover, absolute and relative concentrations of dissolved matter revealed only little temporal variability throughout the sampling period. This was even observed during the formation and disintegration of a dense surface film (slick), which confines a previous study, where concentrations of dissolved matter were constant in a transition zone from a "clean" SML to a dense slick (Carlson 1982b).

Reinthaler et al. (2008) reported that only a minor part (~ 6 %) of the total organic carbon pool in the SML of the Atlantic Ocean consisted of particulate matter. However, high contributions of particulate organic carbon (POC) and nitrogen (PN) to the total organic matter pool were observed in our field study during and after slick formation. Overall, the enrichment of POC and PN in the SML was consistently higher than the dissolved fraction. Enrichment factors for POC in the visible as well as disintegrating slick were 26.8 and 24.0, respectively and were similar to values reported for coastal slicks,

although 40-fold enrichments have also been reported (Carlson 1983, Garabetian et al. 1993).

In the mesocosm experiments similar proportions of the dissolved and particulate fraction were observed as in the field study. Enrichment factors of DOC and DN ranged between 1.0 and 1.7 and were not different in the artificially calmed mesocosms compared to the naturally wind-influenced SML outside the mesocosms (data not shown). In contrast, enrichment factors for POC and PN ranged between 2.2 and 30.4 and were always higher inside than outside the mesocosms. Furthermore, POC (up to 60 %) and PN (up to 71 %) had major contributions to the total organic matter pool. Again, contributions were much higher in the artificially calmed SML inside the mesocosms. Consequently, although distinction of the dissolved and particulate fraction was only possible in a subset of samples, changing concentrations of particles in the SML were most likely the major driving force of temporal variability in the enrichment of total organic carbon (TOC) and nitrogen (TN). Furthermore, the variability in particulate matter enrichment could have also caused the lacking correlation of TOC and TN concentrations between the SML and the ULW in all mesocosm experiments.

While the origin of organic matter in the SML in the present study can finally not be elucidated, there are some indications for a contribution of the ULW: (1) Concentrations of DOC, DN, POC and PN in the ULW on May 6th were slightly increased compared to the following days (~ 15%, data not shown). The high particulate load in the slick might therefore been arosen from aggregation of dissolved and/or particulate matter in the ULW (Kerner et al. 2003, Azetsu-Scott & Passow 2004) and subsequently transported to the SML by bouyant particles or rising bubbles (Wallace & Duce 1978). (2) Phytoplankton activity can support accumulation of material in the SML by production of transparent exopolymer particles (TEP) or surfactants (Gašparović et al. 2007, Wurl & Holmes 2008). In the present study, slick formation developed during the productive spring season and, therefore, might have been fueled by autotrophic production. Furthermore, lowest enrichment of particulate matter in the mesocosms was measured during the second experiment, which was characterised by constantly low concentrations of chlorophyll-a in the ULW and, thus, reduced influence of phytoplankton activity (data not shown).

Bacterial dynamics in the SML

Absolute bacterial abundance in the SML from the southern Baltic Sea was comparable to a recent study in this area (Chapter 1). Considering the strong increase in particulate organic matter in the slick, bacteria could have been passively transported to the SML by existing or newly-formed particles in the ULW and subsequent attachment and, thus, might have caused the increase of bacterial cells in the slick. Similarily, import from the bulk water was suggested to be the major source of enrichment of bacterial cells and virus-like particles in coastal and open ocean SML-samples (Kuznetsova et al. 2004). Comparable to slick formation, abundance of total and CTC-positive cells as well as productivity in the SML changed during the first and third experiment inside the mesocosms. High variability in the enrichment of CTC-positive cells was previously reported (Chapter 1) and might explain why this parameter showed solely significantly different enrichments among all SML-samples. Overall, highest enrichment factors of bacterial parameters followed the patterns of TOC and TN concentrations, suggesting that there was a coupling between new material accumulating in the SML and the responses of the bacterioneuston.

High concentrations of organic matter could explain previous reports of enhanced exo-enzymatic activites in the SML (Münster et al. 1998, Kuznetsova & Lee 2001, Santos et al. 2009). However, low bacterial growth efficiencies in the SML of the open Atlantic Ocean indicate that high bacterioneuston metabolism does not reflect enhanced cell growth (Reinthaler et al. 2008). These observations might indicate stressfull conditions in the SML (Dietz et al. 1976). Similar conclusions can be drawn from the present study: Besides the strong increase in absolute bacterioneuston productivity in the slick, cell-specific activity (^{3}H-TdR incorporation/cell) remained smaller compared to the ULW (data not shown). This was also true for cell-specific activity in the the SML of the mesocosm experiments, except on the third day of each experiment (data not shown). This might at least partially be due to less inhibitory influence of UV-radiation in the mesocosm experiments as samples were taken around sunrise. The SML receives high doses of UV-radiation compared to the ULW (Maki 1993). Generally, low radiation-levels due to the season might also explain why ^{3}H-TdR incorporation was not as decreased as in SML-samples from the Baltic Sea of this and a previous study (Chapter 1). However, there is

contradictory evidence whether UV-radiation generally causes diel patterns of bacterioneuston activity (Carlucci et al. 1986, Santos et al. 2009). Nonetheless, cell-specific activity might even been overestimated as particle-attached bacteria were not counted due to methodological reasons. Particle-attached bacteria have been reported in high numbers in the SML (Aller et al. 2005) and could explain the extensive enrichment of bacteria up to several orders of magnitude in slick samples (Hardy & Apts 1984). Furthermore, relative bacterial productivity was found to be smallest in highest concentrations of particulate matter (Obernosterer et al. 2008). This implies that attachement to particles does not shelter bacteria from stress factors in the SML, although general high activity of particle-attached bacteria in surface water is known (Grossart et al. 2007). The attachment of bacteria to particulate matter in the SML could also explain the decrease in bacterial abundance and productivity during slick disintegration. Increasing wind speed and a collapse of the SML due to increasing surface pressure and convergent forces (Wheeler 1975) most likely caused a transport of material from the SML into the ULW and might explain the strong increase in bacterioplankton productivity on May 8th.

Studies of bacterioneuston community composition are still scarce and to the best of our knowledge no attempt has been made to to distinguish the non-attached and the particle attached members of bacterioneuston communities. Strong variability in the bacterioneuston community has been reported from one day to the next (Agogué et al. 2005a). Similarily, the community composition of non-attached bacteria in the SML changed strongly upon slick formation and disintegration. In the meantime, bacterioplankton community composition was stable over the sampling period. Contrarily, differences in the particle-attached community compositions were stronger and resisted longer. Again, the strongest effect was observed in the bacterioneuston upon slick formation. However, dynamic pattern of the particle-attached community were also observed in the ULW between May 6th (pre-slick) and May 7th (slick). This implies that movement and transformation of particles had strong impacts on overall community structure. In the mesocosm experiments, the changing patterns of total bacterial community composition followed the dynamic enrichment of TOC and TN. Therefore, our results suggest that dynamic variability in community composition was strongly influenced by the particle-attached community. Moreover, the bacterioneuston was found to be strongly influenced by the ULW as strong changes of bacterioplankton community

composition in the second experiment were immediately recognizable in the SML. In this experiment, general changes of the bacterioneuston inside the mesocosms were small. Interestingly, this was accompanied by lowest concentrations of particulate organic matter in the SML, additionally indicating the importance of particles in the SML.

Succession of the SML

Generally, the SML is physically stable due to surface tension forces. Wave breaking disrupts the sea surface, however, reassamblage of the SML is very fast (Hardy 1982). Decreasing wind speed and increasing accumulation of matter in the SML reduces surface tension, which finally dampens capillary waves and induces slick formation. Slicks are frequently observed in marine systems (Romano 1996). Therefore, the SML transforms in between these extremes and our understanding of underlying processes is still small. Thereby, wind induces both removal and supply processes in the SML and contributes strongly to its temporal and spatial variability, already on very short time scales (Kuznetsova & Lee 2002). Still, a simple relation between wind speed and changing concentrations of compounds in the SML can not be drawn (Kuznetsova et al. 2004, Reinthaler et al. 2008).

The present study indicates that very low wind speed induces a succession of the SML and its inhabiting bacterial community (Fig. 3.5). This succession is characterised by an uncoupling of bacterial parameters in the SML from the ULW as well as an increasing coupling of organic matter and bacteria in the SML under desreasing wind-induced surface mixing. Total bacterial abundance as well as productivity in the SML outside the mesocosms correlated very well with changing concentrations in the ULW. In contrast, this correlation was absent in the artificially calmed sea-surfaces inside the mesocosms. There is contradictory evidence whether matter in the SML is related to the ULW (Dietz et al. 1976, Williams 1986, Joux et al. 2006). Our study suggests that an uncoupling of the SML is favoured under low wind conditions. This is especially pronounced during slick formation, where bacterial parameters underwent strong changes.

Under these conditions, the contribution of the particulate fraction to the dynamic enrichment of organic matter and bacterial parameters in the SML increases. This is

supported by strong correlations between the enrichment of POC as well as PN and the enrichment of bacterial abundances in the SML (Table 3.4). Considering all data from this and a previous study (Chapter 1), patterns of TOC and TN enrichment explain changes of bacterial abundances to a lesser extent. Community respiration was found to correlate with the enrichment of TOC (Obernosterer et al. 2005). In this study, patterns of bacterioneuston productivity were not or only weakly linked to changing quantities of organic matter in the SML. These results also underline previous suggestions that the bacterioneuston community is not specifically adapted to this unusual habitat and that it most likely does not have major contributions to the cycling of organic matter in the SML (Obernosterer et al. 2008).

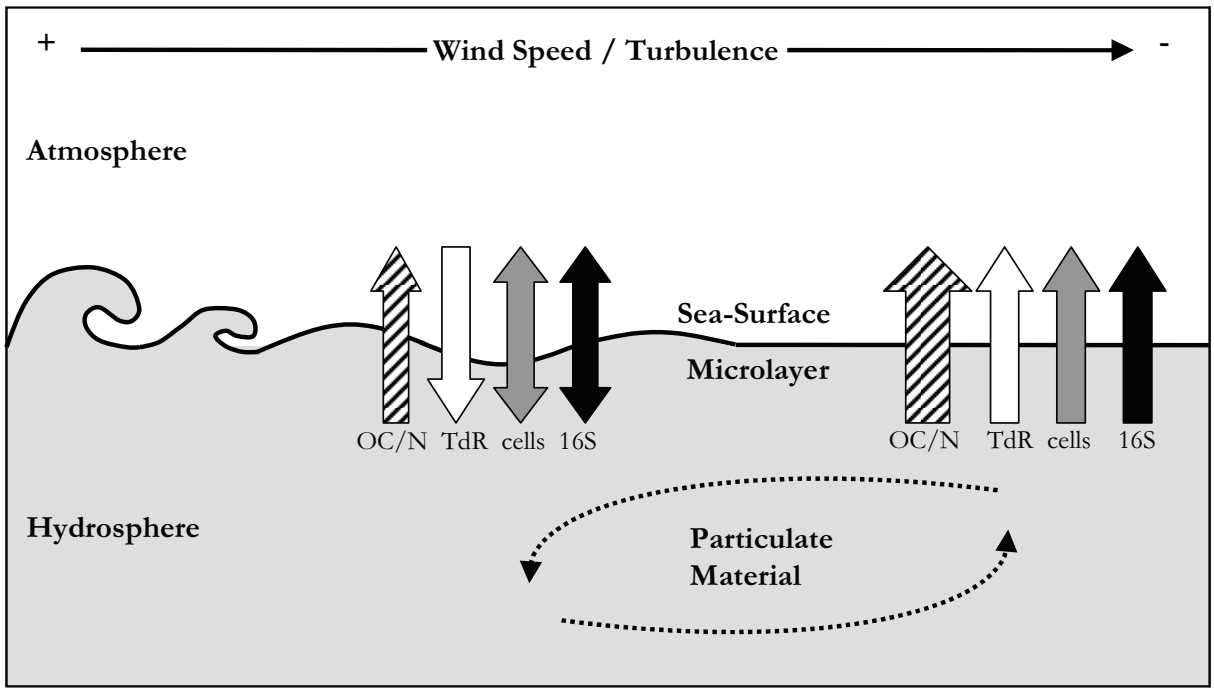

Fig. 3.5 Model of the effect of decreasing wind speed on organic matter enrichment and bacterioneuston parameters in the sea-surface microlayer (SML) compared to the underlying water. The present study indicates that slick formation and an artificially calming of the sea surface show an increasing importance of the particulate fraction in the SML under lower wind conditions. (OC/N = organic carbon and nitrogen, TdR = ^{3}H-thymidine incorporation, cells = bacterial abundance, 16S = differences in community composition based on 16S rRNA fingerprints).

3.6 Conclusions

Taken together, high variability in the physical and chemical environment of the SML makes general characterisations of SML properties and assumptions about processes in this habitat a challenging task. The present study implies that further knowledge of particle transformation is essential to understand patterns of the bacterioneuston community. Finally, calm meteorological conditions in the SML harbour the potential to induce a succession of the bacterial community, which in turn might be important for specific processes, e.g. gas exchange, especially during extensive surface phytoplankton blooms (Sieburth & Conover 1965, Ploug 2008).

Summary and Future Perspectives

Summary

The present thesis investigated bacterial communities in the sea-surface microlayer (SML), which is defined as the top layer of any water body, located at the air-water interface. Main emphasis has been placed on a description of the abundance, activity and community composition of bacterial assemblages in the SML (bacterioneuston) of the Baltic Sea and the comparison of these parameters to the bacterioplankton community of the underlying bulk water (ULW). Special attention has been drawn to potential structuring factors of the bacterioneuston community, particularly meteorological conditions and the accumulation of organic material in the SML. For these analyses, the establishment of a suitable SML-sampling technique was necessary. Briefly, the results of this work can be summarized as follows:

- An appropriate sampling technique (glass-plate sampler) was established, allowing unbiased measurements of all bacterial parameters of interest (Chapter 1)
- Bacterial cell production, i.e. incorporation of ^{3}H-thymidine, in the SML is generally impaired despite strong enrichments of organic material (Chapter 1, 3)
- The non-attached, but not the particle-attached bacterial communities are very similar in the SML and ULW as revealed by 16S rRNA and 16S rRNA gene fingerprints (Chapter 2, 3)
- The differences of the bacterial community composition between the SML and ULW are enhanced with decreasing wind speed and increasing radiation levels (Chapter 2)
- Active bacterioneuston members show high 16S rRNA gene sequence similarities to environmental clones of diverse habitats (bulk water, soil, endosymbionts) (Chapter 2)
- Minimized turbulence at the sea surface in natural and artificial systems induces strong changes ('succession') of the bacterioneuston compared to the bacterioplankton community (Chapter 3)

It is noteworthy to mention that there are two major limitations to the work presented:

(I) The bacterioneuston is defined as the bacterial community which inhabits the air-water interface. Models hypothesize that the bacterioneuston colonises the uppermost 10 μm of the water column (Norkrans 1980, Sieburth 1983). These models have been mainly proposed upon the chemical characteristics of the SML, but yet need to be verified. Consequently, the term bacterioneuston, as it has been used in most literature as well as in the present thesis, refers to the bacterial community which is collected by a particular sampling device (Wangersky 1976) and it remains unclear how a 'real' neuston community is constituted. Any SML-sample might therefore give error-prone characteristics of the bacterioneuston due to dilution with the ULW. The upper 50 μm of the water column were described to be physico-chemical stable, but different from the ULW (Zhang et al. 2003). The glass-plate sampling device used in the present study collected SML-samples of < 50 μm layer thickness, i.e. within this stable layer. Considering that the glass-plate sampler was found to be non-selective for most of the bacterial parameters measured, the data presented give a reliable characterisation of the bacterioneuston in the Baltic Sea. However, the unclarity of SML organisation causes uncertainties about distinctions between neustonic and planktonic organisms. Whether these distinctions can be made at all or upon determination of water depth, physico-chemical conditions, physiological stress, etc., warrants further studies.

(II) The analysis of the bacterioneuston abundance and activity in the present thesis was based on bulk measurements, i.e. the whole community was treated as an entity. This approach is useful to understand processes on a broad scale, e.g. measurements of bacterial productivity to estimate carbon fluxes. However, specific community members might be highly active whereas the bulk activity is unchanged or even decreased. Single-cell analysis would provide a resolution to unravel such possible hidden dynamics of metabolic complexity (Del Giorgio & Gasol 2008). The merit of distinguishing different parts of the bacterial communities becomes evident by the data presented, where particle- and non-attached as well as the present and presumably active members were differentiated for phylogenetic analyses and showed distinct relations to several environmental factors.

The strong temporal and spatial variability in the SML is one of the most challenging tasks to characterise its inhabiting bacterial community. Meteorological forcing and diverse import, export, and transformation processes constantly change the physico-chemical properties of the SML (Liss & Duce 1997) and consequently, influence bacterial life in the air-water interface. However, it is unclear whether a 'successful' bacterioneuston exists (Maki 1993). The major aim of the present thesis was a better understanding of how environmental factors structure and control the bacterioneuston. Figure B summarizes the results of the preceding chapters on how different meteorological conditions and changing quantities of organic material shaped bacterioneuston abundance, activity, as well as diversity in the Baltic Sea. In the following these results will be combined to draw a picture of our current understanding of bacterial assemblages in Baltic Sea sea-surface microlayers.

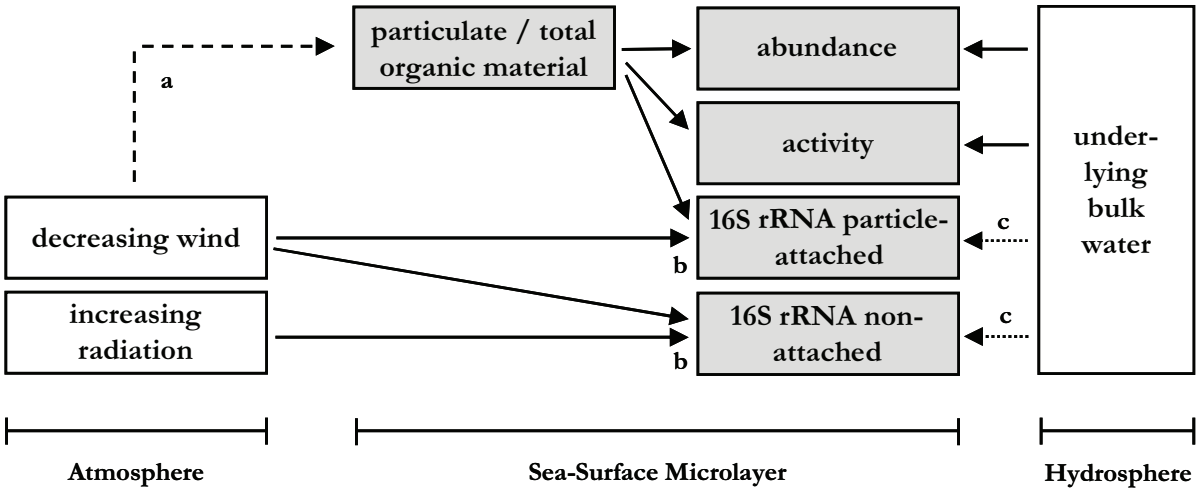

Fig. B The influence of meteorological conditions (atmosphere), the ULW (hydrosphere), and the enrichment of organic material on different bacterial parameters in the SML (grey boxes) is shown. All positive correlations (straight arrows), which have been found and described in the present work, are illustrated; e.g. increasing enrichment of particulate organic material is correlated to increasing bacterioneuston activity. a = the relation between decreasing wind speed and the enrichment of organic material was not statistically assured, but is deduced from the observations in natural and artificially calmed sea-surfaces. b = the parameter '16S rRNA' refers to the differences, i.e. pairwise dissimilarities, between the community composition of the SML and ULW. c = refers to the high sequence similarity of SML-specific SSCP-bands to environmental clones from diverse habitats, especially the bulk water.

Cycling of organic material in the SML

The present thesis supports previous findings that the SML is generally enriched in organic material (Hunter 1997). Despite increased concentrations of potential substrate supply, the bacterioneuston of the Baltic Sea showed generally low productivity, i.e. ^{3}H-thymidine incorporation, but highly variable and often increased abundances of CTC-positive cells compared to the ULW. This implies that the bacterioneuston is metabolically active, but cell growth is not enhanced, which underlines previous suggestions that bacterial growth in the SML is limited (Norkrans 1980, Reinthaler et al. 2008) and that the bacterioneuston has only small importance in the cycling of organic material in aquatic systems (Obernosterer et al. 2008). Whether general reduction of bacterioneuston activity is mainly determined by physiological stress or by an unfavourable alteration of substrates in the SML, however, remains unclear. Most likely a combination of both processes, e.g. due to increased radiation exposure, is responsible. Indirect evidence comes from the data presented, that the enrichment of particulate organic material correlated with bacterial abundances and activity in the SML, indicating a stimulating effect of fresh material entering the air-water interface. Increasing concentrations of total organic carbon correlated with increasing differences between particle-attached communities of the bacterioneuston and bacterioplankton, further implying structuring effects of organic material on bacterioneuston populations.

Influence of the ULW

The present thesis shows that the SML of the Baltic Sea is strongly influenced by the ULW, which has been previously suggested in marine and limnic habitats (Joux et al. 2006, Baastrup-Spohr & Staehr 2009). Positive correlations of bacterial abundance, activity and of organic material between the SML and ULW were found. Overall strong similarities of bacterioneuston and bacterioplankton community composition, especially in the non-attached size fraction, further support this conclusion. Chemical conditions in the SML may favour the occurrence of specific bacterial organisms, e.g. *Gammaproteobacteria* and *Bacteriodetes*, which are able to cope with increasing concentrations

and complexity of their substrates (Kirchman 2002, Pinhassi & Berman 2003). Nonetheless, the majority of all exclusively detectable, active members of the bacterioneuston were very similar (≥ 97% 16S rRNA gene sequence similarity) to environmental clones from diverse aquatic habitats, mainly from the bulk water, and thus, do not represent novel or specifically adapted SML-species. The identification of a close relative to the gas-vacuole containing *Polaribacter filamentus* implies that bacteria from the ULW can control their duration of stay in the SML and thus, the exposure to potential physiological stress.

Influence of meteorological conditions and succession of the SML

Changing meteorological conditions were related to differences between bacterioneuston and bacterioplankton community composition, especially in the 16S rRNA fingerprints, i.e. the active parts of the communities. Increasing UV- and global solar radiation was positively correlated to community differences in the non-attached size fraction, supporting the above suggested influence of radiation exposure on bacterial populations in the SML. Wind affected community composition dynamics, as differences between bacterioneuston and bacterioplankton populations increased with decreasing wind speed. Under minimized turbulent conditions a succession of the bacterioneuston occurs. This is especially pronounced in slicks, where high enrichments of organic material were found and strong changes of the bacterial community composition in the SML were observed. Similar changes were inducible in mesocosm experiments, in which the sea-surface was artificially calmed.

Thus, low wind speed and high concentrations of organic material are most likely the dominant factors under which a succession of the bacterioneuston is driven. Although SML properties change within minutes (Kuznetsova et al. 2004), a succession of the bacterioneuston in the present thesis needed at least several hours to occur. These conditions (continuous low wind, high productivity) do not reflect dominant situations in marine habitats. However, knowledge of a succeeding bacterioneuston community may increase our understanding of biogeochemical cycles in limnic systems, which are less influenced by wind forcing, as well as in slicks, which frequently occur in coastal and open

oceans (Romano 1996), and are enhanced during surface phytoplankton blooms (Sieburth & Conover 1965, Ploug 2008).

Future Perspective

Figure B does obviously not provide a complete picture of interactions between bacterial parameters and physico-chemical conditions in the SML. Nonetheless, the present thesis may provide a link for the opposing results reported in the literature so far, especially regarding bacterial activity and diversity (see Tables B, C in Introduction). However, far more knowledge is needed to fully understand and appreciate the role of bacteria in the SML and their function in aquatic systems.

The present study shows, that slick formation induces a succession of the bacterioneuston. A prerequisite for this to occur is the minimization of turbulent conditions, providing sufficient time for the establishment of the bacterial assemblages in the SML. Under these conditions, the most drastic effects of the bacterioneuston on material cycling between the atmosphere and hydrosphere will be probably observed, especially during high autotrophic production. An analysis of all three compartments (SML, ULW, and atmosphere) is therefore unavoidable in future studies to better understand fluxes across the air-water interface. Thereby, atmospheric deposition, especially its contribution to bacterial community composition, has been found to be important in limnic systems (Hervas & Casamayor 2009) and needs complementary studies in marine and brackish habitats.

Our mesocosm experiments show that a combined study of natural and artificial systems is a promising approach. Only few previous studies have used undisturbed samples to investigate the activity of neustonic organisms, e.g. in Petri dishes (Hermansson & Dahlbaeck 1983) or beakers (Hardy & Apts 1989). This, however, has only limited resolution due to small sample volumes. Cunliffe et al. studied bacterioneuston dynamics during artificially induced phytoplankton blooms, which

however, was restricted to closed mesocosms (Cunliffe et al. 2009b, Cunliffe et al. 2009c). The advantage of the mesocosms in the present study was their employment under *in situ* conditions and provides a reasonable setup for future studies.

The influence of wind speed as well as the enrichment of organic material on bacterioneuston parameters in the present study may be interrelated, because wind speed was found to determine enrichments of organic carbon (Obernosterer et al. 2008). A better understanding of these interactions is definitively needed. More important, however, will be the description of substrate quality in the SML in order to elucidate its availability for bacterial uptake. Contradictory results of diurnal cycles of organic material transformations and bacterioneuston activity (Carlucci et al. 1986, Falkowska 2001, Kuznetsova et al. 2004) underline, that one of the most promising factor to study is the influence of UV-radiation. Photochemical driven alterations of material in the SML and detailed insights of bacterial adaptations, e.g. pigmentation (Hermansson et al. 1987) or SOS-repair systems, which oppose UV-induced physiological stress, are needed. For a deeper understanding, future studies have to link phylogenetic as well as metabolic diversity. Initial results show that different phylogenetic groups in the SML and ULW are responsible for leucine-incorporation (Obernosterer et al. 2008) and thus, warrant further studies to determine functional traits of bacterial assemblages in the SML. Increasing efforts to isolate neustonic organisms could additionally support these approaches.

Taken together, the present thesis is a further step to better understand bacterioneuston assemblages and its structuring factors and will help, with accompanying future studies, to answer Makis' question of a 'successful' bacterioneuston in more detail (Maki 1993).

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Abbreviations

A	Adenine
ATP	Adenosine triphosphate
BLAST	Basic Local Alignement Tool
bp	Base pair
C	Cytosine
cDNA	Complementary DNA
CFU	Colony forming unit
DGGE	Denaturing gradient gel elektrophoresis
DN	Dissolved nitrogen
DNA	Deoxyribonucleic acid
DOC	Dissolved organic carbon
EF	Enrichment factor
Fig(s)	Figure(s)
G	Guanine
GP	Glass plate
HCl	Hydrochloric acid
MS	Metal screen
NA	Non-attached
n	Number of samples
NCBI	National Center for Biotechnology Information
nd	Not determined
n.s.	Not significant
OTU	Operational taxanomic unit
PA	Particle-attached
PCR	Polymerase chain reaction
PN	Particulate nitrogen
POC	Particulate organic carbon
r, r_s, R^2	Level of significance
RD	Rotating drum

rDNA	Ribosomal deoxyribonucleic acid
RNA	Ribonucleic acid
RT-PCR	Reverse-transcription PCR
r.u.	Relative unit
SD	Standard deviation
SML	Sea-surface microlayer
SSCP	Single strand conformation polymorphism
T	Thymine
TdR	Thymidine
TEP	Transparent exopolymer particles
TN	Total nitrogen
TOC	Total organic carbon
T-RFLP	Terminal restriction fragment length polymorphism
ULW	Underlying bulk water
UV	Ultraviolet

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Erklärung

Ich versichere hiermit an Eides statt, dass ich die vorliegende Arbeit selbstständig angefertigt und ohne fremde Hilfe verfasst habe, keine außer den von mir angegebenen Hilfsmitteln und Quellen dazu verwendet habe und die den benutzten Werken inhaltlich und wörtlich entnommenen Stellen als solche kenntlich gemacht habe.

Rostock, den 16.02.2010