

## RESEARCH ARTICLE

# High wind speeds prevent formation of a distinct bacterioneuston community in the sea-surface microlayer

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**One sentence summary:** Bacteria inhabiting the sea-surface microlayer are enriched and prone to community changes in response to wind speed conditions and pCO<sub>2</sub> levels.

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## ABSTRACT

The sea-surface microlayer (SML) at the boundary between atmosphere and hydrosphere represents a demanding habitat for bacteria. Wind speed is a crucial but poorly studied factor for its physical integrity. Increasing atmospheric burden of CO<sub>2</sub>, as suggested for future climate scenarios, may particularly act on this habitat at the air–sea interface. We investigated the effect of increasing wind speeds and different pCO<sub>2</sub> levels on SML microbial communities in a wind-wave tunnel, which offered the advantage of low spatial and temporal variability. We found that enrichment of bacteria in the SML occurred solely at a U<sub>10</sub> wind speed of  $\leq 5.6$  m s<sup>-1</sup> in the tunnel and  $\leq 4.1$  m s<sup>-1</sup> in the Baltic Sea. High pCO<sub>2</sub> levels further intensified the bacterial enrichment in the SML during low wind speed. In addition, low wind speed and pCO<sub>2</sub> induced the formation of a distinctive bacterial community as revealed by 16S rRNA gene fingerprints and influenced the presence or absence of individual taxonomic units within the SML. We conclude that physical stability of the SML below a system-specific wind speed threshold induces specific bacterial communities in the SML entailing strong implications for ecosystem functioning by wind-driven impacts on habitat properties, gas exchange and matter cycling processes.

**Keywords:** air–sea interface; bacterioneuston; enrichment factor; wind-wave tunnel; Baltic Sea, pCO<sub>2</sub>

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## INTRODUCTION

The sea-surface microlayer (SML) spans more than two thirds of the Earth's surface. The SML forms a boundary layer between the lower atmosphere and the ocean with a typical thickness of  $<250 \mu\text{m}$  (Carlson 1982). Despite its thinness, the SML has unique biological and physicochemical properties compared to the underlying water (ULW), a profound role in gas-exchange processes (Liss and Duce 1997) and cycling of matter (Wotton and Preston 2005). By providing stability through high surface tension and presence of surface-active material (Wurl, Miller and Vagle 2011), the SML favors accumulation of organic matter, which in turn promotes metabolic activity of bacterial communities (Cunliffe et al. 2013). Consequently, under certain conditions, e.g. calm sea surfaces, the SML may be considered as a biofilm-like habitat (Wurl et al. 2016). Bacteria in the SML, also referred to as bacterioneuston (Naumann 1917), can be enriched in abundance by a factor of up to 13 compared to the ULW (Agogué et al. 2004). The bacterioneuston is often exposed to harsh environmental conditions such as wave dynamics, ultraviolet and solar radiation (Agogué et al. 2005), strong gradients of temperature and salinity (Zhang et al. 2003a), as well as accumulation of pollutants (Wurl and Obbard 2004). *Bacteroidetes* and *Gammaproteobacteria* are dominant and active members of the bacterioneuston communities probably due to their ability to respond quickly to substrate availability and their higher tolerance to solar radiation (Agogué et al. 2005; Alonso-Saéz et al. 2006).

Wind-induced wave dynamics influence the SML and, thus, most likely the bacterioneuston community. However, a coherent study on the effects of increasing wind speed on bacterioneuston is missing to date, mainly due to the challenge of sampling the SML from a small boat at higher wind conditions as well as due to high spatial and temporal variabilities of field sites. Several field studies reported different conclusions about the effect of wind speed on the SML and its inhabitants. Obernosterer et al. (2008) found that low wind speed had a positive effect on the accumulation of particulate organic material. However, low wind speed was related to inhibited bacterial carbon production in the SML compared to ULW. Reduced bacterial growth reflects the idea of the SML as a challenging habitat (Reinthal, Sintes and Herndl 2008), but wind speed may not solely explain patterns of bacterial activity in the SML. Nevertheless, low wind speed favored the development of distinctive bacterial populations in the SML, when bacterioneuston communities were compared to bacterioplankton communities, i.e. bacteria living in the ULW. These differences between SML and ULW were found among the particle-attached and free-living communities in relation to wind speed (Stolle et al. 2011). It remained however an open question whether SML-specific populations occurred only due to low wind speed or additional factors contributing to the spatial heterogeneity of the habitat, e.g. solar radiation or accumulation of organic matter.

The bacterioneuston is thought to actively participate in gas exchange processes of climate-relevant gases such as methane (Upstill-Goddard et al. 2003), nitrous oxide, carbon monoxide and hydrogen (Conrad and Seiler 1988). Gross primary production and community respiration of planktonic organisms in proximity to the ocean's surface are known to influence air-sea carbon dioxide ( $\text{CO}_2$ ) anomalies (Calleja et al. 2005). Under increasing anthropogenic  $\text{CO}_2$  emissions (Jarvis and Hewitt 2014) and associated ongoing ocean acidification, the metabolic activity of SML and ULW-inhabiting bacteria is likely to be affected. Whereas the effects of ocean acidification on bacterioplankton (Liu et al. 2010;

Engel et al. 2014; Siu, Apple and Moyer 2014) and coastal biofilm communities (Lidbury et al. 2012) have been intensively studied, only little is known about the effects on bacterioneuston.

The effects of decreasing pH on bacterioplankton abundance were dependent on prevailing conditions such as the presence of phytoplankton blooms (Engel et al. 2014; Bunse et al. 2016), availability of nutrients (Roy et al. 2013) or viral dynamics (Brussaard et al. 2013; Sperling et al. 2013). For the SML, Galgani et al. (2014) reported a positive impact of increasing partial pressure of  $\text{CO}_2$  ( $p\text{CO}_2$ ) on bacterial abundance in a mesocosm study. Furthermore, the effects of high  $p\text{CO}_2$  levels on bacterial metabolism are poorly understood. Some studies reported higher degradation rates of organic matter by extracellular enzymes of bacterial origin at high  $p\text{CO}_2$  levels (Grossart et al. 2006; Piontek et al. 2010). Roslev et al. (2004) assumed that heterotrophic  $\text{CO}_2$  assimilation is used for biosynthesis of fatty acids, *de novo* synthesis of glucose, amino acids and nucleotides and contributes up to 6.5% of bacterial biomass production. However, the extent of heterotrophic  $\text{CO}_2$  fixation at elevated  $p\text{CO}_2$  (Dijkhuizen and Harder 1985; Teira et al. 2012) is not known, but could be relevant in the SML where heterotrophic processes are especially dominant (Obernosterer et al. 2005; Reinthal, Sintes and Herndl 2008).

Our aim for this study was to investigate the abundance and community composition of the bacterioneuston in an annular wind-wave tunnel (WWT) during controlled induction of wind speeds ranging from 1.5 to 21.9  $\text{m s}^{-1}$ . This range strongly exceeds wind speed  $>8 \text{ m s}^{-1}$ , above which sampling of bacterioneuston in the field becomes an extremely challenging task. The WWT allowed us to follow the same bacterial community over successive days while spatial and temporal variability of abiotic factors was kept at minimum. The results obtained from the WWT were compared to field samples from the Baltic Sea. In parallel to the impacts of wind speed, we investigated the influence of different  $p\text{CO}_2$  levels in the water of the WWT on the bacterioneuston. We hypothesized that the physical stability of the SML and its function as a microbial habitat is strongly dependent on sea state and, therefore, on wind speed. Our further hypothesis was that high  $p\text{CO}_2$  levels have the potential to shift the abundance and composition of bacterioneuston communities.

## MATERIALS AND METHODS

### Conditions in the WWT: wind speed, $p\text{CO}_2$ levels, light and bubbling

The Small-Scale Air-Sea Interaction Facility, the 'Aeolotron' at the University of Heidelberg, comprises an annular WWT (Mesarchaki et al. 2015), filled with 18 000 L of seawater  $\sim 2$  weeks prior sampling. About 12 000 L of seawater originated from off-shore Norway ( $\sim 55 \text{ m}$  depth, Sula Reef Complex ( $64^\circ 4.90' \text{ N}$ ,  $8^\circ 2.03' \text{ E}$ )) and were mixed with 6000 L of coastal surface water ( $\sim 5 \text{ m}$  depth) taken near the island of Sylt, Germany.

The facility allows wave generation at controlled wind speeds ranging from 1.5 to 21.9  $\text{m s}^{-1}$ . On each of the five experimental days, six different wind speeds were applied, always increasing from low (1.3–2.9  $\text{m s}^{-1}$ ) to high (17.5–21.9  $\text{m s}^{-1}$ ) (Fig. 1A, Table 1). Duration of each wind speed setting varied from 30 min to 2 h with longer durations for the lower wind speeds. Bubbling was induced to enhance bubble bursting additionally to the effect of breaking waves at one of the high wind speeds on each experimental day (Table 1). For this purpose, air from the tank's atmosphere was blown through evenly

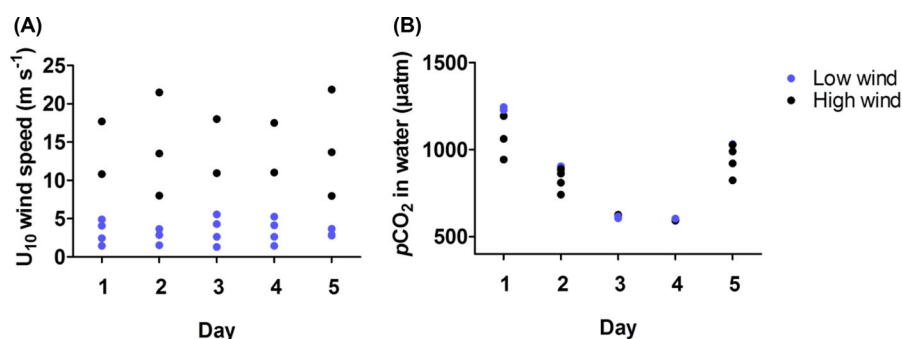


Figure 1. Conditions at the WWT:  $U_{10}$  wind speed ( $\text{m s}^{-1}$ ) categorized into 'low' ( $<8.0 \text{ m s}^{-1}$ ) and 'high' ( $\geq 8.0 \text{ m s}^{-1}$ ) (A), and ranges of  $p\text{CO}_2$  levels in water (B) on the five experimental days.

Table 1. Wind speeds, wind speeds during bubbling,  $p\text{CO}_2$  and light conditions during experiments in the WWT. Fourth column indicates wind speed at which bubbling was applied.

Date	Day	$U_{10}$ wind speed range ( $\text{m s}^{-1}$ )	$U_{10}$ wind speed ( $\text{m s}^{-1}$ ) plus bubbling	$p\text{CO}_2$ range in water ( $\mu\text{atm}$ )	Light/dark
19 November 2014	1	1.5, 2.5, 4.1, 4.9, 10.8 (2x)	10.8	1245–943	Dark
21 November 2014	2	1.5, 2.9, 3.7, 8.0, 13.5, 21.5 (2x)	21.5	883–741	Dark
24 November 2014	3	1.3, 2.6, 4.3, 5.6, 10.9 (2x), 18.0	10.9	624–607	Light
26 November 2014	4	1.5, 2.6, 4.1, 5.3, 11.0 (2x), 17.5	11.0	604–594	Light
28 November 2014	5	2.8 (2x), 3.7, 8.0, 13.7, 21.9 (2x)	21.9	1032–824	Light

distributed submerged aerators at the bottom of the WWT. The wind speed refers to  $U_{10}$  wind speed, because it corresponds to the wind speed in 10 m height. Friction velocity,  $U^*$ , was measured using the momentum balance method described by Bopp (2011). The friction velocity was converted to  $U_{10}$  using the drag coefficient measured by Edson et al. (2013).

A volume of 200 L tank water was externally saturated with  $\text{CO}_2$  on 18 and 27 November 2014 and added into the tank  $\sim 12$  h before the first (low wind speed) sample was taken. This led to particularly high  $p\text{CO}_2$  ranges at days 1 and 5 (Fig. 1B, Table 1). The broad range of  $p\text{CO}_2$  levels for each condition is the result of  $p\text{CO}_2$  flux from water to air at higher wind speeds. The determination of the water  $p\text{CO}_2$  was based on the use of a bubble-type equilibrator that was supplied with a continuous flow of water from inside the tunnel. Infrared spectroscopy was used to detect  $\text{CO}_2$  gas phase equilibrium generated by circulating air in a closed loop through the water column (LI-6262, LI-COR). The IR  $\text{CO}_2$  analyzer was calibrated before and after the experiment, and we estimate the uncertainty of our data being  $<5 \mu\text{atm}$ . More details about the instrumentation and the calculation of the final  $p\text{CO}_2$  are given in Schneider et al. (2014).

On 14 November, nutrients were added to the WWT as follows:  $10 \mu\text{mol L}^{-1}$  nitrate,  $10 \mu\text{mol L}^{-1}$  silicate and  $0.6 \mu\text{mol L}^{-1}$  of phosphate. In addition, 1 L of coccolithophorid algae culture (*Emiliana huxleyi*,  $4.6 \times 10^5$  cells  $\text{mL}^{-1}$ ) and 6.3 L of biogenic SML sample containing a natural phytoplankton community were added to the tank on 22 November and 23 November, respectively. These additions served the purpose to increase primary productivity in the WWT. From 19 to 21 November, the tank was kept dark. From 22 until 28 November, light was switched on with a 12 Light:12 Dark regime (LED SmartArrays, Lumitronix).

### Sample collection in the WWT

After the end of each wind condition, the WWT was opened and the SML was sampled with the glass plate technique (Harvey and Burzell 1972) using a withdrawal rate of  $5\text{--}6 \text{ cm s}^{-1}$  as

suggested by Carlson (1982). The glass plate collects the SML with a thickness of about  $30\text{--}60 \mu\text{m}$  (Carlson 1982), which is consistent with experimentally determined SML thicknesses of  $50 \pm 10 \mu\text{m}$  using pH microelectrodes (Zhang et al. 2003b). The glass plate was cleaned with 70% ethanol and rinsed with sample before use. Adhering water was removed by wiping the plate with a squeegee into a sample-rinsed brown bottle. The procedure was repeated until the required volume of 100 mL was collected ( $\sim 20$  dips).

Samples from the ULW were taken at a depth of 50 cm from a tap connected to the tunnel. A pair of SML and ULW sample was taken at each wind condition.

### Sample collection in the Baltic Sea

SML and ULW samples were obtained from the central and the northeastern Baltic Sea (Gotland Basin and Gulf of Finland) in August 2015 during the cruise M117 with R/V Meteor (Fig. 2, Table 2). Data for the cruise are stored at PANGAEA (Rahlff et al. 2016). Stations were presented using Ocean Data View (Schlitzer 2015). We used the glass plate sampler to collect SML samples from the bow of a small boat using the protocol described above. Glass plate dips affected by splashing water were rejected. We used a syringe connected to a polypropylene tube for the collection of ULW samples at  $\sim 1$  m depth. Wind data were taken from the meteorological station on R/V Meteor and converted into the wind speed for 10 m height to facilitate comparison with the WWT conditions. For conversion, we used Eq. (1)

$$U_{10} = \left( \frac{h_2}{h_1} \right)^g * U_{35.4} \quad (1)$$

with  $U_{10}$  being the wind speed at 10 m height ( $h_2$ ),  $U_{35.4}$  the wind speed that was recorded at 35.4 m height ( $h_1$ ) and the exponent  $g = 0.16$  as suggested for open water by Kleemann and Meliss (1993). The  $U_{10}$  wind speed, ranging from 2.4 to  $7.7 \text{ m s}^{-1}$ , as a

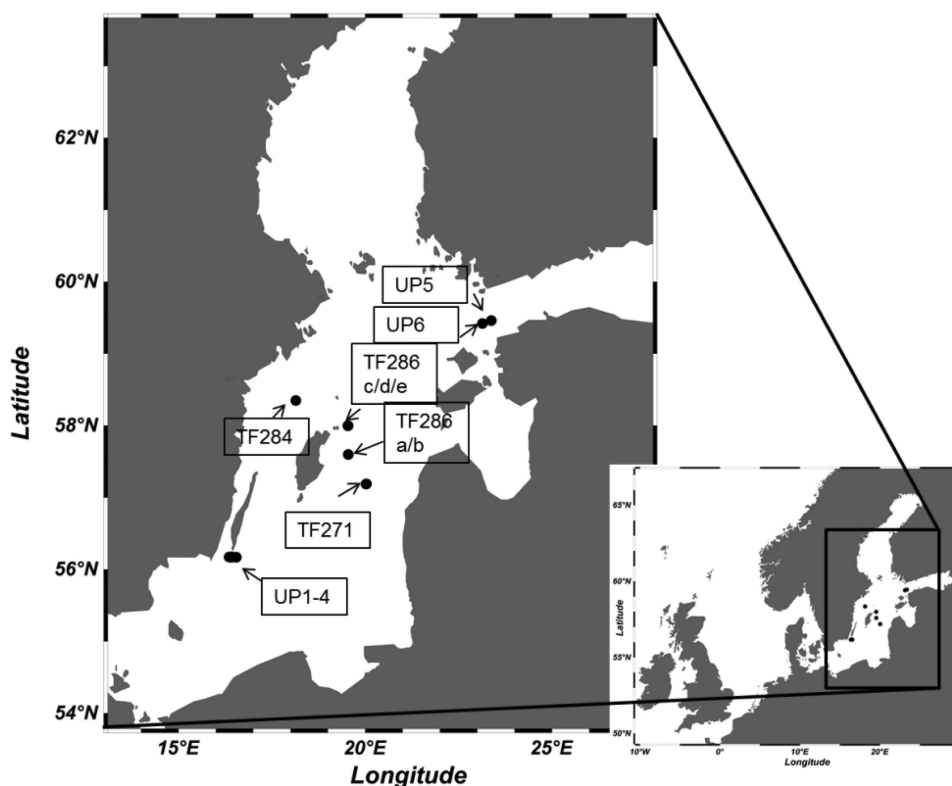


Figure 2. Station map for the Baltic Sea.

Table 2. Stations in the Baltic Sea and field parameters.

Sample	Date	Wind 6 h	Wind 1 h	Temperature (°C)	Upwelling	EF Bacteria
TF271.1	30 July 2015	7.6	8.5	16.1	no	1.08
UP1	01 August 2015	7.7	8.5	8.9	yes	1.05
UP1b	01 August 2015	7.6	6.6	8.1	yes	1.01
UP3	02 August 2015	2.6	3.1	9.6	yes	1.31
UP3b	02 August 2015	2.9	2.2	10.0	yes	1.02
UP2	03 August 2015	3.1	3.3	13.4	yes	1.09
UP2.Foam	03 August 2015	3.1	3.2	13.4	yes	1.37
UP2b	03 August 2015	4.5	4.8	13.6	yes	1.10
UP4	04 August 2015	5.7	5.9	15.9	yes	0.98
TF284	06 August 2015	4.5	2.8	16.2	no	1.03
UP5	08 August 2015	5.9	6.3	17.3	no	1.02
UP5b	08 August 2015	6.2	7.0	15.4	no	0.91
UP6	09 August 2015	4.1	3.8	16.9	no	1.20
TF286a	11 August 2015	2.4	2.7	18.1	no	1.18
TF286b	11 August 2015	2.9	3.6	18.3	no	0.99
TF286c	11 August 2015	3.4	3.8	18.3	no	1.01
TF286d	11 August 2015	3.5	3.8	18.5	no	1.08
TF286e	11 August 2015	3.5	3.4	18.6	no	1.03
TF271.2	12 August 2015	4.2	3.5	19.4	no	0.97

Wind speed is given as mean  $U_{10}$  for 6 h prior sampling and mean of 1 h during sampling ( $\pm 30$  min).

EF = enrichment factor.

6-h mean was used (Obernosterer *et al.* 2008) because we assume it better accounts for temporal variability (Table 2).

### Determination of bacterial abundance

For determination of bacterial cell numbers, water samples from the WWT were fixed with formaldehyde (3.4% final concentration), incubated at room temperature for 1 h, snap-

frozen in liquid nitrogen and stored at  $-18^{\circ}\text{C}$  until further analysis. Field samples from the Baltic Sea were fixed with glutaraldehyde (1% final concentration), incubated for 1 h and frozen on-board at  $-40^{\circ}\text{C}$ . We decided to change for glutaraldehyde as a fixative since it gives a better fluorescence yield (Lunau *et al.* 2005). Prokaryotic cells were stained with SYBR<sup>®</sup> Green I Nucleic Acid Gel Stain (9 $\times$  in final concentration, Molecular Probes, Invitrogen) and incubated in the dark at room temperature for



30 min. Samples were measured on a flow cytometer (C6 Flow Cytometer, BD Bioscience), and cells were counted according to side-scattered light and emitted green fluorescence. We used 1.0  $\mu\text{m}$  beads (Fluoresbrite Multifluorescent, Polysciences) as internal reference to monitor the performance of the device. We calculated the coefficient of variation of replicate measurements to be 2.8% and 4.1% for ULW and SML samples ( $n = 4$  each), respectively. The abundance of bacteria in SML and ULW was used to calculate the enrichment factor (EF), i.e. the relative abundance of bacteria in a SML sample divided by its ULW counterpart. Therefore, an  $\text{EF} > 1$  implies an enrichment of bacteria in the SML, whereas an  $\text{EF} < 1$  shows depletion.

### Nucleic acid extraction and PCR

Filtration of SML and ULW samples from the WWT was conducted on 0.2  $\mu\text{m}$  polycarbonate (PC) filters (Merck Millipore). All filters were initially stored at  $-18^\circ\text{C}$  and at  $-80^\circ\text{C}$  in the home laboratory until analysis.

Nucleic acids (DNA and RNA) were extracted from the filters using the DNA + RNA + Protein Extraction Kit (Roboklon) and a modified protocol. Briefly, filters were treated with 100  $\mu\text{L}$  Lyse All buffer plus 100  $\mu\text{L}$  of 5 mg  $\text{mL}^{-1}$  lysozyme solution (15 min at  $37^\circ\text{C}$ ) with vigorous mixing in between. Afterwards, DRP containing  $\beta$ -mercaptoethanol and 0.1 mm glass beads were added to the filters and intensively vortexed for 3 min. After subsequent centrifugation for 10 min at 13 000 rpm, the supernatant was transferred to a DNA-binding column and the extraction was further conducted as described in the kit's manual.

Polymerase chain reaction (PCR) of the 16S rRNA gene was performed using 2  $\mu\text{L}$  of template DNA, the primer set described by Muyzer et al. (2004), i.e. EU-341 for (5'-CCTACGGGAGGCAGCAG-3') and Uni-907RM rev (5'-CCGTCGAATTCMTTGTAGTTT-3') (Metabion International) and the 2x PCR Super Master Mix (Biotool). A GC clamp with the following sequence was added to 5' end of the forward primer: 'CGCCCGCCGCGCGCGCGCGGGCGGGCGGGGCACGGGGG' (Muyzer, de Waal and Uitterlinden 1993; Muyzer et al. 2004). For PCR, a modified touchdown program of the one described by Muyzer et al. (2004) was applied: 1 cycle of  $94^\circ\text{C}/5$  min,  $65^\circ\text{C}/1$  min,  $72^\circ\text{C}/1$  min; 34 cycles of  $94^\circ\text{C}/1$  min,  $64$ – $55^\circ\text{C}/1$  min (touchdown: decrease of  $0.5^\circ\text{C}$  per cycle for 19 cycles, then  $55^\circ\text{C}/1$  min for 15 cycles); 1 cycle of  $94^\circ\text{C}/1$  min,  $55^\circ\text{C}/1$  min,  $72^\circ\text{C}/10$  min.

To amplify 16S rRNA sequences, nucleic acid extracts were incubated with 3 U of RNase-free DNase (Roboklon) at  $37^\circ\text{C}$  for 60 min, cleaned up using the Universal RNA Purification Kit (Roboklon), and concentrations were determined using the Nanodrop 2000c spectrophotometer (Thermo Scientific). Complete digestion of DNA was tested by PCR on every RNA sample. In case of remaining DNA in the extracts, the DNase treatment was repeated. Samples of RNA (5 ng) were used for cDNA synthesis with the NG dART RT Kit (Roboklon). Reverse transcription was carried out by using the universal primer 1492R (5'-GGTTACCTTGTTACGACTT-3' adapted from Lane 1991) at  $50^\circ\text{C}$  for 50 min and terminated at  $85^\circ\text{C}$  for 5 min as suggested by the manual. The above-mentioned conditions, primer set and 1  $\mu\text{L}$  of cDNA were used for PCR thereafter.

### 16S rRNA and rRNA gene fingerprinting

Analyses of PCR products generated from 16S rRNA genes (reflecting the total bacterial community) or generated from 16S rRNA-derived cDNA (reflecting active members of the bacterial community) was performed by denaturing gradient gel

electrophoresis (DGGE) using the DCode Universal Mutation Detection System (Bio-Rad) with a 40%–70% denaturing gradient and 6% (wt/vol) polyacrylamide content in the gel. As an internal reference for gel analysis and as a marker for band separation, we used a pool of 16S rRNA gene-PCR products derived from different bacterial isolates. Loading volume of PCR products was adjusted according to their band intensity on an agarose gel, i.e. loaded DNA amount on DGGE was  $\sim 200$  ng. Denaturing gradient gels were stained with SYBR Gold (Molecular Probes, Invitrogen) and visualized using the Molecular Imager<sup>®</sup> Gel Doc XR+ System (Bio-Rad) and the software Image Lab.

### Cluster analysis

The banding patterns of single DGGE gels were analyzed using digitized images and GelCompar II (Applied Maths NV) based on densitometric curves, i.e. comparing gray values of specific positions between two gel lanes. Background subtraction, least-square filtering and optimization were carried out according to the manufacturer's instructions. The results are presented as either cluster analyses of whole samples sets from one gel or as pairwise similarities, i.e. the percentage of similarity between the compositions of corresponding bacterioneuston (SML-derived) and bacterioplankton (ULW-derived) communities at each wind speed. Cluster analyses and pairwise comparison were based on the Pearson correlation coefficient implemented in GelCompar II (Rademaker et al. 1999). This curve-based correlation was previously found to be more suitable for the analyses of similar fingerprint data when compared to band-based analyses (Jaccard coefficient—see Stolle et al. 2011 and associated supplement material).

### Sequence analysis

Individual bands from the DGGE gels were excised, resuspended in 50  $\mu\text{L}$  of nuclease-free water and stored overnight at  $4^\circ\text{C}$ . Then DNA of the bands was reamplified using the same primer set as described above but using the forward primer without GC clamp. The PCR program was as follows: initial denaturation at  $95^\circ\text{C}/4$  min, 10 cycles of  $95^\circ\text{C}/50$  s,  $64^\circ\text{C}$ – $55^\circ\text{C}/45$  s (touchdown: decrease of  $1^\circ\text{C}$  per cycle for 10 cycles)  $72^\circ\text{C}/40$  s, 25 cycles of  $95^\circ\text{C}/50$  s,  $55^\circ\text{C}/45$  s,  $72^\circ\text{C}/40$  s, final extension at  $72^\circ\text{C}/7$  min. Success of the PCR was controlled on a 1.2% agarose gel, and PCR products were purified with the Hi Yield<sup>®</sup> Gel/PCR DNA Fragment Extraction Kit (Süd-Laborbedarf). PCR products were sent for sequencing (GATC Biotech) using the above-mentioned forward and reverse primers. Forward and reverse sequences were quality checked and aligned (SeqMan II 5.06, DNASTAR Inc.), and phylogenetic affiliations were determined using the basic local alignment search tool (BLAST, [www.ncbi.nlm.nih.gov/BLAST/](http://www.ncbi.nlm.nih.gov/BLAST/)). Phylogenetic affiliations of partial 16S rRNA and 16S rRNA gene sequences were also checked via Silva database (<http://www.arb-silva.de/>). For each operational taxonomic unit (OTU) determined in this study, a representative partial 16S rRNA gene sequence was deposited in the GenBank database. The sequences were assigned accession numbers KX379571–KX379605 (Table 4).

### Statistical analysis

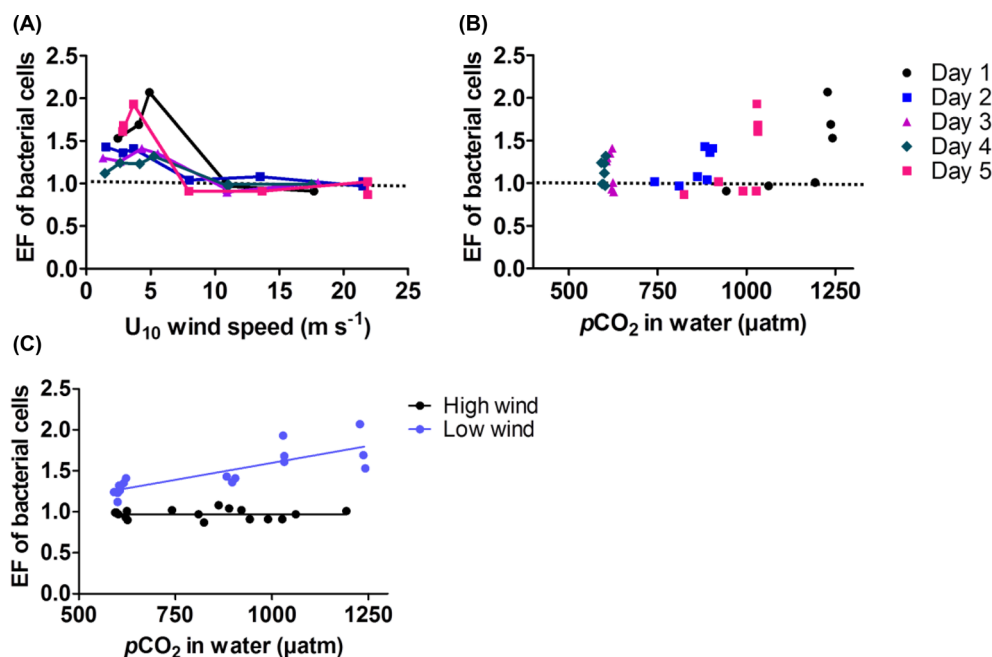
All statistics were carried out using the software R version 3.1.2 (Team 2014). A breakpoint for bacterial enrichment in relation to wind speed was calculated for the dataset of WWT samples by using an iterative searching method with piecewise regressions

**Table 3.** Statistical parameters for linear models describing enrichment of bacteria and pairwise similarities in the WWT in response to  $U_{10}$  wind speed and  $p\text{CO}_2$ .

Explaining variables	p-value	F-value	df	Adjusted R <sup>2</sup>	AIC
<b>Response variable: bacterial EF</b>					
$p\text{CO}_2 \times \text{wind category } (<8.0/\geq 8.0)$	<0.001	88.6	30	0.89	-70.04
Wind category (<8.0/ $\geq$ 8.0)	<0.001	88.7	32	0.73	-41.38
$p\text{CO}_2$	<0.05	5.1	32	0.11	-1.24
<b>Response variable: pairwise similarities</b>					
$p\text{CO}_2 + \text{wind category } (<8.0/\geq 8.0)$	<0.001	11.0	31	0.38	-9.26
Wind category (<8.0/ $\geq$ 8.0)	<0.01	10.6	32	0.23	-2.81
$p\text{CO}_2$	<0.01	8.4	32	0.18	-0.93

Wind speed data were either categorized into 'low' (<8.0 m s<sup>-1</sup>) or 'high' ( $\geq$ 8.0 m s<sup>-1</sup>).

AIC = Akaike's Information Criterion, df = degrees of freedom, EF = enrichment factor.



**Figure 3.** Changes in EF of bacterial cells in response to wind speed (m s<sup>-1</sup>) (A) and high  $p\text{CO}_2$  (B) for the five experimental days as well as to  $p\text{CO}_2$  level with grouped wind categories (C); dashed line indicates EF = 1.

(Crawley 2013). For this calculation, the range for the breakpoint was predefined between 0 and 15 m s<sup>-1</sup>. This breakpoint was used to categorize  $U_{10}$  wind speed into a 'low' and a 'high' category. We then used linear regression to examine the effect of either categorized  $U_{10}$  wind speed or  $p\text{CO}_2$  alone or their interactive effect on log-transformed EF of bacteria (Table 3). To describe the effect of  $U_{10}$  wind speed on bacterial community structure, i.e. the pairwise similarities between SML and ULW, we also applied a linear model with the variables  $U_{10}$  wind speed (categorical) and  $p\text{CO}_2$ . All response variables were log-transformed to ensure variance homoscedasticity. To assess a model's fit, we used adjusted R<sup>2</sup>, Akaike's Information Criterion and residual diagnostics.

## RESULTS

### Bacterial enrichment in the SML of the WWT

From day 1 to day 2, the bacterial abundance in both SML and ULW increased and then constantly declined until the end of the

experiment (Fig. S1, Supporting Information). Irrespective of the time of the experiment and the total cell counts at any day, bacterial cell numbers in the SML were always higher compared to the ULW at low wind speed and compared to the SML and ULW at high wind speed (Fig. S1). Therefore, the EF of bacterial cells was highest at low wind speed with maximum values at day 1 and day 5 (up to 2.1 and 1.9, respectively, Fig. 3A). The enrichment of bacterial cells was always >1.1 up to  $U_{10}$  of  $\leq 5.6$  m s<sup>-1</sup>. At the next highest wind speed applied (i.e. 8.0 m s<sup>-1</sup>), the EF declined to <1.1 and did not increase to values >1.1 at higher wind speeds (i.e. >8.0 m s<sup>-1</sup>). Considering uncertainties of measurements and natural variability, we defined the limit for a consistent enrichment of bacteria as EF > 1.1. Additional bubbling of the water at high wind speed did not further affect the enrichment of bacteria in the SML. The higher enrichment of bacteria in SML over ULW at low wind speed was consistent for all experiments.

In addition, we found the enrichment of bacteria at  $\leq 5.6$  m s<sup>-1</sup>  $U_{10}$  to be even more pronounced at high  $p\text{CO}_2$  levels (days 1

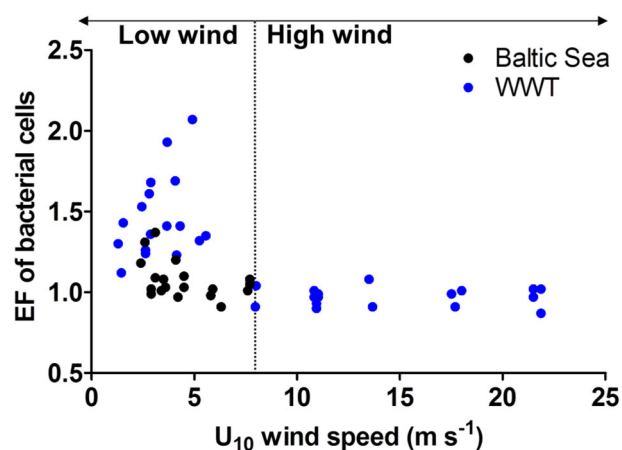


Figure 4. EF of bacterial cells versus  $U_{10}$  wind speed for WWT and field samples from the Baltic Sea. Breakpoint for categorization at  $8.0 \text{ m s}^{-1}$  is indicated by a dashed line.

and 5 in Fig. 3B) than on days with lower  $p\text{CO}_2$  in water (days 2 to 4). Using the piecewise regression approach, we defined a low wind speed category (i.e.  $<8.0 \text{ m s}^{-1}$ ) and a high wind speed category (i.e.  $\geq 8.0 \text{ m s}^{-1}$ ) according to the pattern for bacterial enrichment in the WWT (Fig. 4). The slopes for the relationship between wind speed and bacterial EF below and above the threshold value were both not significant. The overall piecewise model fit the data well ( $F = 22.98$ ,  $df = 29$ ,  $P < 0.001$ , adjusted  $R^2 = 0.73$ ). Applying the wind speed categories to the linear model, we could demonstrate a significant interactive effect of categorized wind speeds and  $p\text{CO}_2$  on EF of bacteria ( $t = 4.17$ ,  $P < 0.001$ ,  $n = 34$ , Fig. 3C, Table 3). At wind speeds of  $\leq 5.6 \text{ m s}^{-1}$ , the enrichment of bacteria was positively affected by increasing levels of  $p\text{CO}_2$ . A comparison of the full model with models including either wind speed or  $p\text{CO}_2$  as single predictor variables showed that the full model explained significantly more variance in the data than either factor on its own (Table 3). Considering wind speed and  $p\text{CO}_2$  alone, we found a significant relationship for bacterial enrichment in response to categorized  $U_{10}$  wind speed ( $t = 9.42$ ,  $P < 0.001$ ,  $n = 34$ ) and  $p\text{CO}_2$  ( $t = 2.25$ ,  $P = 0.031$ ,  $n = 34$ ) and showed that wind speed is the more relevant variable for bacterial enrichment according to the model.

### Bacterial enrichment in the SML of the Baltic Sea and comparison with WWT

Overall, the EFs of bacterial cells in the Baltic Sea were lower than for the WWT samples and never raised above 1.4 (Table 2). The enrichment of bacteria was also  $<1.0$  for low wind speeds in some cases (minimum  $EF = 0.9$ ). The highest EF was found in a SML sample associated with sea foam taken at a  $U_{10}$  wind speed of  $3.1 \text{ m s}^{-1}$  (Table 2). At wind speeds  $>4.1 \text{ m s}^{-1}$ , the EF of bacterial cells in the field was never  $>1.1$ . However, we could not find any statistically significant relationship for bacterial EF and wind speed in the field. We found that bacterial enrichment  $>1.1$  could be detected until a  $U_{10}$  wind speed of  $5.6 \text{ m s}^{-1}$  in the WWT whereas in the Baltic Sea it could only be found until a  $U_{10}$  wind speed of  $4.1 \text{ m s}^{-1}$  (Fig. 4).

### Bacterioneuston community composition in response to wind speed and $p\text{CO}_2$ in the WWT

Cluster analysis of the 16S rRNA gene fingerprints revealed that SML samples collected during low wind conditions ( $\leq 5.6 \text{ m s}^{-1}$ ) generally formed distinctive clusters on each of the experimental days, which differed from SML samples during high wind conditions and ULW samples (Fig. S2, Supporting Information). These SML clusters at low wind speed were especially distinct on days 1, 3 and 5, with 58.9%, 62.4% and 48.5% similarity to all other samples, respectively. SML clusters at low wind speeds on days 2 and 4 were more similar to all other SML and ULW samples from these experimental days (75.8% and 88.0%, respectively).

For determination of wind speed effects on community differentiation between SML and ULW, the similarity between the composition of bacterioneuston and bacterioplankton communities during each observed wind speed was analyzed and expressed as a pairwise similarity. Grouping the data according to the wind speed categories that were determined for bacterial enrichment and applying a linear regression model, we did not find a significant interaction between categorized  $U_{10}$  wind speeds and  $p\text{CO}_2$  on the pairwise similarities. However, we could find significant effects of the individual variables, i.e. categorized wind speed ( $t = -3.32$ ,  $P < 0.01$ ,  $n = 34$ , Fig. S3A and C, Supporting Information) and  $p\text{CO}_2$  ( $t = -2.96$ ,  $P < 0.01$ ,  $n = 34$ , Fig. S3B and C). It follows that both the enrichment of bacteria and the pairwise similarities between SML and ULW community composition showed a strong dependence on  $U_{10}$ . Particularly at low wind speeds, higher enrichment of bacteria was associated with decreasing similarities between the SML and ULW community (Fig. S4, Supporting Information).

Sequencing revealed that members of the bacterial communities in the SML and ULW were related to bacterial taxa that had been previously described for marine habitats (Table 4). Some of these OTUs were present at all experimental days, e.g. one OTU sharing high identity with the marine alga symbiont *Phaeodactylibacter xiamenensis* (Genbank accession no: KF986715.1). Another OTU related to *Legionella impletisoli* strain OA1-1 (NR.041321.1) was detected during the light period on days 3–5. Some OTUs appeared solely at low wind speeds in the SML. They were closely related to *Alteromonas* sp. (AB526338.1) (day 1), *Limnobacter thiooxidans* (LN774750.1) (day 2) or *Pseudoalteromonas* sp. (AF239705.1) (day 2, Fig. 5). On day 5, we identified one OTU related to *Flavobacterium bacterium* Yb008 (AB496663.1), which was present in the ULW at all applied wind speeds, but occurred in the SML only at high wind speed conditions, i.e. it disappeared between  $3.7$  and  $8.0 \text{ m s}^{-1}$  (Fig. 6).

For examination whether SML-specific OTUs during low wind could also be considered as being active, we further investigated bacterial community composition of day 1 based on 16S rRNA fingerprints (Fig. S5, Supporting Information). We identified a specific SML and low-wind OTU related to *Alteromonas* sp. (AB526338.1), which was also abundant in the SML at the same day according to the 16S rRNA gene-based sequencing results (Fig. S5, Table 4). We detected an OTU with high identity to *F. bacterium* Yb008 on the DNA-based DGGE, which was also active on day 1 and similarly absent from the SML at low wind speed on the RNA-based gel. Moreover, an OTU associated with *Mariobacter* sp. (KM979164.1) was active and abundant in SML and ULW at all times.

**Table 4.** Phylogenetic affiliation and BLAST results for 16S rRNA gene sequences, OTU = operational taxonomic unit, OTU name = SML/ULW.WS( = wind speed).D+No = (Day + Number).RNA (if RNA-based).

Taxon and OTU	Name of closest phylogenetic relative	Accession number	16S rRNA sequence similarity (%)	SML-specific at low wind (S)/absent from SML (A)	Phylogenetic affiliation (SINA)	Genbank accession no. (this study)
<i>Alphaproteobacteria</i>						
SML.WS2.9.D2.H	<i>Sphingobium</i> sp. FO10	KC478083.1	100		<i>Sphingomonadaceae</i> (99.8%)	KX379584
SML.WS3.7.D2.I	<i>Sphingobium</i> sp. X-b4	JX997857.1	96		<i>Sphingomonadaceae</i> (98.4%)	KX379585
ULW.WS13.5.D2.G	<i>Pseudoruegeria aquimaris</i> strain SW-255	NR_043932.1	99		<i>Rhodobacteraceae</i> (99.4%)	KX379587
SML.WS8.0.D5.J	<i>Alpha proteobacterium</i> C54	AB302371.1	99		<i>Rhodobacteraceae</i> (99.3%)	KX379577
ULW.WS3.7.D5.L	<i>Pseudoruegeria aquimaris</i> strain SW-255	NR_043932.1	99		<i>Rhodobacteraceae</i> (98.8%)	KX379601
<i>Betaproteobacteria</i>						
SML.WS4.1.D1.K	<i>Limnobacter thiooxidans</i> isolate 0312MAR12L4	LN774750.1	98		<i>Burkholderiales</i> (98.6%)	KX379578
SML.WS2.9.D2.F	<i>Limnobacter thiooxidans</i> isolate 0312MAR12L4	LN774750.1	100	S	<i>Burkholderiaceae</i> (100%)	KX379573
<i>Gammaproteobacteria</i>						
SML.WS1.5.D1.G	<i>Alteromonas</i> sp. JAM-GA15	AB526338.1	99	S	<i>Alteromonadaceae</i> (99.5%)	KX379571
SML.WS4.9.D1.P	<i>Oleibacter marinus</i> strain NBRC 105760	NR_114287.1	95		<i>Oceanospirillaceae</i> (96.7%)	KX379579
ULW.WS17.7.D1.K	<i>Marinobacter adhaerens</i> strain G4	KU058181.1	99		<i>Alteromonadaceae</i> (99.8%)	KX379596
SML.WS2.9.D2.D	<i>Alteromonas</i> sp. JAM-GA15	AB526338.1	100	S	<i>Alteromonadaceae</i> (99.7%)	KX379582
SML.WS3.7.D2.B	<i>Methylophaga</i> sp. VSW109	KC534309.1	96		<i>Piscirickettsiaceae</i> (96.8%)	KX379586
SML.WS3.7.D2.O	<i>Pseudoalteromonas</i> sp. SA12	KM033244.1	96	S	<i>Alteromonadaceae</i> (99.5%)	KX379572
ULW.WS21.5.D2.E	<i>Marinobacter adhaerens</i> strain Bsw-39b	KP336737.1	96		<i>Alteromonadaceae</i> (98.6%)	KX379595
SML.WS1.3.D3.D	<i>Legionella</i> sp. LH-SW	LN899829.1	92		Unclassified_Gammaproteobacteria (95.4%)	KX379597
SML.WS2.6.D3.C	<i>Legionella impletisoli</i> strain OA1-1	NR_041321.1	92	S	<i>Legionellaceae</i> (96.0%)	KX379574
ULW.WS5.6.D3.D	<i>Legionella</i> sp. LH-SW	LN899829.1	92		Unclassified_Gammaproteobacteria (95.6%)	KX379598
SML.WS1.5.D4.H	<i>Legionella</i> sp. LH-SW	LN899829.1	92		<i>Legionellaceae</i> (95.6%)	KX379592
SML.WS1.5.D4.J	<i>Marinobacter</i> sp. H2-43	KM979164.1	100		<i>Alteromonadaceae</i> (100%)	KX379600
ULW.WS11.0.D4.C	<i>Legionella impletisoli</i> strain OA1-1	NR_041321.1	84		<i>Legionellaceae</i> (94.8%)	KX379591
SML.WS2.8.D5.F	<i>Legionella impletisoli</i> strain OA1-1	NR_041321.1	92		<i>Legionellaceae</i> (95.9%)	KX379594
SML.WS3.7.D5.C	<i>Gamma proteobacterium</i> HLSB119	FJ999568.1	99	S	<i>Haliaceae</i> (98.9%)	KX379599
SML.WS1.5.D1.A.RNA	<i>Alteromonas</i> sp. JAM-GA15	AB526338.1	94	S	<i>Alteromonadaceae</i> (94.0%)	KX379602
SML.WS1.5.D1.B.RNA	<i>Oleibacter marinus</i> strain 5	KT906692.1	95	S	<i>Oceanospirillaceae</i> (92.6%)	KX379603
SML.WS4.9.D1.F.RNA	<i>Marinobacter</i> sp. H2-43	KM979164.1	97		<i>Alteromonadaceae</i> (92.5%)	KX379604
<i>Bacteroidetes</i>						
SML.WS4.9.D1.D	<i>Crocinitomix catalasitica</i>	AB681013.1	96		<i>Cryomorphaceae</i> (95.7%)	KX379583
ULW.WS4.9.D1.H	<i>Phaeodactylibacter xiamenensis</i> strain KD52	KF986715.1	98		<i>Saprospiraceae</i> (86.5%)	KX379580
ULW.WS21.5.D2.C	<i>Phaeodactylibacter xiamenensis</i> strain KD52	KF986715.1	99		<i>Saprospiraceae</i> (87.5%)	KX379581
SML.WS1.3.D3.H	<i>Phaeodactylibacter xiamenensis</i> strain KD52	KF986715.1	99		<i>Saprospiraceae</i> (97.7%)	KX379588
SML.WS2.6.D4.D	<i>Phaeodactylibacter xiamenensis</i> strain KD52	KF986715.1	98		<i>Saprospiraceae</i> (97.5%)	KX379590



Table 4. (continued).

Taxon and OTU	Name of closest phylogenetic relative	Accession number	16S rRNA sequence similarity (%)	SML-specific at low wind (S)/absent from SML (A)	Phylogenetic affiliation (SINA)	Genbank accession no. (this study)
SML.WS11.0.D4.E	<i>Phaeodactylibacter xiamenensis</i> strain KD52	KF986715.1	99		Saprospiraceae (98.2%)	KX379589
SML.WS21.9.D5.D	<i>Flavobacteria bacterium</i> Yb008	AB496663.1	100	A	Flavobacteriaceae (99.1%)	KX379576
SML.WS2.8.D5.I	<i>Phaeodactylibacter xiamenensis</i> strain KD52	KF986715.1	99		Saprospiraceae (98.1%)	KX379593
SML.WS4.9.D1.C.RNA	<i>Flavobacteria bacterium</i> Yb008	AB496663.1	99	A	Flavobacteriaceae (95.4%)	KX379605
Cyanobacteria						
SML.WS3.7.D5.A	<i>Amphora coffeaeformis</i> isolate C107 chloroplast	FJ002183.1	98	S	Cyanobacteria; Chloroplast (97.4%)	KX379575

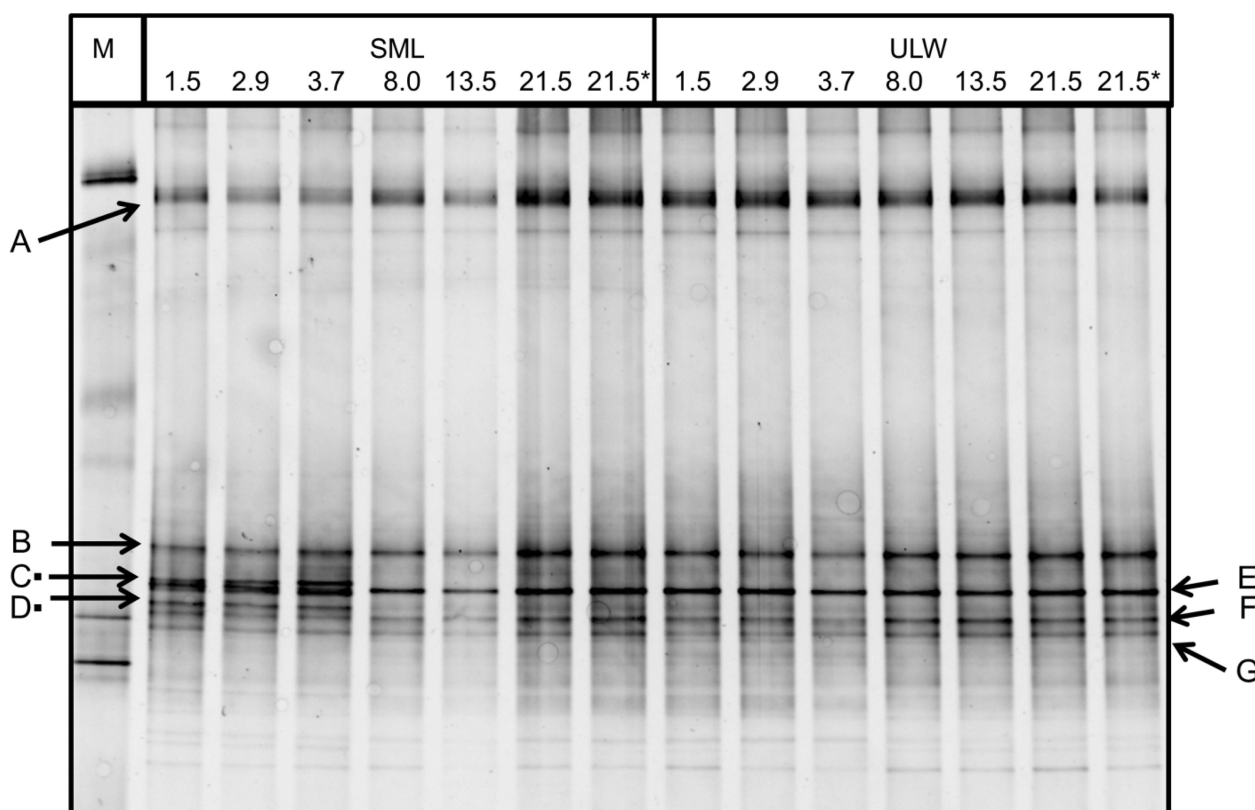


Figure 5. DGGE gel shows WWT SML and ULW 16S rRNA gene samples under increasing  $U_{10}$  wind speed ranging from 1.5 to 21.5  $\text{m s}^{-1}$  from experimental day 2. OTUs derived from sequenced bands are A = *Flavobacteria bacterium* Yb008, B = *Phaeodactylibacter xiamenensis*, C = *Pseudoalteromonas* sp., D = *Limnobacter thiooxidans*, E = *Marinobacter* sp., F = *Pseudoruegeria aquimaris*, G = *Sphingopyxis* sp.; SML/low wind speed-specific OTU on a denaturing gradient gel (marked with filled squares). M = marker, SML = sea-surface microlayer, ULW = underlying water, asterisk indicates additional aeration at that wind speed.

## DISCUSSION

### Impact of wind on bacterioneuston enrichment

We detected enrichments of bacterial cells in the SML of the WWT and in the field at a  $U_{10}$  of  $\leq 5.6$  and  $\leq 4.1$   $\text{m s}^{-1}$ , respectively, but not above those thresholds. Since flow cytometry does not detect cells attached to particles, and particulate matter frequently occurs within the SML (Aller et al. 2005), enrichment of bacterial cell numbers was probably even underestimated. Webster and Hutchinson (1994) proposed a critical wind speed range

of  $>2\text{--}3$   $\text{m s}^{-1}$ , at which mixing processes at the sea surface favor the downward transport of floating phytoplankton cells, much larger than bacterial cells, in a lake. They concluded that surface films, i.e. SML, would decrease the capability of wind to stir buoyant organisms into the ULW. Wurl et al. (2011) suggested that surfactant accumulation persists in the oceanic SML at the global average wind speed of 6.6  $\text{m s}^{-1}$ , but SML thickness was found to decrease at wind speeds  $>8$   $\text{m s}^{-1}$  (Falkowska 1999). Our thresholds match the observation of Romano (1996) that sea-surface slicks, sea surface phenomena formed by wave-damping

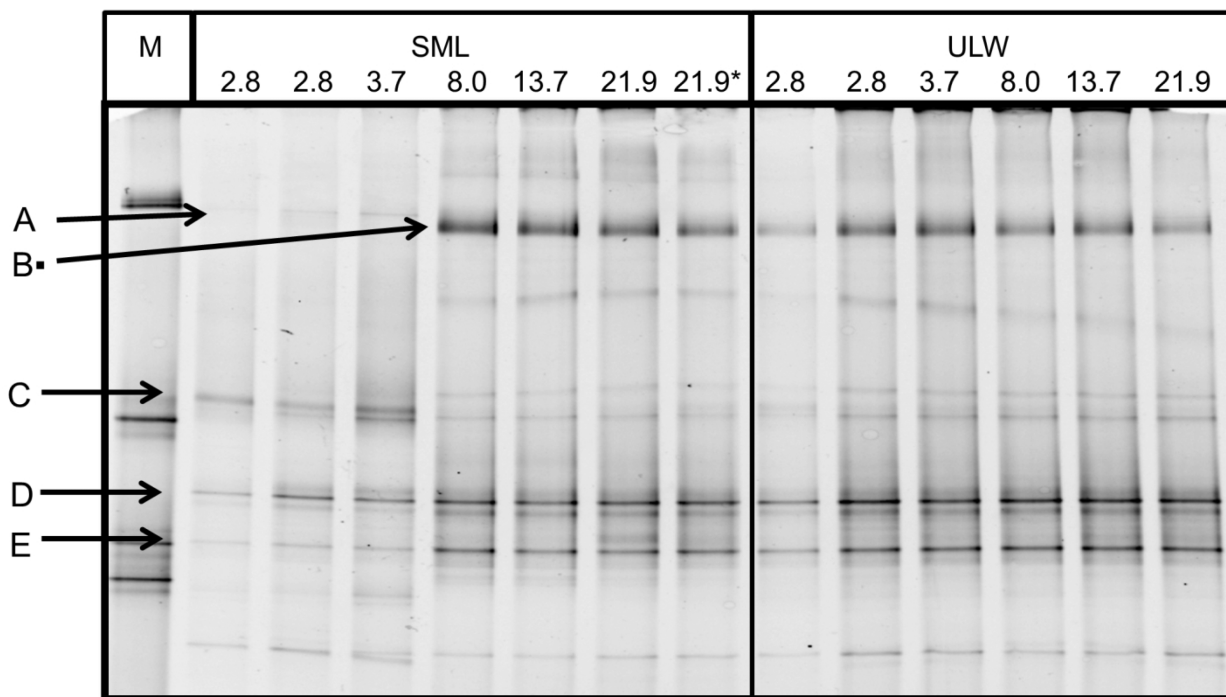


Figure 6. DGGE gel shows WWT SML and ULW 16S rRNA gene samples under increasing  $U_{10}$  wind speed ranging from 2.8 to 21.9  $\text{m s}^{-1}$  for experimental day 5. OTUs derived from sequenced bands are A = *Amphora coffeaeformis*, B = *Flavobacterium bacteria* Yb008, C = *Legionella* sp., D = *Phaeodactylibacter xiamenensis*, E = *Marinobacter* sp., SML-avoiding OTU under low wind conditions on denaturing gradient gel (marked with filled squares), M = marker, SML = sea-surface microlayer, ULW = underlying water, asterisk indicates additional bubbling at that wind speed.

action of high concentrations of surface-active organic material, only persist at wind speeds lower than  $5 \text{ m s}^{-1}$ .

Disturbance of the sea surface by strong wind forces and wave breaking generates vigorous mixing of the SML and sea spray formation. This leads to the distribution of bacteria to the ULW or to aerosols at higher wind speeds (Wotton and Preston 2005). For instance, wind-driven breakdown of the SML and increased formation of aggregates has been recently reported for freshwater lakes (Drudge and Warren 2014). The authors identified sinking aggregates as a transport vector for microorganisms to the ULW, which supports our observation of declining EFs of bacterial cells with increasing wind speeds.

We found that bacterial EFs were generally higher in the WWT (mean EF  $\pm$  standard deviation (SD) =  $1.22 \pm 0.31$ ) than in the field (mean EF  $\pm$  SD =  $1.08 \pm 0.12$ ), and our EFs from the field are similar to previous studies (Stolle et al. 2010; mean EF  $\pm$  SD =  $1.18 \pm 0.29$ ). Significant enrichment of bacteria during calm sea states, similar to conditions in the WWT at  $U_{10} = 0 \text{ m s}^{-1}$ , is well known for the SML (Stolle et al. 2010; Wurl et al. 2016). No disturbance for a prolonged time ( $\sim 12 \text{ h}$  between two measurement days) at zero wind speed probably allowed bacteria to become more enriched in the WWT-SML compared to the field. However, even after periodic disturbance, e.g. by the breaking of small waves at moderate wind speeds close to our thresholds, re-establishment of the SML and re-accumulation of organic material occurs within seconds (Dragcevic and Pravdic 1981). In addition, higher EFs observed in the WWT compared to the field could be the result of a less disturbed habitat lacking solar radiation, the overall low abundance and diversity of phytoplankton as well as the absence of any higher trophic levels exerting grazing pressure. However, no stimulatory or inhibitory effect of either solar or UV radiation on bacterial EFs in the Baltic Sea samples could be detected (data not shown).

Whereas enrichment of bacteria was only impaired by higher wind speeds in the WWT, in the Baltic Sea bacterial EFs were sometimes even  $<1$  (indicating depletion) at calm sea states. In addition, enrichment of bacterial cells in field samples occurred overall at lower wind speeds ( $\leq 4.1 \text{ m s}^{-1}$ ) when compared to the WWT ( $\leq 5.6 \text{ m s}^{-1}$ ) (Fig. 4). Factors other than wind, e.g. patchiness of substrate or other meteorological variations might have prevented the accumulation of bacteria under certain conditions. For example, high enrichment of bacteria at low wind conditions ( $<4.1 \text{ m s}^{-1}$ ) was mainly observed in the coastal upwelling areas (Gotland Basin, UP1-4, Fig. 2, Table 2) being characterized by cold, nutrient and  $\text{CO}_2$ -enriched water that rises to the surface (Norman et al. 2013). Considering our finding of the interactive low wind-high  $p\text{CO}_2$  effect in the WWT (see below), we suggest that upwelled  $\text{CO}_2$  and nutrients contributed to the stronger enrichment of bacteria at those stations. The observed uncoupling of the bacterioneuston (abundance and community composition) at low wind conditions could have been additionally favored by the occurrence of slicks as previously demonstrated (Stolle et al. 2010). Microbial biomass is highly abundant in natural slicks at calm sea states (Wurl et al. 2016), which has most likely important implications for nourishing higher trophic levels such as protists (Cunliffe and Murrell 2010) and therefore the overall functioning of the food web (Taylor 2007; Dewar et al. 2008).

Due to the challenge of collecting SML at higher wind speeds, studies on the SML, including bacterioneuston communities, have been limited to a wind speed range of up to  $\sim 9 \text{ m s}^{-1}$  ( $=U_{10}$  of  $7.4 \text{ m s}^{-1}$ ) (Reinthal, Sintes and Herndl 2008). For this reason, the application of controlled increasing wind speed in this study provided insights into the integrity of the SML and its ecological role as a microbial habitat at higher wind speeds. From the results of the WWT experiment, we hypothesize that a

critical wind speed threshold for the enrichment of bacteria probably exists but might not be easily transferable to the field. Our results confirm that uncoupling of bacterioneuston is strongly related to wind speed as observed by Stolle et al. (2010), but additional effects of other biotic or abiotic forces (e.g. grazing pressure, rain or turbulences) remain to be determined. A more complete data set, especially between  $U_{10}$  in the range of 5.6–8.0  $m\ s^{-1}$  (data gap in our observations), is needed to refine our understanding of bacterial enrichment. To elucidate whether EFs at wind speeds  $>8.0\ m\ s^{-1}$  remain comparably low in the field as in the WWT, we suggest that establishing methods for SML sampling at higher wind speeds in the field needs further attention.

### Bacterial taxa in response to different wind speeds

We identified that low wind conditions ( $\leq 5.6\ m\ s^{-1}$ ) are a major prerequisite for increasing differentiation of SML and ULW communities. We found formation of distinctive clusters of bacterioneuston communities collected at low wind speeds, especially at days 1, 3 and 5 (Fig. S2). Although the WWT is an artificial system, the majority of identified OTUs was related to bacterial taxa known from marine habitats and hence resembles a natural marine bacterial community.

In our study, OTUs related to *Pseudoalteromonas* sp. and *Alteromonas* sp. occurred exclusively in the SML at low wind speed, and were dominant species in this habitat in earlier studies (Franklin et al. 2005; Cunliffe et al. 2008, 2009). These OTUs were neither detected in the ULW at any wind speed nor in the SML at high wind speeds in our study. We assume that the respective organisms thrive under calm conditions in the SML but are diluted at higher wind speeds below the detection limit of the DGGE analysis within the bulk community. Yamada et al. (2016) showed that isolates of *Pseudoalteromonas* spp. enhance gel particle coagulation and similar to *Alteromonas* sp. can rapidly out-grow other members of the bulk bacterial community in response to dissolved organic carbon availability (Pedler, Aluwihare and Azam 2014). Thus, both OTUs could have populated the SML as it offered them favorable conditions such as high physical stability or the accumulation of transparent exopolymer particles. The latter cannot only be produced by bacteria (Ortega-Retuerta, Duarte and Reche 2010) but can also serve as a habitat for them (Alldredge, Passow and Logan 1993; Wurl et al. 2016).

Some OTUs were overall highly abundant in the SML and ULW at all wind speeds and  $pCO_2$  ranges, e.g. one being related to *Phaeodactylibacter xiamenensis*. The ubiquitous presence of some OTUs provides an indication that some bacteria are either well adapted to populate the SML even at higher wind speeds or that they are extremely abundant and therefore detectable in both SML and ULW. Moreover, the SML might actively select against certain OTUs. For example, an OTU related to *Flavobacterium bacterium* Yb008 was absent from the SML at low wind speed but was present at high wind speeds (Figs S2 and S5). Such a behavior could for instance be attributed to unfavorable abiotic conditions or the presence of competitors within the SML.

16S rRNA fingerprints confirmed that OTUs related to *Alteromonas* sp., *F. bacterium* Yb008 and *Marinobacter* sp. were active members of the community. The presence of active SML-specific bacteria shows that not only passive transport to the SML, e.g. by buoyant particles, is responsible for the establishment of the bacterial community, but that actively metabolizing bacteria colonize and use this habitat.

### Impact of $pCO_2$ on bacterial enrichment and community structure

In the WWT, highest  $pCO_2$  levels on days 1 and 5 (943–1245 and 824–1032  $\mu atm$ , respectively) were associated with highest enrichment of bacterial cells (EF up to 2.1) in the SML. It should be noticed that even our lowest  $pCO_2$  treatment on days 3 (607–624  $\mu atm$ ) and 4 (594–604  $\mu atm$ ) exceeded present summer Atlantic Ocean  $pCO_2$  levels ( $\sim 350\ \mu atm$ , Takahashi et al. (2014)). Nevertheless, low (594–624  $\mu atm$ ) and medium (741–883  $\mu atm$ )  $pCO_2$  levels were not associated with bacterial EFs  $> 1.4$ .

Nutrients and chlorophyll concentration were low in the WWT during the whole experiment. Low photosynthetic activity and depletion of nutrients could have further contributed to an enhanced  $pCO_2$  effect on bacterial EF as previously reported for the SML in a mesocosm experiment (Galvani et al. 2014). We showed a statistically significant interaction between  $pCO_2$  level and wind speed on the EF of bacteria in the SML. During low wind speed, the SML is physically more stable and gas transfer becomes limited by diffusion. In this study,  $CO_2$  was added to the water of the WWT causing a  $CO_2$  flux into the atmosphere potentially creating  $CO_2$  gradients in the WWT with decreasing concentration in the SML. Similar observations were previously reported by Zhang et al. (2003b), who discovered pH microgradients within the SML. Lower  $CO_2$  concentrations in the SML compared to the ULW could have provided a slightly less acidified 'sanctuary' for the bacteria. This might explain their higher enrichment in the SML, although total bacterial cell counts were lower at days of high  $pCO_2$  (Fig. S1). For instance, Chauhan et al. (2015) reported that high  $pCO_2$  environments at natural vent sites were associated with a decline *Gammaproteobacteria*, compared to reference sites of low  $pCO_2$ .

While  $pCO_2$  intensified enrichment of bacteria in the SML, we could also detect an influence of increasing  $pCO_2$  on the composition of bacterial communities between SML and ULW, which corresponded to decreasing pairwise similarities. Similarly, a laboratory ocean acidification experiment suggested that reduced pH caused shifts in community composition (Krause et al. 2012). However, the  $pCO_2$  effect on bacterial communities might have been masked by the stronger acting impact of wind speed in our experimental set-up.

Some OTUs were solely discovered at high and/or medium  $pCO_2$  in the dark (days 1 and 2) such as those being related to *Limnobacter thiooxidans*, *Alteromonas* sp. or *Pseudoalteromonas* sp. Assimilation of  $CO_2$  during heterotrophic growth has been reported for the order *Burkholderiales* to which the chemolitho-heterotrophic *L. thiooxidans* belongs (Roslev et al. 2004). The effective use of  $CO_2$  as an alternative carbon source depends on the carbon content of the medium used for growth (Dijkhuizen and Harder 1985). As the availability of organic material in the SML might sometimes be limited for heterotrophic growth (Reinthal, Sintes and Herndl 2008), bacterial taxa capable of heterotrophic  $CO_2$  fixation could have been stimulated by increased availability of  $CO_2$ .

Doubtlessly, studying the effect of high  $pCO_2$  on bacteria needs a thorough understanding of the prevailing conditions and composition of community members. For a reliable assessment of the consequences of increasing  $pCO_2$  levels on bacterioneuston communities, we urge on future work considering factors such as  $CO_2$ -driven changes in the availability of nutrients or microgradients of carbonate chemistry variables forming within the SML.



## CONCLUSIONS

We found that low wind speed and high  $p\text{CO}_2$  together induced the enrichment of bacterial cells in the SML over ULW. We detected high enrichment of bacterial cells at a  $U_{10}$  of  $\leq 5.6 \text{ m s}^{-1}$  in the WWT and at  $\leq 4.1 \text{ m s}^{-1}$  in the field. In addition, both factors induced changes in SML bacterial community structure. Our results indicate that considering wind speed limits for bacterial enrichment and community composition in the SML is fundamental to assess its ecological function as a microbial habitat. In our study, we could find first evidence that low wind speed may select certain bacterial taxa to use or avoid the SML as a habitat. Future studies will need a higher phylogenetic resolution, e.g. by next-generation sequencing methods, to fully address these ideas. A better understanding of the microbial community at the physically highly influenced air–sea boundary will improve our knowledge on air–sea gas exchange processes, biogeochemical cycling of organic matter and the importance of the SML in the trophic network. We suggest that additional factors concerning the spatial and temporal variability of the natural habitat such as slick formation or substrate availability must be considered, as should be a physiological characterization of the bacterial members that can cope with the challenging life in the SML.

## SUPPLEMENTARY DATA

Supplementary data are available at [FEMSEC](#) online.

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## REFERENCES

- Agogué H, Casamayor EO, Joux F et al. Comparison of samplers for the biological characterization of the sea surface microlayer. *Limnol Oceanogr-Meth* 2004;2:213–25.
- Agogué H, Joux F, Obernosterer I et al. Resistance of marine bacterioneuston to solar radiation. *Appl Environ Microb* 2005;71:5282–9.
- Allredge AL, Passow U, Logan BE. The abundance and significance of a class of large, transparent organic particles in the ocean. *Deep-Sea Res Pt I* 1993;40:1131–40.
- Aller JY, Kuznetsova MR, Jahns CJ et al. The sea surface microlayer as a source of viral and bacterial enrichment in marine aerosols. *J Aerosol Sci* 2005;36:801–12.
- Alonso-Saéz L, Gasol JM, Lefort T et al. Effect of natural sunlight on bacterial activity and differential sensitivity of natural bacterioplankton groups in northwestern Mediterranean coastal waters. *Appl Environ Microb* 2006;72:5806–13.
- Bopp M. Messung der Schubspannungsgeschwindigkeit am Heidelberger Aeolotron mittels der Impulsbilanzmethode. Bachelor Thesis, Institut für Umweltp Physik, Fakultät für Physik und Astronomie, Univ Heidelberg 2011.
- Brussaard C, Noordeeloes A, Witte H et al. Arctic microbial community dynamics influenced by elevated  $\text{CO}_2$  levels. *Biogeosciences* 2013;10:719–31.
- Bunse C, Lundin D, Karlsson CM et al. Response of marine bacterioplankton pH homeostasis gene expression to elevated  $\text{CO}_2$ . *Nat Clim Change* 2016;6:483–7.
- Calleja ML, Duarte CM, Navarro N et al. Control of air–sea  $\text{CO}_2$  disequilibria in the subtropical NE Atlantic by planktonic metabolism under the ocean skin. *Geophys Res Lett* 2005;32:L08606.
- Carlson DJ. A field-evaluation of plate and screen microlayer sampling techniques. *Mar Chem* 1982;11:189–208.
- Chauhan A, Pathak A, Rodolfo-Metalpa R et al. Metagenomics reveals planktonic bacterial community shifts across a natural  $\text{CO}_2$  gradient in the Mediterranean Sea. *Genome Announc* 2015;3:e01543–14.
- Conrad R, Seiler W. Influence of the surface microlayer on the flux of nonconservative trace gases ( $\text{CO}$ ,  $\text{H}_2$ ,  $\text{CH}_4$ ,  $\text{N}_2\text{O}$ ) across the ocean–atmosphere interface. *J Atm Chem* 1988;6:83–94.
- Crawley MJ. *The R Book* Second edition. Chichester, UK: John Wiley & Sons, 2013.
- Cunliffe M, Engel A, Frka S et al. Sea surface microlayers: A unified physicochemical and biological perspective of the air–ocean interface. *Progr Oceanogr* 2013;109:104–16.
- Cunliffe M, Murrell JC. Eukarya 18S rRNA gene diversity in the sea surface microlayer: implications for the structure of the neustonic microbial loop. *ISME J* 2010;4:455–8.
- Cunliffe M, Salter M, Mann PJ et al. Dissolved organic carbon and bacterial populations in the gelatinous surface microlayer of a Norwegian fjord mesocosm. *FEMS Microbiol Lett* 2009;299:248–54.
- Cunliffe M, Schafer H, Harrison E et al. Phylogenetic and functional gene analysis of the bacterial and archaeal communities associated with the surface microlayer of an estuary. *ISME J* 2008;2:776–89.
- Dewar H, Mous P, Domeier M et al. Movements and site fidelity of the giant manta ray, *Manta birostris*, in the Komodo Marine Park, Indonesia. *Mar Biol* 2008;155:121–33.
- Dijkhuizen L, Harder W. Microbial metabolism of carbon dioxide. In: Moo-Young M, (ed). *Comprehensive Biotechnology: The Principles, Applications, and Regulations of Biotechnology in Industry, Agriculture, and Medicine*. Oxford, NY: Pergamon Press. 1985.
- Dragcevic D, Pravdic V. Properties of the seawater–air interface. 2. Rates of surface film formation under steady state conditions. *Limnol Oceanogr* 1981;26:492–9.
- Drudge CN, Warren LA. Diurnal floc generation from neuston biofilms in two contrasting freshwater lakes. *Environ Sci Technol* 2014;48:10107–15.
- Edson JB, Jampana V, Weller RA et al. On the exchange of momentum over the open ocean. *J Phys Oceanogr* 2013;43:1589–610.
- Engel A, Piontek J, Grossart HP et al. Impact of  $\text{CO}_2$  enrichment on organic matter dynamics during nutrient induced coastal phytoplankton blooms. *J Plankton Res* 2014;36:641–57.
- Falkowska L. Sea surface microlayer: a field evaluation of teflon plate, glass plate and screen sampling techniques. Part 1. Thickness of microlayer samples and relation to wind speed. *Oceanologia* 1999;41:211–21.
- Franklin MP, McDonald IR, Bourne DG et al. Bacterial diversity in the bacterioneuston (sea surface microlayer): the bacterioneuston through the looking glass. *Environ Microbiol* 2005;7:723–36.



- Galgani L, Stolle C, Endres S et al. Effects of ocean acidification on the biogenic composition of the sea-surface microlayer: results from a mesocosm study. *J Geophys Res-Oceans* 2014;**119**:7911–24.
- Grossart H-P, Allgaier M, Passow U et al. Testing the effect of CO<sub>2</sub> concentration on dynamics of marine heterotrophic bacterioplankton. *Limnol Oceanogr* 2006;**51**:1–11.
- Harvey GW, Burzell LA. A simple microlayer method for small samples. *Limnol Oceanogr* 1972;**17**:156–7.
- Jarvis A, Hewitt C. The 'Business-As-Usual' growth of global primary energy use and carbon dioxide emissions—historical trends and near-term forecasts. *Earth Syst Dynam Discuss* 2014;**5**:1143–58.
- Kleemann M, Meliss M. *Renewable Energy Sources*. Berlin-Heidelberg, Springer, 1993.
- Krause E, Wichels A, Gimenez L et al. Small changes in pH have direct effects on marine bacterial community composition: a microcosm approach. *PLoS One* 2012;**7**: e47035.
- Lane D. 16S/23S rRNA sequencing. In Stackebrandt E, Goodfellow M, (eds). *Nucleic Acid Techniques in Bacterial Systematics*. Chichester, UK: John Wiley & Sons, 1991, 115–75.
- Lidbury I, Johnson V, Hall-Spencer JM et al. Community-level response of coastal microbial biofilms to ocean acidification in a natural carbon dioxide vent ecosystem. *Mar Pollut Bull* 2012;**64**:1063–6.
- Liss PS, Duce RA. *The Sea Surface and Global Change*. Cambridge, UK: Cambridge University Press, 1997.
- Liu JW, Weinbauer MG, Maier C et al. Effect of ocean acidification on microbial diversity and on microbe-driven biogeochemistry and ecosystem functioning. *Aquat Microb Ecol* 2010;**61**:291–305.
- Lunau M, Lemke A, Walther K et al. An improved method for counting bacteria from sediments and turbid environments by epifluorescence microscopy. *Environ Microbiol* 2005;**7**: 961–8.
- Mesarchaki E, Kräuter C, Krall K et al. Measuring air-sea gas-exchange velocities in a large-scale annular wind-wave tank. *Ocean Science* 2015;**11**:121–38.
- Muyzer G, Brinkhoff T, Nübel U et al. Denaturing gradient gel electrophoresis (DGGE) in microbial ecology. In: Kowalchuk GA, de Bruijn FJ, Head IM et al. (eds). *Molecular Microbial Ecology Manual*, 2nd edn. Dordrecht, The Netherlands: Kluwer academic publishers, 2004, 743–70.
- Muyzer G, de Waal EC, Uitterlinden AG. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl Environ Microb* 1993;**59**: 695–700.
- Naumann E. Über das Neuston des Süßwassers. *Biol Centralblatt* 1917;**37**:98–106.
- Norman M, Parampil SR, Rutgersson A et al. Influence of coastal upwelling on the air-sea gas exchange of CO<sub>2</sub> in a Baltic Sea Basin. *Tellus B* 2013;**65**:21831.
- Obernosterer I, Catala P, Lami R et al. Biochemical characteristics and bacterial community structure of the sea surface microlayer in the South Pacific Ocean. *Biogeosciences* 2008;**5**:693–705.
- Obernosterer I, Catala P, Reinthaler T et al. Enhanced heterotrophic activity in the surface microlayer of the Mediterranean Sea. *Aquat Microb Ecol* 2005;**39**:293–302.
- Ortega-Retuerta E, Duarte CM, Reche I. Significance of bacterial activity for the distribution and dynamics of transparent exopolymer particles in the Mediterranean sea. *Microb Ecol* 2010;**59**:808–18.
- Pedler BE, Aluwihare LI, Azam F. Single bacterial strain capable of significant contribution to carbon cycling in the surface ocean. *P Natl Acad Sci USA* 2014;**111**:7202–7.
- Piontek J, Lunau M, Handel N et al. Acidification increases microbial polysaccharide degradation in the ocean. *Biogeosciences* 2010;**7**:1615–24.
- Rademaker J, Louws F, Rossbach U et al. Computer-assisted pattern analysis of molecular fingerprints and database construction. In: Akkermans ADL, van Elsas JD, de Bruijn FJ, (eds). *Molecular Microbial Ecology Manual*, 7.1.3, Dordrecht, The Netherlands: Kluwer Academic Publishers, 1999, 1–33.
- Rahlff J, Stolle C, Giebel H-A et al. Sea-surface microlayer characteristics during METEOR cruise M117. *Baltic Sea* 2016, DOI: 10.1594/PANGAEA.861664.
- Reinthal T, Sintes E, Herndl GJ. Dissolved organic matter and bacterial production and respiration in the sea-surface microlayer of the open Atlantic and the western Mediterranean Sea. *Limnol Oceanogr* 2008;**53**:122–36.
- Romano JC. Sea-surface slick occurrence in the open sea (Mediterranean, Red Sea, Indian Ocean) in relation to wind speed. *Deep-Sea Res Pt I* 1996;**43**:411–23.
- Roslev P, Larsen MB, Jørgensen D et al. Use of heterotrophic CO<sub>2</sub> assimilation as a measure of metabolic activity in planktonic and sessile bacteria. *J Microbiol Meth* 2004;**59**:381–93.
- Roy AS, Gibbons SM, Schunck H et al. Ocean acidification shows negligible impacts on high-latitude bacterial community structure in coastal pelagic mesocosms. *Biogeosciences* 2013;**10**:555–66.
- Schlitzer R. *Ocean Data View*. 2015, <http://odv.awi.de>.
- Schneider B, Gülzow W, Sadkowiak B et al. Detecting sinks and sources of CO<sub>2</sub> and CH<sub>4</sub> by ferrybox-based measurements in the Baltic Sea: three case studies. *J Mar Syst* 2014;**140**:13–25.
- Siu N, Apple JK, Moyer CL. The effects of ocean acidity and elevated temperature on bacterioplankton community structure and metabolism. *Open J Ecol* 2014;**4**:434–55.
- Sperling M, Piontek J, Gerds G et al. Effect of elevated CO<sub>2</sub> on the dynamics of particle-attached and free-living bacterioplankton communities in an Arctic fjord. *Biogeosciences* 2013;**10**:181–91.
- Stolle C, Labrenz M, Meeske C et al. Bacterioneuston community structure in the southern Baltic sea and its dependence on meteorological conditions. *Appl Environ Microb* 2011;**77**:3726–33.
- Stolle C, Nagel K, Labrenz M et al. Succession of the sea-surface microlayer in the coastal Baltic Sea under natural and experimentally induced low-wind conditions. *Biogeosciences* 2010;**7**:2975–88.
- Takahashi T, Sutherland SC, Chipman D et al. Climatological distributions of pH, pCO<sub>2</sub>, total CO<sub>2</sub>, alkalinity, and CaCO<sub>3</sub> saturation in the global surface ocean, and temporal changes at selected locations. *Mar Chem* 2014;**164**:95–125.
- Taylor JG. Ram filter-feeding and nocturnal feeding of whale sharks (*Rhincodon typus*) at Ningaloo Reef, Western Australia. *Fish Res* 2007;**84**:65–70.
- Team RC. R: *A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing, 2013: ISBN 3-900051-07-0, 2014.
- Teira E, Fernandez A, Alvarez-Salgado XA et al. Response of two marine bacterial isolates to high CO<sub>2</sub> concentration. *Mar Ecol Prog Ser* 2012;**453**:27–36.

- Upstill-Goddard RC, Frost T, Henry GR et al. Bacterioneuston control of air-water methane exchange determined with a laboratory gas exchange tank. *Global Biogeochem Cy* 2003;**17**:1108.
- Webster IT, Hutchinson PA. Effect of wind on the distribution of phytoplankton cells in lakes revisited. *Limnol Oceanogr* 1994;**39**:365–73.
- Wotton RS, Preston TM. Surface films: Areas of water bodies that are often overlooked. *Bioscience* 2005;**55**:137–45.
- Wurl O, Miller L, Vagle S. Production and fate of transparent exopolymer particles in the ocean. *J Geophys Res* 2011;**116**:C00H13.
- Wurl O, Obbard JP. A review of pollutants in the sea-surface microlayer (SML): a unique habitat for marine organisms. *Mar Pollut Bull* 2004;**48**:1016–30.
- Wurl O, Stolle C, Van Thuoc C et al. Biofilm-like properties of the sea surface and predicted effects on air-sea CO<sub>2</sub> exchange. *Progr Oceanogr* 2016;**144**:15–24.
- Wurl O, Wurl E, Miller L et al. Formation and global distribution of sea-surface microlayers. *Biogeosciences* 2011;**8**:121–35.
- Yamada Y, Fukuda H, Tada Y et al. Bacterial enhancement of gel particle coagulation in seawater. *Aquat Microb Ecol* 2016;**77**:11–22.
- Zhang Z, Liu L, Liu C et al. Studies on the sea surface microlayer. II. The layer of sudden change of physical and chemical properties. *J Colloid Interface Sci* 2003a;**264**:148–59.
- Zhang ZB, Cai WJ, Liu LS et al. Direct determination of thickness of sea surface microlayer using a pH microelectrode at original location. *Sci China Ser B* 2003b;**46**:339–51.