Comparison of Bacterioneuston and Bacterioplankton Dynamics during a Phytoplankton Bloom in a Fjord Mesocosm[∇]

Michael Cunliffe,¹ Andrew S. Whiteley,² Lindsay Newbold,² Anna Oliver,²
Hendrik Schäfer,³ and J. Colin Murrell^{1*}

Department of Biological Sciences, University of Warwick, Gibbet Hill Road, Coventry CV4 7AL, United Kingdom¹;
Centre for Ecology and Hydrology, Mansfield Road, Oxford OX1 3SR, United Kingdom²; and Warwick HRI,
University of Warwick, Wellesbourne CV35 9EF, United Kingdom³

Received 12 June 2009/Accepted 18 September 2009

The bacterioneuston is the community of *Bacteria* present in surface microlayers, the thin surface film that forms the interface between aquatic environments and the atmosphere. In this study we compared bacterial cell abundances and bacterial community structures of the bacterioneuston and the bacterioplankton (from the subsurface water column) during a phytoplankton bloom mesocosm experiment. Bacterial cell abundance, determined by flow cytometry, followed a typical bacterioplankton response to a phytoplankton bloom, with *Synechococcus* and high-nucleic acid content (HNA) bacterial cell numbers initially falling, probably due to selective protist grazing. Subsequently HNA and low-nucleic acid content bacterial cells increased in abundance, but *Synechococcus* cells did not. There was no significant difference between bacterioneuston and bacterioplankton cell abundances during the experiment. Conversely, distinct and consistent differences between the bacterioneuston and the bacterioplankton community structures were observed. This was monitored simultaneously by *Bacteria* 16S rRNA gene terminal restriction fragment length polymorphism and denaturing gradient gel electrophoresis. The conserved patterns of community structure observed in all of the mesocosms indicate that the bacterioneuston is distinctive and nonrandom.

Determining and understanding both spatial and temporal patterns in bacterioplankton community structure are a core aim of marine microbial ecology (15). Distributions of bacterioplankton over space and time can be correlated to environmental parameters, and subsequent links can therefore be made to ecosystem function. A broad range of spatial studies made on macro- (34), meso- (20), and microscales (27) have shown clear patterns in distribution of the bacterioplankton.

The sea surface microlayer is part of the air-sea interface and is generally considered to be the top 1 mm or less of the ocean (26). Surface microlayers have a fundamental role in regulating transport processes between the ocean and the atmosphere (26) and are often referred to as the neuston (28, 31). For more than 25 years it has been hypothesized that the sea surface microlayer is a hydrated gelatinous layer (40) that contains surface-active organic compounds such as carbohydrates, proteins, lipids, and humic substances in relatively high concentrations (17, 45, 48). Recently, gel-like transparent exopolymer particles (TEP) have been shown to be enriched in the surface microlayer, supporting the concept of a gelatinous interfacial layer (46).

Bacteria present in surface microlayers or the neuston are regarded as the bacterioneuston. There are relatively few studies which have directly compared the community structure of the bacterioneuston with that of the cognate subsurface (bacterioplankton) in the marine environment. Analysis of *Bacteria* 16S rRNA gene clone libraries constructed using DNA iso-

lated from surface microlayer and subsurface water (<1 m) samples from the North Sea revealed that the bacterioneuston was dominated by two operational taxonomic units which accounted for 81% of clones analyzed (13). Community structure profiling using denaturing gradient gel electrophoresis (DGGE) of the bacterioneuston at three sites around Oahu Island in the Pacific Ocean showed that the bacterioneuston forms consistent and distinct community structures. Conversely, the archaeal community structure of the same samples using *Archaea* 16S rRNA gene DGGE analysis did not show the same surface microlayer-specific response, indicating that bacteria and archaea respond to their environment in fundamentally different ways in the neuston (7).

Other studies have, however, reported no consistent differences between the bacterioneuston and the bacterioplankton. Samples collected from two separate sites in the Mediterranean Sea were analyzed using single-strand conformation polymorphism of *Bacteria* 16S rRNA genes (1). The authors did not report any significant differences between the surface microlayer and subsurface samples using this community profiling method.

Nonmarine studies of the bacterioneuston and *Archaea* communities in estuarine (10) and freshwater (5, 19) environments have also shown distinct microbial community structures present in the surface microlayer compared to those in subsurface water ≤1 m below.

Recurring phytoplankton blooms are a key feature of coastal waters and strongly influence bacterioplankton community structure and succession (4, 14, 38). Phytoplankton blooms stimulate the bacterioplankton by the release of dissolved organic matter (22) or affect bacterioplankton negatively by direct competition for resources (6). Bacterioplankton commu-

* Corresponding author. Mailing address: Department of Biological Sciences, University of Warwick, Gibbet Hill Road, Coventry CV4 7AL, United Kingdom. Phone: 44 24 7652 3553. Fax: 44 24 7652 3568. E-mail: j.c.murrell@warwick.ac.uk.

[∇] Published ahead of print on 25 September 2009.

FIG. 1. (A) Photograph showing the mesocosms used in this study. Twelve mesocosms were divided into three larger containers. (B) Each mesocosm was filled sequentially, from A to L. Control mesocosms were A, C, E, G, I, and K. The phytoplankton bloom was induced in nutrient-amended mesocosms B, D, F, H, J, and L.

nity structure may also be influenced by grazing flagellates or viral lysis (47).

Mesocosm experiments have been used to study plankton ecology for many decades (33). Mesocosms facilitate study of the effects of key environmental parameters, such as temperature, on plankton communities and allow the succession of natural plankton communities that resemble those found in the marine environment (11). The enclosed water mass means that experiments can be designed which manipulate physicochemical parameters to observe biological effects. Furthermore, with replicated mesocosms, the data collected can be analyzed with statistics rigorously. In this study we monitored the dynamics of the bacterioplankton and the bacterioplankton in mesocosms of fjord surface water during an artificially induced phytoplankton bloom and compared bacterial abundances and bacterial community structures in the surface microlayer and subsurface water.

MATERIALS AND METHODS

Mesocosm setup and sampling. The experiment was carried out at the Marine Biological Field Station, Espeland, Norway (20 km south of Bergen, Norway) from 21 May 2008 to 1 June 2008. Twelve land-based mesocosms (1.5-m diameter and 1.5-m deep) were each filled (2,474 liters) with prefiltered ($\sim 300 \mu\text{m}$) water from the Raunefjorden. The water in the mesocosms was constantly mixed by means of submerged aquarium pumps. The mesocosms were contained in three larger open containers (Fig. 1A) that were filled and circulated constantly with pumped fjord water to maintain the mesocosms at ambient fjord temperature. The 12 mesocosms were divided into two treatment groups, control and nutrient amended, allowing six replicate mesocosms for each treatment. Each of the larger containers held two control mesocosms and two nutrient-amended mesocosms (Fig. 1B). Addition of nitrate and phosphate according to the Redfield stoichiometry (N/P = 16:1) (35), as $16 \mu\text{M NaNO}_3$ and $1 \mu\text{M KH}_2\text{PO}_4$, was used to induce the phytoplankton bloom at 2100 h on day zero.

Sampling took place every day for 11 days at 0900 h. Subsurface waters were sampled from a depth of 0.75 m in the center of the mesocosms using a siphon.

The surface microlayer was sampled using two different methods, a mesh screen (Garrett screen) and polycarbonate membranes taken from the center of the mesocosms. The methods sample two different depths: the mesh screen removes the top $\sim 400 \mu\text{m}$, and the polycarbonate membrane removes the top $\sim 40 \mu\text{m}$ of the surface microlayer (7). The mesh screen (16-mesh stainless steel screen; size, 275 by 275 mm) was placed below the surface water and lifted horizontally through the surface microlayer, and the water was collected into a sterile bottle. A total of 250 ml was then filtered using a peristaltic pump through a Sterivex-GS filter unit (pore size, $0.2 \mu\text{m}$; Millipore). After all the water had been evacuated from the filter unit, 1.6 ml of RNAlater (Ambion) was added, and the filter unit was stored at 4°C . Polycarbonate membranes (47-mm diameter; pore size, $0.2 \mu\text{m}$; Isopore; Millipore) were placed onto the water surface using forceps and left for 10 s before being removed and stored in 2-ml screw-cap tubes at -20°C .

Dissolved inorganic nutrients. Subsurface water samples were filtered (Sterivex-GS; pore size, $0.2 \mu\text{m}$; Millipore) before being stored in polyethylene vials at -20°C until nitrate, nitrite, phosphate, and silicate quantities were determined using standard segmented flow analysis with photometric procedures (18).

Phytoplankton and bacterial cell counts. Phytoplankton and bacterial cells in the mesocosms were enumerated with a Becton Dickinson FACSCalibur benchtop flow cytometer (BD Bioscience) equipped with a 488-nm laser line. Cells were enumerated in samples collected from the subsurface and mesh screens only since membrane-collected samples do not remove enough water for flow cytometry analysis. Two analyses were performed per sample to determine both phytoplankton and bacterial cell counts. Briefly, phytoplankton (picoeukaryotes, coccolithophorids, and small and large nanoplankton) and *Synechococcus* cell counts were enumerated on fresh unstained samples using modified flow rates (ca. $100 \mu\text{l min}^{-1}$) and pre- and postaspiration sample weighing together with timed acquisition (5 min) (42). Bacterial cell counts (total count and subsets for high-nucleic acid content [HNA] and low-nucleic acid content [LNA] bacterial cells) were determined on paraformaldehyde-fixed, citrate-treated samples stained with SYBR Green I (Invitrogen) using timed acquisition (2 min) in concert with pre- and postaspiration weighing (50). For pre- and postaspiration weighing, all samples were weighed before and after analysis to determine sample volumes aspirated during the sample analysis, and internal $0.49\text{-}\mu\text{m}$ reference beads were used to account for flow and machine drift. All analyzed samples were exported as list mode files and analyzed using Cyflogic to gate major populations and calculate absolute cell concentrations from aspirated volumes.

Extraction of DNA for bacterial community structure analysis. DNA was extracted from subsurface, mesh screen, and membrane samples collected on day 2, day 5, and day 10. DNA was extracted from three control mesocosms (replicates A, E, and K) and three nutrient-amended mesocosms (replicates B, F, and L) (Fig. 1B). DNA was extracted in a sucrose buffer using lysozyme, proteinase K, sodium dodecyl sulfate, and phenol-chloroform as described by Cunliffe et al. (10). The resuspended DNA was quantified using a spectrophotometer (ND-1000; NanoDrop) before all DNA samples were diluted to a concentration of $30 \text{ ng} \cdot \mu\text{l}^{-1}$ and stored at -20°C .

Bacterial community structure analysis. PCR amplification of *Bacteria* 16S rRNA genes for terminal restriction fragment length polymorphism (T-RFLP) analysis was performed using the fluorescently labeled primer, (6FAM)27F (5'-AGA GTT TGA TCM TGG CTC AG-3'; 6FAM is 6-carboxyfluorescein), and primer 536R (5'-GWA TTA CCG CGG CKG CTG-3') (41). For PCR, a total volume of $50 \mu\text{l}$ contained 0.5 mM each of the deoxynucleoside triphosphates, $0.5 \mu\text{M}$ of each primer, 2 units of *Taq* DNA polymerase (Sigma), and 30 ng of template DNA. The PCR program consisted of initial denaturation at 94°C for 2 min, followed by 30 cycles of 94°C for 1 min, annealing at 52°C for 1 min, and elongation at 72°C for 3 min, with a final elongation step at 72°C for 10 min. PCR products were verified by agarose gel electrophoresis and stored at -20°C .

PCR products were purified using a QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions. A total of $20 \mu\text{l}$ of purified PCR product was digested for 4 h at 37°C using the restriction enzyme *MspI* (Promega). The digestion product ($0.5 \mu\text{l}$) was combined with $0.5 \mu\text{l}$ of denatured LIZ600 size standard (Applied Biosystems) and formamide before being run on a 3730 DNA sequencer (Applied Biosystems). The sizes of the terminal restriction fragments (T-RFs) were calculated and binned using Genemarker (Softgenetics). Bin widths were checked and manually adjusted to encompass all concordant peaks. To differentiate signal from background, a fluorescence unit threshold of 40 units was used to determine which T-RFs to include. Relative abundance was calculated for each T-RF by dividing individual T-RF fluorescence by total sample fluorescence.

PCR amplification of 16S rRNA genes from *Bacteria* for DGGE analysis was performed using primers 341F (5'-CCT ACG GGA GGC AGC AG-3') and

FIG. 2. Dissolved inorganic nutrient concentration changes in control (□) and nutrient-amended mesocosms (■). Mean values were plotted ($n = 6$), with the error bars representing the standard errors.

primer 518R (5'-ATT ACC GCG GCT GCT GG-3') (30). The same PCR was set up as before for T-RFLP but using the different primers. The PCR program for DGGE consisted of an initial denaturation at 94°C for 5 min, followed by 35 cycles of 95°C for 1 min, annealing at 65 to 55°C for 20 cycles (reduction of -0.5°C per cycle) and at 55°C for 15 cycles, elongation at 72°C for 1 min, and then a final elongation step at 72°C for 10 min.

DGGE was performed with a DCode system (Bio-Rad). Gels were prepared with 10% (vol/vol) acrylamide-bisacrylamide with a 30 to 70% linear denaturant gradient (100% denaturant solution contains 6.9 M urea and 11.5 M formamide). The gel was run in 1× Tris-acetate-EDTA buffer at 60°C for a total of 1,008 volt-hours (constant voltage of 63 V for 16 h). Gels were stained with SYBR Gold nucleic acid stain (Invitrogen) before the image was captured on a UV transilluminator (Syngene).

DGGE bands that were relatively more abundant in the surface microlayer samples were selected and excised. The excised bands were washed in sterile molecular-grade water before being crushed in 20 μl of molecular-grade water and incubated at 4°C for 2 h. The eluted DNA was used to reamplify the DGGE band using the same PCR primers and conditions as before. DGGE band DNA sequences were obtained using the University of Warwick Molecular Biology Services Laboratory.

Statistical and ordination analysis. Analysis of variance was used to identify statistical significance in the phytoplankton and bacterial cell count data ($n = 6$; $P < 0.05$). Where significant differences were seen, a Tukey test was used to compare data within a defined set. Both analysis of variance and Tukey's test were performed using SPSS statistical software (SPSS). Principal component analysis (PCA) was used to visualize the relationships between bacterial community structures from the T-RFLP data and was carried out using MINITAB statistical software (Minitab). PCA is used to reduce the complexity of multivariate data (T-RF relative abundance) by producing new variables that account for most of the variation in the original data (39). DGGE profiles of 16S rRNA genes from *Bacteria* were compared using GelCompare II (Applied Maths) by calculating similarity coefficients using a curve-based Pearson correlation, followed by the construction of unweighted pair group method with arithmetic mean dendrograms from the calculated similarity coefficients.

Nucleotide sequence accession numbers. DGGE band DNA sequences determined in this study were deposited in the GenBank under accession numbers GQ902042 to GQ902046.

RESULTS

Phytoplankton abundance. The phytoplankton bloom succession in the mesocosms progressed generally as expected based on previous experience from earlier experiments with water collected from Raunefjorden (6, 29). The nitrate and phosphate added to the nutrient-amended mesocosms were steadily depleted, and levels returned to background concentrations by day 9 (Fig. 2). The concentration of silicate remained constant throughout the experiment. Nitrite increased in the nutrient-amended mesocosms to $0.19 \pm 0.01 \mu\text{M}$ at day 5 before returning to background levels by day 10 (Fig. 2).

Phytoplankton cells were divided into four groups by flow cytometry analysis: picoeukaryotes, large nanoplankton, small nanoplankton, and coccolithophorids (see Materials and Methods). Picoeukaryote numbers increased in both control and nutrient-amended mesocosms at the start of the experiment (Fig. 3). By day 5 a significant increase in picoeukaryote numbers was detected in the nutrient-amended mesocosms compared to control mesocosms. The artificially induced picoeukaryote bloom peaked on day 7 with a median cell density of $\sim 2 \times 10^5 \text{ cells} \cdot \text{ml}^{-1}$. There was no detectable significant difference between picoeukaryote cell counts in the surface microlayer and their cognate subsurface water samples.

Phytoplankton cells designated as large nanoplankton showed

FIG. 3. Changes in abundances of phytoplankton and bacterial cells in the surface microlayer (▲) and subsurface water (■). The surface microlayer was sampled using a mesh screen. Open symbols, control mesocosm samples; filled symbols, nutrient-amended mesocosm samples. Mean values are plotted ($n = 6$), with the error bars representing the standard errors.

a significant increase in numbers in the nutrient-amended mesocosms from day 5 onwards (Fig. 3). As with picoeukaryotes, there was no significant difference between numbers in the surface microlayer and subsurface water.

Small nanoplankton showed more variable cell counts during the time of the experiment than picoeukaryotes and large nanoplankton (Fig. 3). After day 6, a significant difference was detected between the counts in the nutrient-amended meso-

cosms and cell counts in control mesocosms. The bloom of small nanoplankton peaked on day 7 before returning to cell numbers similar to those in the control mesocosms by day 9.

As with the small nanoplankton, coccolithophorid abundance appeared stochastic in contrast to the picoeukaryotes and large nanoplankton cell counts and had no distinct trend. The intravariation between mesocosms was high for coccolithophorid counts, and this subsequently affected statistical analysis. At day 7 there was a significant difference between cell counts in the subsurface samples from the control and nutrient-amended mesocosms. For the remainder of the experiment the coccolithophorid counts were significantly higher in the nutrient-amended mesocosms. There was also some indication of weak enrichment of coccolithophorids in the surface microlayer (Fig. 3).

Bacterial abundance. Flow cytometry was used to separate three bacterial cell groups: HNA bacterial cells, LNA bacterial cells, and *Synechococcus* cells. The dynamics of the three groups was different during the experiment (Fig. 3).

HNA bacterial cells showed a marked decrease in abundance at the start of the experiment, with the rate of decrease accelerating rapidly on day 3. On day 5 the HNA bacterial cell numbers had dropped from an initial $\sim 6 \times 10^5$ cells $\cdot$ ml $^{-1}$ to $\sim 1 \times 10^5$ cells $\cdot$ ml $^{-1}$. After day 5 the abundance of HNA bacterial cells began to increase in all mesocosms, and a significant difference between HNA bacterial cell counts in the nutrient-amended mesocosms and counts in the control mesocosms for the remainder of the experiment was detected (Fig. 3). At the end of the experiment HNA bacterial cell numbers reached similar levels to those at the start of the experiment. There was no significant difference in HNA bacterial cell abundances between surface microlayer and subsurface water samples.

Unlike the HNA bacterial cells, LNA bacterial cells did not show a drastic drop in abundance (Fig. 3). LNA bacterial cell abundance fluctuated from day 0 to day 8 with no overall pattern. At day 2 and day 3 there was a significant difference between subsurface and surface microlayer LNA bacterial cell abundances, with fewer cells in the surface microlayer sample. LNA bacterial cell abundance fluctuated until day 9, when there was a significant increase in the nutrient-amended mesocosms, peaking at $\sim 7 \times 10^5$ cells $\cdot$ ml $^{-1}$.

As with the HNA bacterial cell abundance, *Synechococcus* cell abundance declined at two rates at the start of the experiment. Initially, cell abundance dropped slowly up to day 3 and then rapidly down to $\sim 4 \times 10^3$ cells $\cdot$ ml $^{-1}$ on day 6 (Fig. 3). Unlike HNA bacterial cell numbers, *Synechococcus* cell abundance did not recover and remained low for the remainder of the experiment. There were no significant differences in abundances of *Synechococcus* cells between treatments or between surface microlayer and subsurface water.

Bacterial community structure. We used two *Bacteria* 16S rRNA gene profiling methods (T-RFLP and DGGE) to monitor changes in the bacterial community structures in surface microlayer and subsurface water samples collected on day 2, day 5, and day 10.

PCA ordination of the structures of the bacterial communities from T-RFLP analysis of subsurface and surface microlayer DNA samples is shown in Fig. 4. On day 2, the samples collected from the subsurface and from the surface microlayer

FIG. 4. Ordination diagram from PCA of bacterial T-RFLP profiles. Samples were collected on day 2 (black), day 5 (dark grey), and day 10 (light grey). Subsurface water (■) was collected using a siphon, and the surface microlayer was sampled using two methods: a mesh screen (▲) and polycarbonate membranes (●). Open symbols, control mesocosm samples; filled symbols, nutrient-amended mesocosm samples.

using the mesh screen clustered closely together relative to the surface microlayer samples collected using polycarbonate membranes. As the mesocosm blooms progressed, this pattern changed drastically. At day 5, samples from the subsurface showed a distinct cluster that was separate from the mesh screen samples. As with day 2, the membrane-collected surface microlayer samples remained distinct from the subsurface samples. Near the end of the experiment on day 10, bacterial community structure in the samples collected with the mesh screen clustered with the samples collected with membranes and not subsurface water samples. Ordinance analysis of the T-RFLP data in this experiment showed no evidence of bacterial community structural differences as a result of the induced phytoplankton bloom (Fig. 4).

DGGE analysis of the bacterial community structures showed similar results to those of the T-RFLP analysis. At day 2, subsurface and mesh screen-collected samples were similar, and membrane-collected samples showed some differences (Fig. 5). This was less pronounced with DGGE than with T-RFLP at day 2. By day 5, the membrane-collected samples were distinctly different from mesh screen and subsurface samples, forming a separate clade in the dendrogram. Also at day 5, some mesh screen collected-samples were different from their associated subsurface samples. By day 10, both the membrane- and mesh screen-collected samples were distinctly different from the subsurface samples, corroborating the results from the T-RFLP analysis. As with the T-RFLP analysis, DGGE analysis confirmed that the bacterial community structures were not affected by the phytoplankton bloom.

Five relatively dominant DGGE bands from the surface microlayer samples were excised and sequenced (Fig. 5). All five DGGE band DNA sequences were very similar ($\geq 98\%$) to 16S rRNA gene sequences from isolated bacterial strains (Table 1). DGGE bands 1 and 2 were identical to the 16S rRNA gene sequences of *Dokdonia donghaensis* PRO95 (FJ627052) and *Krokinobacter genikus* Cos-13 (AB198086), respectively,

TABLE 1. Sequence similarities of excised 16S rRNA gene DGGE bands shown in Fig. 5

Band	BLAST match (accession no.)	% Similarity (no. of bases)	Taxonomic grouping (class, order, family)
1	<i>Dokdonia donghaensis</i> PRO95 (FJ627052)	100 (158)	<i>Flavobacteria</i> , <i>Flavobacteriales</i> , <i>Flavobacteriaceae</i>
2	<i>Krokinobacter genikus</i> Cos-13 (AB198086)	100 (158)	<i>Flavobacteria</i> , <i>Flavobacteriales</i> , <i>Flavobacteriaceae</i>
3	<i>Alteromonas</i> sp. strain BCw006 (FJ889589)	100 (163)	<i>Gammaproteobacteria</i> , <i>Alteromonadales</i> , <i>Alteromonadaceae</i>
4	<i>Alteromonas</i> sp. strain Oct07-MA-2BB-3 (GQ215064)	100 (163)	<i>Gammaproteobacteria</i> , <i>Alteromonadales</i> , <i>Alteromonadaceae</i>
5	<i>Glaciecola nitratireducens</i> FR1064 (AY787042)	98 (161)	<i>Gammaproteobacteria</i> , <i>Alteromonadales</i> , <i>Alteromonadaceae</i>

from the *Flavobacteria* family *Flavobacteriaceae*. DGGE DNA sequences 3, 4, and 5 were almost identical to two genera, *Alteromonas* and *Glaciecola*, of the *Alteromonadaceae* (Table 1).

DISCUSSION

Bacterial abundance. Results show that the three bacterial cell types quantified in the mesocosms responded in three different ways (Fig. 3). Both HNA bacterial cells and *Synechococcus* cells decreased in numbers drastically at the start of the experiment. HNA bacterial cells and LNA bacterial cells then increased in numbers in the phytoplankton bloom.

An abrupt decrease, followed by an increase in bacterioplankton cell abundance, is a characteristic response frequently observed during phytoplankton blooms (4, 6, 29, 36). A previous *Emiliania huxleyi*-dominated mesocosm experiment using Raunefjorden fjord water showed a very similar bacterial cell response (6). Other mesocosm experiments at Raunefjorden also reported the same reduction in *Synechococcus* cell abundance during an induced bloom (29), thus indicating that *Synechococcus* cells are not successful under these conditions and/or are out-competed.

One of the principal sources of bacterial mortality in the water column is protist predation, with many protists grazing selectively (32). Significantly, some protists target rapidly growing and dividing bacterial cells, such as HNA cells (16, 44). Furthermore, recent evidence suggests that the concomitant drop in bacterial numbers and bloom of small phytoplankton may be due to mixotrophic growth of phytoplankton (49). This may therefore account for the mortality of HNA bacterial cells and *Synechococcus* cells, whereas the LNA bacterial cells did not appear to be affected (Fig. 3).

In this study, cell numbers in the bacterioneuston and the bacterioplankton were not significantly different, indicating that there was no enrichment of cells in the surface microlayer. Surface microlayer and subsurface water samples collected from two sites in the Mediterranean Sea also showed that the numbers of *Synechococcus* cells in the surface microlayer were the same as those in subsurface (0.5 m) samples (23). Bacterial cell counts by flow cytometry analysis from the same samples did have low levels of enrichment in the surface microlayer, yet the enrichment of cultivable bacterial cells was much more

variable, with enrichment factors ranging from 0.5 to 191 (23). High numbers of cultivable bacterial cells in the surface microlayer compared to subsurface waters are often reported (1, 2, 43).

Bacterial community structure. Unlike bacterial cell abundance, bacterial community structure in the surface microlayer was consistently different from that of the subsurface water. Surface microlayer samples collected using both membranes and a mesh screen showed a reproducibly distinct bacterioneuston in the mesocosms. Previous studies have characterized the marine bacterioneuston and cognate subsurface bacterioplankton in the North Sea (13), the Mediterranean Sea (1), and Pacific Ocean (7). In the North Sea and Pacific Ocean studies, the bacterioneuston community structure was distinct compared to that of the bacterioplankton 1 m below the surface (7, 13). Conversely, the Mediterranean Sea study reported no consistent differences between communities (1).

The method of surface microlayer sampling is important in the study of the bacterioneuston (7). Even though the sea surface microlayer is considered the top 1 mm of the ocean, it is operationally defined by sampling depth (26). We used a mesh screen (sampling depth of ~400 μm) and membranes (sampling depth of ~40 μm) to determine bacterial community structure. Previous comparison of membrane-collected and mesh screen-collected samples from an estuarine surface microlayer showed that samples collected using a mesh screen underrepresent the bacterioneuston because samples also contain subsurface water, which “dilutes” the bacterioneuston sample (7). In this study, at the start of the experiment, the mesh screen-collected bacterial community structures were more similar to the subsurface (bacterioplankton) than to the membrane-collected samples (bacterioneuston). This, however, changed during the experiment, with mesh screen-collected samples becoming more similar to the membrane-collected samples (Fig. 4 and 5). This indicated an enrichment effect in the surface microlayer, causing the bacterial communities sampled using the mesh screen to change from bacterioplankton-like to bacterioneuston-like during the experiment.

The proposed enrichment of the surface microlayer and bacterioneuston may be due to the physical nature of the mesocosms used in this experiment. Even though the mesocosms were mixed continuously, they were calmer than the

FIG. 5. Bacterial 16S rRNA gene DGGE profiles from day 2, day 5, and day 10. DGGE profiles show each replicate from the subsurface water (SS) and from the surface microlayer sampled using a mesh screen (MS) and polycarbonate membranes (PC). Beside each DGGE profile is the associated unweighted pair group method with arithmetic mean dendrogram showing the similarity of the lanes in the DGGE profiles. The arrows show which DGGE bands were excised and sequenced (Table 1).

open fjord. Examination of surface microlayer samples offshore of Barcelona showed that under calm conditions (low wind speed and cloudless skies), the enrichment of several parameters in the surface microlayer, including heterotrophic *Bacteria* counts, chlorophyll-*a*, and suspended particle matter, increases substantially (23), supporting our observations in the mesocosms.

The methodological approaches used to compare the community structures of the bacterioneuston and the bacterioplankton can also influence data interpretation. Agogue et al. (1) used similarity values based upon Jaccard coefficients of single-strand conformation polymorphism profiles from surface microlayer and subsurface water samples collected in the Mediterranean Sea. Jaccard coefficients are absence/presence based and do not consider relative abundances (21). Franklin et al. (13) and Cunliffe et al. (7) used 16S rRNA gene clone libraries and DGGE profiles assessed using Pearson correlations, both of which take into account the relative abundances between samples. In this study we also included changes in relative abundances (T-RFs and DGGE bands). The increased resolution of community structure comparisons made using relative abundances versus comparisons made using absence/presence data may, in part, account for the conclusions of Agogue et al. (1).

In this study, bacterial community structure dynamics in each mesocosm were synchronous, showing consistent patterns between replicates (Fig. 4 and 5). The bacterioneuston communities at two sites on either side of Oahu Island were more similar to each other than to their cognate subsurface water bacterioplankton communities just 0.4 m below, also indicating nonrandom assembly of the surface microlayer community (7). Synchronicity of discrete bacterial communities, although poorly understood, is very important, as concordant community dynamics suggest that the community structure patterns that emerge are controlled and are not random (24). Therefore, if the bacterioneuston community structure is controlled by the environment and is not random, as our data suggest, then the sea surface microlayer is, indeed, an important ecological zone of the water column.

Five dominant DGGE bands in the surface microlayer were sequenced and identified (Fig. 5 and Table 1). The bands were very similar to just two families, *Flavobacteriaceae* (bands 1 and 2) and *Alteromonadaceae* (bands 3, 4 and 5). The genera *Alteromonas* and *Glaciecola* (order *Alteromonadales*, family *Alteromonadaceae*) were also prevalent in surface microlayer samples collected from the marine end of Blyth Estuary on the Northeast Coast of the United Kingdom (10). A previous study has also showed that the closely related genus *Pseudoalteromonas* (order *Alteromonadales*, family *Pseudoalteromonadaceae*) dominated surface microlayer samples collected from the North Sea, close to the coast of the United Kingdom (13).

Bacterial cell abundance compared to community structure. Bacterioplankton in the water column include both free-living cells and cells attached to several possible surfaces, including phytoplankton (25) and marine gels (3). Marine gels are a significant component of the sea surface microlayer, giving it a gelatinous structure (8, 40, 46). Surface microlayer samples collected from the same mesocosms in this study were enriched with TEP (9). Therefore, in the sea surface microlayer more microorganisms may be attached than are free living (8). Anal-

ysis of free-living and attached bacterioplankton communities cooccurring in the water column show that both temporal variability and diversity in the attached community are higher than in the free-living bacterial community (37), and specific attached bacterial communities can develop (12).

The two standard marine microbial ecology approaches used in this study, flow cytometry and community profiling (T-RFLP and DGGE), inherently analyze different components of the free-living and attached bacterial cell pools. We filtered the water samples for DNA extraction and subsequent community profiling; therefore, all particles in the water sample of >0.2 µm were analyzed by T-RFLP and DGGE in both free-living and attached bacterial cell pools. However, flow cytometry counts only the free-living bacterial cells. This may contribute to the observations that there are no differences in bacterial cell abundances between the surface microlayer and subsurface water (free-living cells only), yet there are distinct and consistent differences in the bacterial community structures (free-living and attached cells). This may also be responsible for the differences reported between flow cytometry bacterial cell counts and bacterial CFU counts in samples collected in the Mediterranean Sea by Joux et al. (23).

Conclusions. The similar dynamics of bacterial cell numbers and community structure between replicate mesocosms described in this study show how conserved patterns can emerge in bacterial systems such as the sea surface microlayer. These data indicate that the bacterial community structure patterns witnessed in the sea surface microlayer are determined by environmental forces and are not idiosyncratic. This has important implications for marine microbiological research as it is empirical evidence that supports the hypothesis that the surface ocean, particularly the sea surface microlayer, is much more structured than previously thought.

ACKNOWLEDGMENTS

This work was supported by the Natural Environment Research Council (United Kingdom) through the project SOLAS Bergen Mesocosm Experiment (NE/E011446/1), which is part of the NERC-Surface Ocean Lower Atmosphere Study (SOLAS)-directed program.

We thank all the people involved in the project who helped with the preparation and sampling of the mesocosms, including Agnes Aadnesen (University of Bergen). We thank Mikal Heldal, Jorun Egge (University of Bergen), Gill Malin (University of East Anglia), and Ian Joint (Plymouth Marine Laboratory) for invaluable advice concerning the setup and management of mesocosm experiments at Espeland. We also thank Linda Fonnes at the Institute of Marine Research, Bergen, Norway, for inorganic nutrient analysis.

REFERENCES

1. Agogue, H., E. O. Casamayor, M. Bourrain, I. Obernosterer, F. Joux, G. J. Herndl, and P. Lebaron. 2005. A survey on bacteria inhabiting the sea surface microlayer of coastal ecosystems. *FEMS Microbiol. Ecol.* **54**:269–280.
2. Agogue, H., E. O. Casamayor, F. Joux, I. Obernosterer, C. Dupuy, F. Lantoin, P. Catala, M. G. Weinbauer, T. Reinthal, G. J. Herndl, and P. Lebaron. 2004. Comparison of samplers for the biological characterization of the sea surface microlayer. *Limnol. Oceanogr. Methods* **2**:213–225.
3. Alldredge, A. L., U. Passow, and B. E. Logan. 1993. The abundance and significance of a class of large, transparent organic particles in the ocean. *Deep-Sea Res.* **40**:1131–1140.
4. Allers, E., L. Gomaz-Consarnau, J. Pinhassi, J. M. Gasol, K. Simek, and J. Pernthaler. 2007. Response of *Alteromonadaceae* and *Rhodobacteriaceae* to glucose and phosphorus manipulation in marine mesocosms. *Environ. Microbiol.* **9**:2417–2429.
5. Auguet, J. C., and E. O. Casamayor. 2008. A hotspot for cold crenarchaeota in the neuston of high mountain lakes. *Environ. Microbiol.* **10**:1080–1086.

6. Castberg, T., A. Larsen, R. A. Sandaa, C. P. D. Brussaard, J. K. Egge, M. Heldal, R. Thyrhaug, E. J. van Hannen, and G. Bratbak. 2001. Microbial population dynamics and diversity during a bloom of the marine coccolithophorid *Emiliania huxleyi* (Haptophyta). *Mar. Ecol. Prog. Ser.* **221**:39–46.
7. Cunliffe, M., E. Harrison, M. Salter, H. Schafer, R. C. Upstill-Goddard, and J. C. Murrell. 2009. Comparison and validation of sampling strategies for the molecular microbial ecological analysis of surface microlayers. *Aquat. Microb. Ecol.* **57**:69–77.
8. Cunliffe, M., and J. C. Murrell. 2009. The sea surface microlayer is a gelatinous biofilm. *ISME J.* **3**:1001–1003.
9. Cunliffe, M., M. Salter, P. J. Mann, A. S. Whiteley, R. C. Upstill-Goddard, and J. C. Murrell. 2009. Dissolved organic carbon and bacterial populations in the gelatinous surface microlayer of a Norwegian fjord mesocosm. *FEMS Microbiol. Lett.* **299**:248–254.
10. Cunliffe, M., H. Schafer, E. Harrison, S. Cleave, R. C. Upstill-Goddard, and J. C. Murrell. 2008. Phylogenetic and functional gene analysis of the bacterial and archaeal communities associated with the surface microlayer of an estuary. *ISME J.* **2**:776–789.
11. Davis, C. O. 1982. The importance of understanding phytoplankton life strategies in the design of enclosure experiments p. 323–332. *In* G. D. Grice and M. R. Reeve (ed.), *Marine mesocosms: biological and chemical research in experimental ecosystems*. Springer-Verlag, New York, NY.
12. DeLong, E. F., D. G. Franks, and A. L. Alldredge. 1993. Phylogenetic diversity of aggregate-attached vs. free-living marine bacterial assemblages. *Limnol. Oceanogr.* **38**:924–934.
13. Franklin, M. P., I. R. McDonald, D. G. Bourne, N. J. Owens, R. C. Upstill-Goddard, and J. C. Murrell. 2005. Bacterial diversity in the bacterioneuston (sea surface microlayer): the bacterioneuston through the looking glass. *Environ. Microbiol.* **7**:723–736.
14. Fuhrman, J. A., I. Hewson, M. S. Schwalbach, M. S. Steele, J. A. Brown, and S. Naeem. 2006. Annually reoccurring bacterial communities are predictable from ocean conditions. *Proc. Natl. Acad. Sci. USA* **103**:13104–13109.
15. Fuhrman, J. A., and J. A. Steele. 2008. Community structure of marine bacterioplankton: patterns, networks, and relationships to function. *Aquatic Microb. Ecol.* **53**:69–81.
16. Gasol, J. M., U. L. Zweifel, F. Peters, J. A. Fuhrman, and A. Hagstrom. 1999. Significance of size and nucleic acid content heterogeneity as measured by flow cytometry in natural planktonic bacteria. *Appl. Environ. Microbiol.* **65**:4475–4483.
17. Gasparovic, B., Z. Kozarac, A. Saliot, B. Cosovi, and D. Mobius. 1998. Physicochemical characterization of natural and ex-situ reconstructed sea-surface microlayers. *J. Colloid Interface Sci.* **208**:191–202.
18. Grasshoff, K., K. Kremling, M. Ehrhardt, and L. G. Anderson. 1999. *Methods of seawater analysis*. Wiley-VCH, Weinheim, Germany.
19. Hervas, A., and E. O. Casamayor. 2008. High similarity between bacterioneuston and airborne bacterial community compositions in a high mountain lake area. *FEMS Microbiol. Ecol.* **67**:219–228.
20. Hewson, I., J. A. Steele, D. G. Capone, and J. A. Fuhrman. 2006. Temporal and spatial scales of variation in bacterioplankton assemblages of oligotrophic waters. *Mar. Ecol. Progr. Ser.* **311**:67–77.
21. Jaccard, P. 1908. Nouvelles recherches sur la distribution florale. *Bull. Soc. Vaudoise Sci. Nat.* **44**:223–270.
22. Jensen, L. M. 1983. Phytoplankton release of extracellular organic carbon, molecular weight composition and bacterial assimilation. *Mar. Ecol. Progr. Ser.* **11**:39–48.
23. Joux, F., H. Agogue, I. Obernosterer, C. Dupuy, T. Reinthaler, G. J. Herndl, and P. Lebaron. 2006. Microbial community structure in the sea surface microlayer at two contrasting coastal sites in the northwestern Mediterranean Sea. *Aquat. Microb. Ecol.* **42**:91–104.
24. Kent, A. D., A. C. Yannarell, J. A. Rusak, E. W. Triplett, and K. D. McMahon. 2007. Synchrony in aquatic microbial community dynamics. *ISME J.* **1**:38–47.
25. Kogure, K., U. Simidu, and N. Taga. 1981. Bacterial attachment to phytoplankton in sea water. *J. Exp. Mar. Biol. Ecol.* **56**:197–204.
26. Liss, P. S., and R. A. Duce. 2005. *The sea surface and global change*. Cambridge University Press, Cambridge, United Kingdom.
27. Long, R. A., and F. Azam. 2001. Microscale patchiness of bacterioplankton assemblage richness in seawater. *Aquat. Microb. Ecol.* **26**:103–113.
28. Marshall, H. G., and L. Burchardt. 2005. Neuston: its definition with a historical review regarding its concept and community structure. *Arch. Hydrobiol.* **164**:429–448.
29. Martinez-Martinez, J., S. Norland, T. F. Thingstad, D. C. Schroeder, G. Bratbak, W. H. Wilson, and A. Larsen. 2006. Variability in microbial population dynamics between similarly perturbed mesocosms. *J. Plankton Res.* **28**:1–9.
30. Muyzer, G., E. C. de Waal, and A. G. Uitterlinden. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.* **59**:695–700.
31. Naumann, E. 1917. Beiträge zur Kenntnis des Teichnanoplanktons. II. Über das Neuston des Süßwasser. *Biol. Zentralbl.* **37**:98–106.
32. Pernthaler, J. 2005. Predation on prokaryotes in the water column and its ecological implications. *Nat. Rev. Microbiol.* **3**:537–546.
33. Petersson, H., F. Gross, and F. Koczy. 1939. Large-scale plankton culture. *Nature* **144**:332–333.
34. Pommier, T., B. Canback, L. Riemann, K. H. Bostrom, K. Simu, P. Lunberg, A. Tunlid, and Å. Hagstrom. 2007. Global patterns of diversity and community structure in marine bacterioplankton. *Mol. Ecol.* **16**:867–880.
35. Redfield, A. C. 1934. On the proportions of organic derivations in sea water and their relation to the composition of plankton, p. 177–192. *In* R. J. Daniel (ed.), *James Johnstone memorial volume*. University Press of Liverpool, Liverpool, United Kingdom.
36. Riemann, L., G. F. Steward, and F. Azam. 2000. Dynamics of bacterial community composition and activity during a mesocosm diatom bloom. *Appl. Environ. Microbiol.* **66**:578–587.
37. Rooney-Varga, J. N., M. W. Giewat, M. C. Savin, S. Sood, M. LeGresley, and J. L. Martin. 2005. Links between phytoplankton and bacterial community dynamics in a coastal marine environment. *Microb. Ecol.* **49**:163–175.
38. Sapp, M., A. Wichels, K. H. Wiltshire, and G. Gerdt. 2007. Bacterial community dynamics during the winter-spring transition in the North Sea. *FEMS Microb. Ecol.* **59**:622–637.
39. Shaw, P. J. A. 2003. *Multivariate statistics for the environmental sciences*. Arnold, London, United Kingdom.
40. Sieburth, J. M. 1983. Microbiological and organic-chemical processes in the surface and mixed layers, p. 121–172. *In* P. S. Liss and W. G. N. Slinn (ed.), *Air-sea exchange of gases and particles*. Reidel Publishers Co., Hingham, MA.
41. Suzuki, M., M. S. Rappe, and S. J. Giovannoni. 1998. Kinetic basis in estimates of coastal picoplankton community structure obtained by measurements of small-subunit rRNA gene PCR amplicon length heterogeneity. *Appl. Environ. Microbiol.* **64**:4522–4529.
42. Tarran, G. A., J. L. Heywood, and M. V. Zubkov. 2006. Latitudinal changes in the standing stock of nano- and picoeukaryotic phytoplankton in the Atlantic Ocean. *Deep Sea Res. II* **53**:1516–1529.
43. Tsyban, A. V. 1971. Marine bacterioneuston. *J. Oceanogr. Soc. Jpn.* **27**:56–66.
44. Vaque, D., E. O. Casamayor, and J. M. Gasol. 2001. Dynamics of whole community bacterial production and grazing losses in seawater incubations as related to the changes in the proportions of bacteria with different DNA content. *Aquat. Microb. Ecol.* **25**:163–177.
45. Williams, P. M., A. F. Carlucci, S. M. Henrichs, E. S. Vanvleet, S. G. Horrigan, F. M. H. Reid, and K. J. Robertson. 1986. Chemical and microbiological studies of sea-surface films in the southern gulf of California and off the west-coast of Baja-California. *Mar. Chem.* **19**:17–98.
46. Wurl, O., and M. Holmes. 2008. The gelatinous nature of the sea-surface microlayer. *Mar. Chem.* **110**:89–97.
47. Zhang, R., M. Weinbauer, and P. Qian. 2007. Viruses and flagellates sustain apparent richness and reduce biomass accumulation of bacterioplankton in coastal marine waters. *Environ. Microbiol.* **9**:3008–3018.
48. Zhengbin, Z., L. Liansheng, W. Zhijian, L. Jun, and D. Haibing. 1998. Physicochemical studies of the sea surface microlayer. *J. Colloid Interface Sci.* **204**:294–299.
49. Zubkov, M. V., and G. A. Tarran. 2008. High bacterivory by the smallest phytoplankton in the North Atlantic Ocean. *Nature* **455**:224–226.
50. Zubkov, M. V., G. A. Tarran, I. Mary, and B. M. Fuchs. 2008. Differential microbial uptake of dissolved amino acids and amino sugars in surface waters of the Atlantic Ocean. *J. Plankton Res.* **30**:211–220.