Antonie van Leeuwenhoek (2012) 101:819–835
DOI 10.1007/s10482-012-9697-z

ORIGINAL PAPER

Spatial and temporal analysis of estuarine bacterioneuston and bacterioplankton using culture-dependent and culture-independent methodologies

Juliana S. N. Azevedo · Isabel Ramos · Susana Araújo · Cláudia S. Oliveira · António Correia · Isabel S. Henriques

Received: 4 October 2011 / Accepted: 3 January 2012 / Published online: 19 January 2012

Abstract Bacterioneuston may play a key role in water–air exchange of gases and in processing organic matter and pollutants that accumulate at the sea-surface microlayer (SML). However, the phylogenetic diversity of bacterioneuston has been poorly characterized. We analyzed 24 samples each from the SML and underlying water (UW) at three sites in the *Ria de Aveiro* estuary, Portugal. Cultivation and culture-independent techniques were used to compare bacterioneuston and bacterioplankton. Culturable heterotrophic bacteria were enriched in the SML. The culturable community was dominated by *Psychrobacter* and *Acinetobacter*. The presence of high numbers of *Psychrobacter* was a notable result. Differences were confined to a few genera overrepresented in UW samples (*Kocuria*, *Agrococcus* and *Vibrio*). 16S rDNA DGGE profiles were highly stable in terms of number and position of bands between sampling sites and dates but cluster analysis revealed a slight tendency for grouping according to sampled layer. SML-specific DGGE bands affiliated with *Actinobacteria*, *Cyanobacteria*, *Gammaproteobacteria* and *Bacteroidetes*. Low similarity between nucleotide sequences of DGGE-bands and previously reported sequences suggest the occurrence of SML-specific populations. Enrichment of SML for *Pseudomonas* and *Aeromonas* was questioned and the diversity of both communities was analyzed. Consistent differences between SML and UW aeromonads communities were not identified. In terms of *Pseudomonas*, a culturable operational taxonomic unit was consistently overrepresented within SML samples. Taken together, our results indicate that the similarity between SML and UW communities depends on spatial and temporal factors.

Keywords Phylogenetic diversity biofilm · Sea-surface microlayer · Estuary · DGGE

Electronic supplementary material The online version of this article (doi:10.1007/s10482-012-9697-z) contains supplementary material, which is available to authorized users.

J. S. N. Azevedo · C. S. Oliveira · A. Correia · I. S. Henriques (✉)
CESAM and Department of Biology, University of Aveiro, 3810-193 Aveiro, Portugal
e-mail: ihenriques@ua.pt

I. Ramos · S. Araújo
Department of Biology, University of Aveiro, 3810-193 Aveiro, Portugal

I. Ramos
Department of Microbiology, Faculty of Medicine, University of Porto, 4200-319 Porto, Portugal

Introduction

A thin (roughly 1–1,000 μm) surface film was reported to form at the interface between marine environments and the atmosphere (Liss et al. 1997). This physical boundary has commonly been designated as sea-surface microlayer (SML) albeit similar

films occur at the surface of freshwater and estuarine systems (Cunliffe et al. 2008; Hervàs and Casamayor 2009). SML ecological relevance is well recognized and derives from the fact that it covers about 70% of the Earth's surface and strongly impacts the exchange of gases and matter across the air–water interface (Maki 2002; Zemmelink et al. 2005). Specifically, SML has a large effect on water–air exchange of gases directly related to climate changes, such as carbon monoxide, carbon dioxide, methane and dimethyl sulfide (Conrad and Seiler 1988; Upstill-Goddard et al. 2003; Zemmelink et al. 2005; Cunliffe et al. 2008).

The SML is distinct from underlying waters (UW) in terms of its physical and chemical properties (Cunliffe et al. 2011; Liss et al. 1997). For example, this layer has been described as a place of accumulation of organic matter and of a variety of pollutants including hydrocarbons and heavy metals (Cincinelli et al. 2005; Cuong et al. 2008).

Based on these distinct characteristics it has been hypothesized that SML also constitutes a unique ecosystem, which includes distinct biological communities (Maki 2002). The bacterial community present within this layer is usually referred to as bacterioneuston (Naumann 1917). In the past, the SML has been reported to comprise more abundant and active bacterial communities than subjacent waters (Sieburth et al. 1976; Hardy 1982). Regarding phylogenetic composition, while some authors reported considerable compositional differences between bacterioneuston and bacterioplankton (Franklin et al. 2005; Cunliffe et al. 2008) others did not find relevant dissimilarities (Agogué et al. 2005; Obernosterer et al. 2008). Inconsistencies between studies have been related to the use of different SML sampling devices (Cunliffe et al. 2009, 2011). Though, spatial and temporal factors might contribute to the unevenness in the SML formation and thickness and accordingly to the variability in the structure and functional properties of bacterioneuston (Peltzer et al. 1992; Santos et al. 2009).

Estuaries may represent one of the most favorable habitats for the establishment of distinct bacterioneuston communities since they consist on semi-enclosed water bodies, usually strongly influenced by anthropogenic activities, often becoming eutrophicated and concentrating high levels of pollutants (Keddy 2000). In these systems, terrestrially derived material probably contributes significantly to the formation of distinct surface layers. In fact, within estuaries the occurrence of visible surface slicks is a common phenomenon (Liss et al. 1997).

The properties of SML may vary significantly along time and space. Therefore it can be hypothesized that the degree of similarity between adjacent bacterioneuston and bacterioplankton would differ between sampling sites and dates. To confirm this hypothesis, in this study, the phylogenetic composition of bacterioneuston and bacterioplankton communities from a highly polluted estuarine system (*Ria de Aveiro*, Portugal) was compared by combining culture-independent and culture-dependent approaches. Nearly all studies conducted in the last decade examined differences between SML and UW communities using culture-independent methods. However, specific properties of SML, namely accumulation of organic matter at this interface (Cunliffe and Murrell 2009), may conduce to the establishment of a distinct and highly active community of culturable heterotrophic bacteria. Thus, this community was also considered during this study.

Other authors (Cunliffe and Murrell 2009) have hypothesized that microorganisms with ability to form biofilms have a selective advantage in the gelatinous film that SML is. For that reason, efforts were made to verify this hypothesis in what concerns *Aeromonas* and *Pseudomonas*, two genera whose members are commonly found in estuarine waters and have been frequently associated with biofilm formation.

Materials and methods

Site description and sample collection

Ria de Aveiro is a shallow estuary on the north–west coast of Portugal (40°38′N, 8″45′W), about 45 km long and 8.5 km wide (Fig. 1). Samples were collected at three sites: *Cais do Chegado* (CC), where the main contamination sources are industrial effluents, *Costa Nova* (CN), mainly impacted by urban effluents, aquacultures and run-off from agriculture fields and *Cais do Sporting* (CS) subjected to anthropogenic pressure mainly due to the presence of harbor facilities (Fig. 1).

Samples were taken during 4 campaigns in May (C1), June (C2), September (C3) and October 2008 (C4), with gentle weather conditions and wind below 3–4 m/s. For each campaign, SML and UW samples were collected always at low tide, during day (maximum light) and during night (minimum light). A water

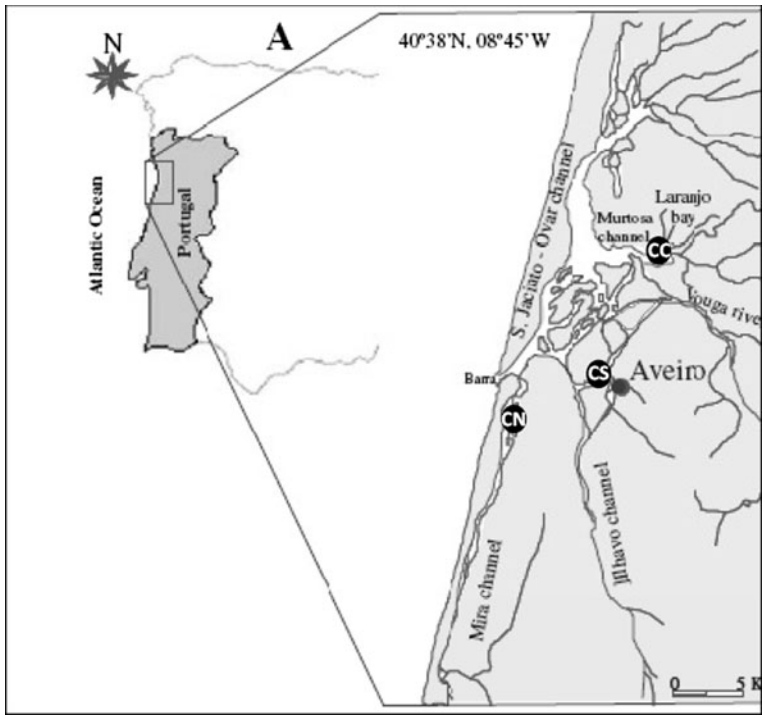


Fig. 1 Map of Ria de Aveiro showing sampling sites in *Costa Nova* (CN), *Cais do Sporting* (CS) and *Cais do Chegado* (CC)

layer of 60–100 μm was collected with a 0.25 m wide × 0.35 m long and 4 mm thick glass plate as previously described (Agogué et al. 2004; Santos et al. 2009). Prior to collection, the plate was washed with ethanol, sterile Milli-Q water and several times with the respective SML water. To minimize the contamination with UW, water draining from the plate for the first 20 s was rejected. Bacterioplankton was collected at approximately 0.4 m depth in 2 L autoclaved bottles. Samples were kept in cold and dark conditions during transport and were processed within 1 h after sampling. Salinity was determined with a WTW Conductivity Meter Model LF 196 (WTW, Weinheim, Germany) and measured using the Practical Salinity Scale.

Enumeration, isolation and identification of culturable heterotrophic bacteria

Water samples were serially diluted in 0.9% NaCl and aliquots of 100 μL of each dilution were spread onto 4 replicate plates of Estuarine Agar (EA; Weiner et al. 1980) and GSP (Glutamate Starch Phenol Red Agar, *Pseudomonas*/*Aeromonas* selective agar) media. Colony-forming units (CFU) were counted after 3 (GSP plates) and 5 days (EA plates) of incubation at 22°C in the dark.

Colonies were randomly selected from each sample, purified and maintained on the same media. Approximately the same number of isolates was selected from each sample and, whenever possible, colonies were picked from plates corresponding to the same dilution. DNA extraction was performed using the Genomic DNA Extraction Kit (MBI Fermentas, Vilnius, Lithuania). Phylogenetic affiliation of the bacterial isolates was established by 16S rRNA gene sequence analysis. Amplification was performed with universal bacterial primers 27F and 1492R as described previously (Lane 1991). PCR products were purified with the Jetquick PCR Product Purification Spin Kit (Genomed, Löhne, Germany) and used as template in the sequencing reaction carried out by the company Stab-Vida (Oeiras, Portugal). The sequence similarity search and phylogenetic affiliation were performed using the BLAST program (Altschul et al. 1997).

The 16S rRNA gene sequences from the SML and UW culture collections were processed by using the analysis pipeline on the ribosomal database project (RDP) website (Cole et al. 2009; http://rdp.cme.msu.edu). Operational taxonomic units (OTUs) were calculated. Classical indices were used to estimate diversity (Shannon-Wiener index) for each culture collection

and to determine the similarity degree between culture collections (Bray-Curtis similarity index). All indices were calculated using the EstimateS software (version 7; available at http://viceroy.eeb.uconn.edu/estimates).

DNA extraction from water samples and community analysis

For DNA extraction 200 mL water samples from SML and UW were filtered through 0.2-μm-pore-size filters (Poretics Products). DNA extraction was performed using the Genomic DNA Extraction Kit (MBI Fermentas, Vilnius, Lithuania) as described previously (Henriques et al. 2004).

For each sample, DGGE was performed on DNA extracts to obtain bacterial and *Aeromonas*-specific molecular fingerprints of the SML and UW. The V3 region of bacterial 16S rRNA gene was amplified using the primers 338F and 518R as previously described (Henriques et al. 2006). A fragment of the *gyr*B gene was amplified with *Aeromonas*-specific primers gyrB-F and gyrB-R following the protocol described by Calhau et al. (2010). For the *gyr*B gene, a reamplification approach was required to obtain sufficient PCR product for subsequent analysis. For this, a second PCR was conducted using 0.5 μL of the first PCR product as template and the same primers and conditions.

A GC clamp was attached to the 5′ end of the forward primers in order to prevent complete melting of the PCR products during subsequent DGGE analysis (Muyzer et al. 1993). The PCR reactions were carried out in a Bio-Rad iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) using *Taq* polymerase, nucleotides and buffers purchased from MBI Fermentas (Vilnius, Lithuania).

PCR products were directly applied onto 8% polyacrylamide gels (37.5:1, acrylamide/bisacrylamide) in 0.5× TAE buffer (20 mmol/L Tris–acetate, pH 7.4, 10 mmol/L sodium acetate, 0.5 mmol/L Na_2EDTA) with urea and formamide as denaturants. Linear denaturing gradients ranged from 37 to 65% for 16S rDNA-DGGE and from 45 to 70% for gyrB-DGGE (100% corresponds to 7 M Urea and 40% formamide). Electrophoresis was performed on a D-Code Universal Mutation Detection System (Bio-Rad, USA) at 60°C; initially a constant voltage of 20 V was applied for 15 min followed by 75 V during 16 h. After electrophoresis, the gels were stained for 5 min with ethidium bromide and then rinsed for 20 min in distilled water. Gel images were acquired using a Molecular Image FX apparatus (Bio-Rad Laboratories, Hercules, California, USA).

Gel images were analyzed with the Diversity Database™ Software (Bio-Rad Laboratories, Hercules, California, USA). Band automatic assignment was carefully checked and, when necessary, manually edited. Taking into account the presence/absence and intensity of individual bands in each lane, a similarity matrix was constructed using the Bray-Curtis measure. Cluster analysis was performed using the PRIMER v6 software (Clarke and Gorley 2001); the same software was used to calculate the Shannon–Wiener index (H'). Dendrograms were generated using the group average method.

Identification of DGGE bands

SML-specific bands, UW-specific bands and bands displaying different intensities in SML and UW profiles were selected for determination of its nucleotide sequence. Bands were aseptically excised, reamplified and checked for accuracy in DGGE gels. The corresponding PCR products were cloned using a TA cloning kit (Invitrogen, California, USA) according to the manufacturer's instructions. Subsequently, at least six inserts were checked by PCR-DGGE and subjected to sequencing analysis as described above, using vector-specific primers. Band sequences were compared to the GenBank nucleotide data library using the BLAST software (Altschul et al. 1997) in order to determine their closest phylogenetic relatives.

Nucleotide sequence accession numbers

Sequences representing culturable OTUs were deposited in GenBank under the following accession numbers: JQ072029–JQ072088. Sequences from DGGE bands were deposited under the following accession numbers: JQ237824–JQ237846.

Results

Abundance and phylogenetic diversity of heterotrophic bacteria

The average number of CFUs per mL was highly variable between sites and campaigns (Fig. 2). However,

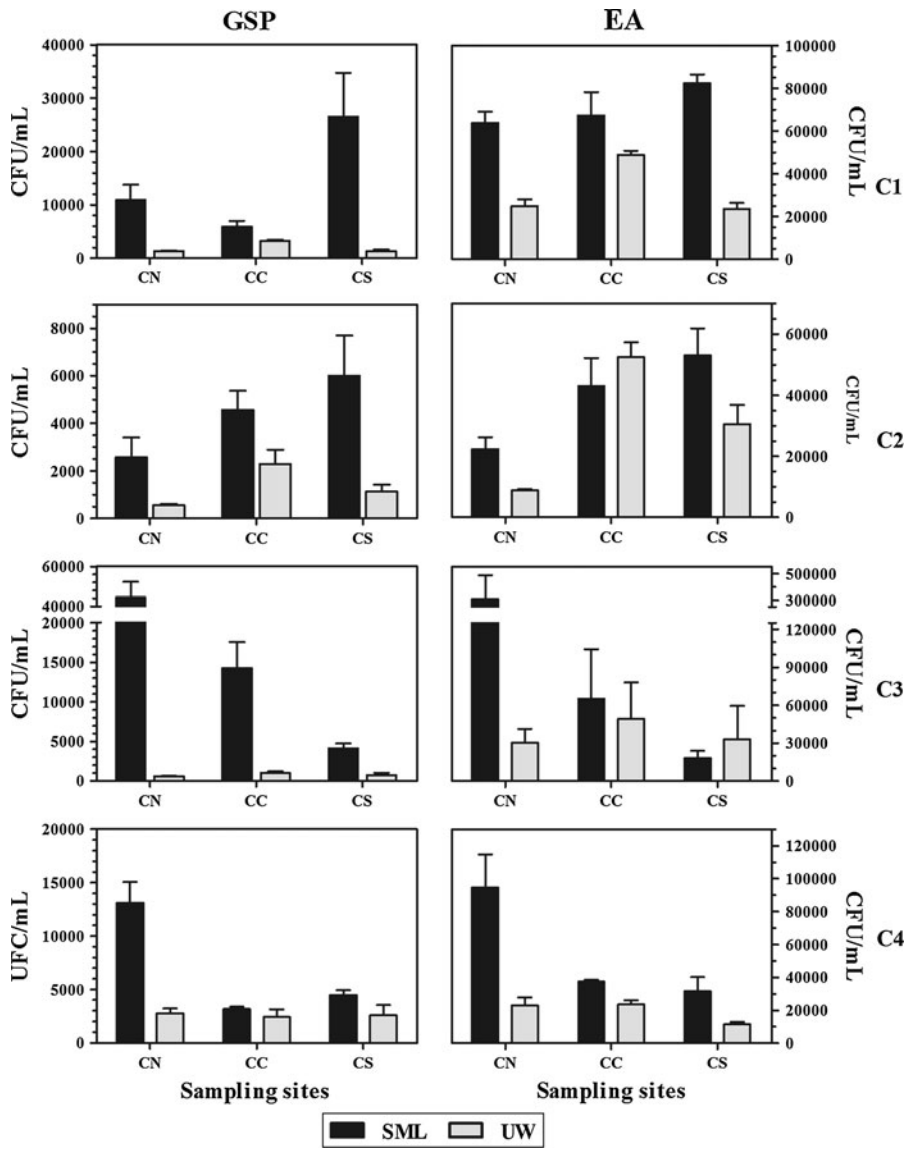


Fig. 2 Fluctuations in mean CFU concentrations (CFU/mL) for SML and UW samples collected in 4 campaigns (C1–C4) and plated in *Pseudomonas/Aeromonas* selective agar (*GSP*) and estuarine agar (*EA*) media

the mean CFU concentrations obtained for the SML samples were usually 2–8 times (and exceptionally up to 75 times) higher than the mean concentration of CFU in the UW samples. These differences were consistently more pronounced in site CN and in the third campaign.

A total of 352 isolates were recovered from water samples, being 168 from SML samples and 184 from UW samples. These isolates, based on identity criteria of >97% at the 16S rRNA gene sequence, could be categorized into 60 operational taxonomic units (OTUs) (Table 1). Two OTUs were dominant and included 39% of the isolates from both the SML and UW samples. OTUs containing a single isolate represented 46.7% of the total OTUs. A similarity value of 69% between the two culture collections was obtained using the Bray-Curtis index.

Based on phylogenetic analysis bacterial isolates were assigned to five phyla within the domain Bacteria, namely *Proteobacteria*, *Bacteroidetes*, *Deinococcus-Thermus*, *Firmicutes*, and *Actinobacteria*, and represented 20 orders (Figs. 3, 4). The positively identified strains belonged to 43 bacterial genera (Table 1). Additionally, isolates included into 3 OTUs (isolated from both layers) could not be identified at genus level and were affiliated with families *Enterobacteriaceae* (2 OTUs) and *Micrococcaceae* (1 OTU).

Table 1 Summary of bacteria isolated from SML and UW samples

Phylogenetic group	Representative isolate[a, b] (accession no)	Closest relatives (accession no)	Sequence identity (%)	Number of isolates in OTU[c]	
				SML	UW
Agrococcus	ENNP5_III (JQ072029)	*Agrococcus citreus* (AB279547)	99	0	12
Arthrobacter	ENDN1_III (JQ072030)	*Arthrobacter agilis* (EU730943.1)	98	1	0
	ENNN6_I (JQ072031)	*Arthrobacter arilaitensis* (EU240951.1)	100	1	1
Brevibacterium	ECNP2_I (JQ072032)	*Brevibacterium* sp. (FN392692.1)	99	0	1
Corynebacterium	ECNN8_I (JQ072033)	*Corynebacterium casei* (DQ361013.1)	99	1	0
Kocuria	ESDP1_III (JQ072034)	*Kocuria* sp. (FJ889675.1)	100	0	5
Microbacterium	ENNP2_I (JQ072035)	*Microbacterium* sp. (FJ765512.1)	99	0	1
Micrococcus	ENDN2_III (JQ072036)	*Micrococcus* sp. (FJ607363.1)	99	2	2
Micrococcaceae	ESNP6_II (JQ072037)	*Micrococcus* sp. (FJ607363)	99	2	6
Aerococcus	GCNN9_I (JQ072038)	Uncultured bacterium (GQ091598.1)	100	1	0
Bacillus	ESDN8_II (JQ072039)	*Bacillus cereus* (EF488087.1)	99	2	0
	ENDP2_I (JQ072040)	*Bacillus pumilus* (GU125637.1)	100	0	1
	ESDN10_III (JQ072041)	*Bacillus* sp. (AF440439.2)	99	1	0
Exiguobacterium	ESDP2_III (JQ072042)	*Exiguobacterium homiense* (FJ999945.1)	100	0	2
Planococcus	ENDN3_II (JQ072043)	*Planococcus* sp. (FJ237405.1)	99	2	0
Staphylococcus	GSDN10_II (JQ072044)	*Staphylococcus equorum* (EU855190.1)	100	1	2
Cyclobacterium	ENNN10_III (JQ072045)	*Cyclobacterium amurskyense* (FJ229465.1)	100	1	0
Algoriphagus	ECNP10_I (JQ072047)	*Algoriphagus aquatilis* (EU313811.1)	97	0	1
Leeuwenhoekiella	ENDP4_III (JQ072048)	Uncultured *Leeuwenhoekiella* (FN433319.1)	98	0	1
Flavobacterium	GNNN5_III (JQ072049)	*Flavobacterium* sp. (AM934639.1)	97	1	0
Olleya	ESDN4_II (JQ072050)	*Olleya marilimosa* (FJ015035.1)	100	1	0
Deinococcus	ESNP7_II (JQ072051)	*Deinococcus radiopugnans* (NR_026403.1)	99	0	1
Brevundimonas	GSDP8_I (JQ072052)	*Brevundimonas* sp. (FJ544245.1)	100	0	1
	GCNP1_II (JQ072053)	*Brevundimonas* sp. (DQ177489.1)	100	0	2
	GNDN8_III (JQ072054)	*Brevundimonas* sp. (DQ310472.1)	99	1	0
Erythrobacter	ENDN8_III (JQ072055)	*Erythrobacter citreus* (EU440970.1)	100	3	1
Agrobacterium	GCNN2_III (JQ072056)	*Agrobacterium tumefaciens* (FJ785222.1)	99	1	0
Pseudorhodobacter	ENDN8_I (JQ072057)	*Pseudorhodobacter incheonensis* (DQ001322.1)	100	2	2
Paracoccus	ESDP1_II (JQ072058)	*Paracoccus* sp. (AY167832.1)	99	0	2
Devosia	ECDN8_I (JQ072046)	*Devosia* sp. (FR731130.1)	97	1	0
Alcaligenes	GSNN1_I (JQ072059)	Uncultured *Alcaligenes* sp. (DQ168833.1)	99	2	1
Achromobacter	GNDN8_I (JQ072060)	*Achromobacter* sp. (GU138383.1)	97	1	0
Comamonas	GCNN4_I (JQ072061)	Uncultured bacterium (EU468035.1)	99	1	0
Delftia	GCNP4_II (JQ072062)	*Delftia* sp. (AB461757.1)	100	0	1
Arcobacter	GCDN6_III (JQ072063)	*Arcobacter* sp. (EF419216.1)	98	1	0
Aeromonas	GNDP3_I (JQ072064)	*Aeromonas hydrophila* (GQ470995.1)	100	14	11
Alteromonas	ESDN8_III (JQ072065)	*Alteromonas* sp. (EF061431.1)	99	1	0
Shewanella	GCDN4_III (JQ072066)	*Shewanella* sp. (FJ025779.1)	99	1	1
	ESDN7_III (JQ072067)	*Shewanella* sp. (EU979479)	98	11	9
Marinobacter	ECDP4_III (JQ072068)	*Marinobacter* sp. (FJ903190.1)	99	0	1
Pseudoalteromonas	ECNP10_III (JQ072069)	Uncultured *Pseudoalteromonas* (FJ695595)	100	1	1
	ESNP3_I (JQ072070)	*Pseudoalteromonas* sp. (AM913917.1)	99	0	1
Rheinheimera	ENNN3_I (JQ072071)	*Rheinheimera* sp. (AM110966.1)	99	2	4

Table 1 continued

Phylogenetic group	Representative isolate[a, b] (accession no)	Closest relatives (accession no)	Sequence identity (%)	Number of isolates in OTU[c]	
				SML	UW
Erwinia	ECDN2_I (JQ072072)	*Erwinia* sp. (AY690711.1)	99	3	0
Halomonas	GSNN9_III (JQ072073)	*Halomonas* sp. (FJ386522.1)	100	2	0
Marinomonas	GNNN6_I (JQ072074)	*Marinomonas* sp. (AY745826.1)	99	1	2
Acinetobacter	ENDN8_II (JQ072075)	Uncultured *Acinetobacter* (DQ234186.2)	99	23	25
	ECDP6_I (JQ072076)	*Acinetobacter* sp. (AF336348.1)	99	0	2
Pseudomonas	GNDP9_III (JQ072077)	*Pseudomonas cf. stutzeri* (AJ244724.1)	99	6	10
	GCDN9_III (JQ072078)	*Pseudomonas* sp. (GQ868355)	100	20	7
	GCNP2_III (JQ072079)	*Pseudomonas* sp. (AB461633.1)	100	0	1
Psychrobacter	GNDP2_II (JQ072080)	*Psychrobacter faecalis* (EU370413.1)	100	43	46
Vibrio	GNNN3_III (JQ072081)	*Vibrio* sp. (AM913925.1)	100	1	5
	GNNP7_III (JQ072082)	*Vibrio diazotrophicus* (NR_026123.1)	100	0	2
	ECNP1_III (JQ072083)	*Vibrio* sp. (AM902263)	99	0	1
Lysobacter	GCNP3_II (JQ072084)	*Uncultured bacterium* (JF168457.1)	99	0	1
Stenotrophomonas	GCDP10_III (JQ072085)	*Stenotrophomonas rhizophila* (GQ359325.1)	100	3	1
	GCDP4_I (JQ072086)	*Stenotrophomonas* sp. (EU073094.1)	100	0	1
Enterobacteriaceae	ENDP9_III (JQ072087)	*Hafnia alvei* (DQ412565.1)	99	3	2
	GNDN3_I (JQ072088)	Uncultured bacterium (GQ069695.1)	99	3	4

[a] Isolates were assigned a code where first letter represents the culture medium (**G**SP or **E**A), the second letter represents the sampling site (C**C**, C**N** or C**S**), the third letter represents the sampling period (**N**ight or **D**ay) and the fourth letter represents the sampled community (**N**euston or **P**lankton). Letters are followed by an arbitrary number (1–10) and the field survey number (I, II or III)

[b] One isolate was chosen to represent each defined OTU and the corresponding 16SrDNA sequence was deposited in the GenBank database

[c] The data represent the number of isolates that were assigned to a particular OTU within SML and UW samples

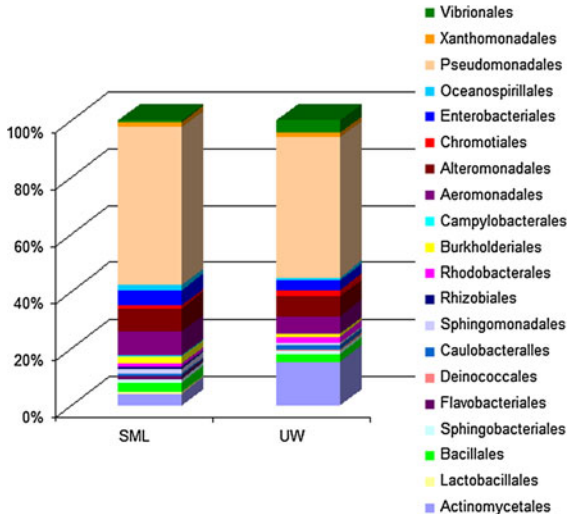


Fig. 3 Stacked columns comparing the contribution at order level to the composition of culture collections representing UW and SML samples

The most common genera were *Psychrobacter* (89 isolates) and *Acinetobacter* (58 isolates). Bacteria belonging to the genera *Pseudomonas* (43 isolates), *Aeromonas* (25 isolates) and *Shewanella* (22 isolates) were also frequently isolated. Most pronounced differences between SML and UW samples were observed for order *Actinomycetales* (Fig. 3; Table 1) namely for genera *Agrococcus* (12 isolates from UW) and *Kocuria* (5 isolates from UW), and for genus *Vibrio* (8 isolates from UW and 1 isolate from SML). On the other hand one of the OTUs assigned to the *Pseudomonas* genus was more abundant in the SML (20 SML isolates and 7 UW isolates) (Table 1).

DGGE analysis of bacterial community structure

DGGE fingerprinting was used to compare the bacterial community structure in SML and UW samples.

During preliminary experiments, DNA extractions and subsequent PCR and DGGE analysis were carried out in triplicate. No detectable differences between profiles were observed. Also, analysis of DGGE profiles revealed negligible variability (<6%) between samples (n = 5) collected within a limited area of approximately 10 m^2 for each sampling site (data not shown).

Subsequently, the sampling sites were visited four times during the warm season (from May to October) and temperature and salinity values for each sampling site/date are presented as supplemental material (Table S1). As previously described for this estuarine system (Henriques et al. 2006), temperature values were relatively stable in time and space while clear differences in salinity values between sampling sites and sampling dates were frequently observed (Table S1).

16S rDNA DGGE profiles are shown in Fig. 5. The number of DGGE bands detected in each profile ranged from 36 to 46 in SML samples and from 35 to 46 in UW samples. Also, overall community diversity did not consistently differ between SML and UW samples: the range of the Shannon–Wiener index values was from 1.52 to 1.61 (mean 1.55) for SML communities and from 1.48 to 1.60 (mean 1.55) for UW communities. Finally, for each campaign, a high number of common bands between all profiles could be identified (Fig. 5).

In fact, cluster analysis showed that the similarity between all profiles was generally high (>50% according to Bray-Curtis measure; Fig. 5). However, in spite of a number of exceptions, for each campaign, samples from the same sampling site clustered primarily by layer (samples collected from each layer during day and night frequently clustered together). Well-defined spatial-driven clusters, which included all samples collected from each site, were observed for the second campaign (sites CC and CN) and for the third campaign (sites CC and CS). On the other hand, temporal compositional shifts were clearly identified for all sampling sites (Fig. S1).

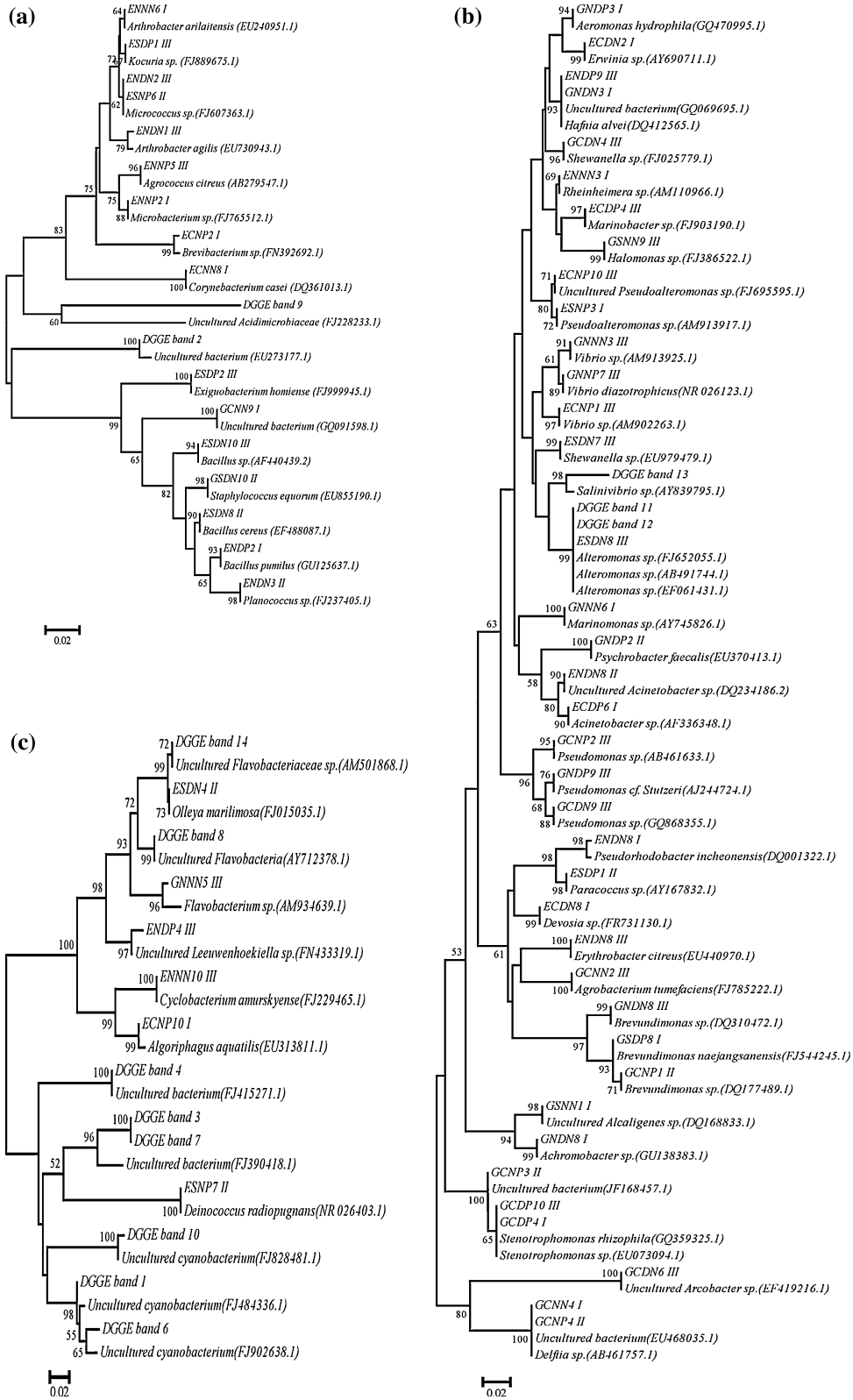
Fourteen bands were excised from the 16S rDNA DGGE profiles obtained from SML samples (Fig. 5). Bands 1, 2, 4, 5, 6, 8 and 12 were also detected in UW profiles but were more pronounced in SML profiles while bands 3, 7, 9, 10, 13 and 14 were only detected in SML samples. Taking into account band intensity, some of the excised bands seem to represent dominant phylotypes (1, 4, 5, 6, 7, 8 and 10). Eight out of 14 bands were excised from profiles from site CS, including 4 SML-exclusive bands (3, 7, 13 and 14). The recovered sequences had high similarity to known bacteria or environmental sequences. However, it was interesting that 4 out of 6 SML-exclusive bands shared only 94% identity with previously reported sequences. Most of the sequenced bands had closest relatives originating from aquatic environments (Table 2). Phylogenetically, the sequences were distributed in four groups: *Cyanobacteria* (3 bands), *Bacteroidetes* (2 bands), *Actinobacteria* (1 band) and *Gammaproteobacteria* (3 bands). Four clones (2, 3, 4 and 7) could only be assigned to domain Bacteria and 1 sequence was most closely related to chloroplast sequences.

Fig. 4 Neighbour joining phylogenetic trees showing the relationships among 16S rRNA gene sequences from bacteria isolated during this study, DGGE bands and sequences obtained from GenBank (accession numbers of these sequences are given in *parentheses*) affiliated with Gram-positive bacteria (**a**), Proteobacteria (**b**) and other phylogenetic groups (**c**). Distances were corrected using the Jukes-Cantor method. Bootstrap values >50% are indicated at the nodes for 1,000 replicates. The *bar* indicates the estimated sequence divergence ▶

DGGE analysis of aeromonads community structure

Aeromonas-specific DGGE profiles were obtained for samples collected during the first, second and fourth campaign (Fig. 6). The number of DGGE bands detected in each profile ranged from 19 to 25 in SML samples and from 15 to 24 in UW samples. According to cluster analysis, the variability between these profiles was higher (>70%) than between bacterial profiles (>50%) (Fig. 6). However, no systematic difference was observed between SML and UW *Aeromonas* communities. Also, no strong spatial-driven differences were observed except for the first campaign where samples from CC and CN formed well-defined clusters.

From *Aeromonas*-specific profiles nine bands were sequenced. Six bands were retrieved from UW and 3 from SML profiles (Fig. 6). Most (5 out of 9) bands were selected from sampling site CS. Two bands (Aer–3, Aer–4) appear to be exclusive from SML samples and 5 bands were putative UW-exclusive (Aer–5, Aer–6, Aer–7, Aer–8 and Aer–9). All band sequences affiliated with members of the *Aeromonas* genus and all sequences were at least 94% identical to the database entries (Table 2).


(a)
ENNN6 I
Arthrobacter arilaitensis (EU240951.1)
ESDP1 III
Kocuria sp. (FJ889675.1)
ENDN2 III
ESNP6 II
Micrococcus sp.(FJ607363.1)
ENDN1 III
Arthrobacter agilis (EU730943.1)
ENNP5 III
Agrococcus citreus (AB279547.1)
ENNP2 I
Microbacterium sp.(FJ765512.1)
ECNP2 I
Brevibacterium sp.(FN392692.1)
ECNN8 I
Corynebacterium casei (DQ361013.1)
DGGE band 9
Uncultured Acidimicrobiaceae (FJ228233.1)
DGGE band 2
Uncultured bacterium (EU273177.1)
ESDP2 III
Exiguobacterium homiense (FJ999945.1)
GCNN9 I
Uncultured bacterium (GQ091598.1)
ESDN10 III
Bacillus sp.(AF440439.2)
GSDN10 II
Staphylococcus equorum (EU855190.1)
ESDN8 II
Bacillus cereus (EF488087.1)
ENDP2 I
Bacillus pumilus (GU125637.1)
ENDN3 II
Planococcus sp.(FJ237405.1)
0.02
(b)
GNDP3 I
Aeromonas hydrophila(GQ470995.1)
ECDN2 I
Erwinia sp.(AY690711.1)
ENDP9 III
GNDN3 I
Uncultured bacterium(GQ069695.1)
Hafnia alvei(DQ412565.1)
GCDN4 III
Shewanella sp.(FJ025779.1)
ENNN3 I
Rheinheimera sp.(AM110966.1)
ECDP4 III
Marinobacter sp.(FJ903190.1)
GSNN9 III
Halomonas sp.(FJ386522.1)
ECNP10 III
Uncultured Pseudoalteromonas sp.(FJ695595.1)
ESNP3 I
Pseudoalteromonas sp.(AM913917.1)
GNNN3 III
Vibrio sp.(AM913925.1)
GNNP7 III
Vibrio diazotrophicus(NR 026123.1)
ECNP1 III
Vibrio sp.(AM902263.1)
ESDN7 III
Shewanella sp.(EU979479.1)
DGGE band 13
Salinivibrio sp.(AY839795.1)
DGGE band 11
DGGE band 12
ESDN8 III
Alteromonas sp.(FJ652055.1)
Alteromonas sp.(AB491744.1)
Alteromonas sp.(EF061431.1)
GNNN6 I
Marinomonas sp.(AY745826.1)
GNDP2 II
Psychrobacter faecalis(EU370413.1)
ENDN8 II
Uncultured Acinetobacter sp.(DQ234186.2)
ECDP6 I
Acinetobacter sp.(AF336348.1)
GCNP2 III
Pseudomonas sp.(AB461633.1)
GNDP9 III
Pseudomonas cf. Stutzeri(AJ244724.1)
GCDN9 III
Pseudomonas sp.(GQ868355.1)
ENDN8 I
Pseudorhodobacter incheonensis(DQ001322.1)
ESDP1 II
Paracoccus sp.(AY167832.1)
ECDN8 I
Devosia sp.(FR731130.1)
ENDN8 III
Erythrobacter citreus(EU440970.1)
GCNN2 III
Agrobacterium tumefaciens(FJ785222.1)
GNDN8 III
Brevundimonas sp.(DQ310472.1)
GSDP8 I
Brevundimonas naejangsanensis(FJ544245.1)
GCNP1 II
Brevundimonas sp.(DQ177489.1)
GSNN1 I
Uncultured Alcaligenes sp.(DQ168833.1)
GNDN8 I
Achromobacter sp.(GU138383.1)
GCNP3 II
Uncultured bacterium(JF168457.1)
GCDP10 III
GCDP4 I
Stenotrophomonas rhizophila(GQ359325.1)
Stenotrophomonas sp.(EU073094.1)
GCDN6 III
Uncultured Arcobacter sp.(EF419216.1)
GCNN4 I
GCNP4 II
Uncultured bacterium(EU468035.1)
Delftia sp.(AB461757.1)
0.02
(c)
DGGE band 14
Uncultured Flavobacteriaceae sp.(AM501868.1)
ESDN4 II
Olleya marilimosa(FJ015035.1)
DGGE band 8
Uncultured Flavobacteria(AY712378.1)
GNNN5 III
Flavobacterium sp.(AM934639.1)
ENDP4 III
Uncultured Leeuwenhoekiella sp.(FN433319.1)
ENNN10 III
Cyclobacterium amurskyense(FJ229465.1)
ECNP10 I
Algoriphagus aquatilis(EU313811.1)
DGGE band 4
Uncultured bacterium(FJ415271.1)
DGGE band 3
DGGE band 7
Uncultured bacterium(FJ390418.1)
ESNP7 II
Deinococcus radiopugnans(NR 026403.1)
DGGE band 10
Uncultured cyanobacterium(FJ828481.1)
DGGE band 1
Uncultured cyanobacterium(FJ484336.1)
DGGE band 6
Uncultured cyanobacterium(FJ902638.1)
0.02

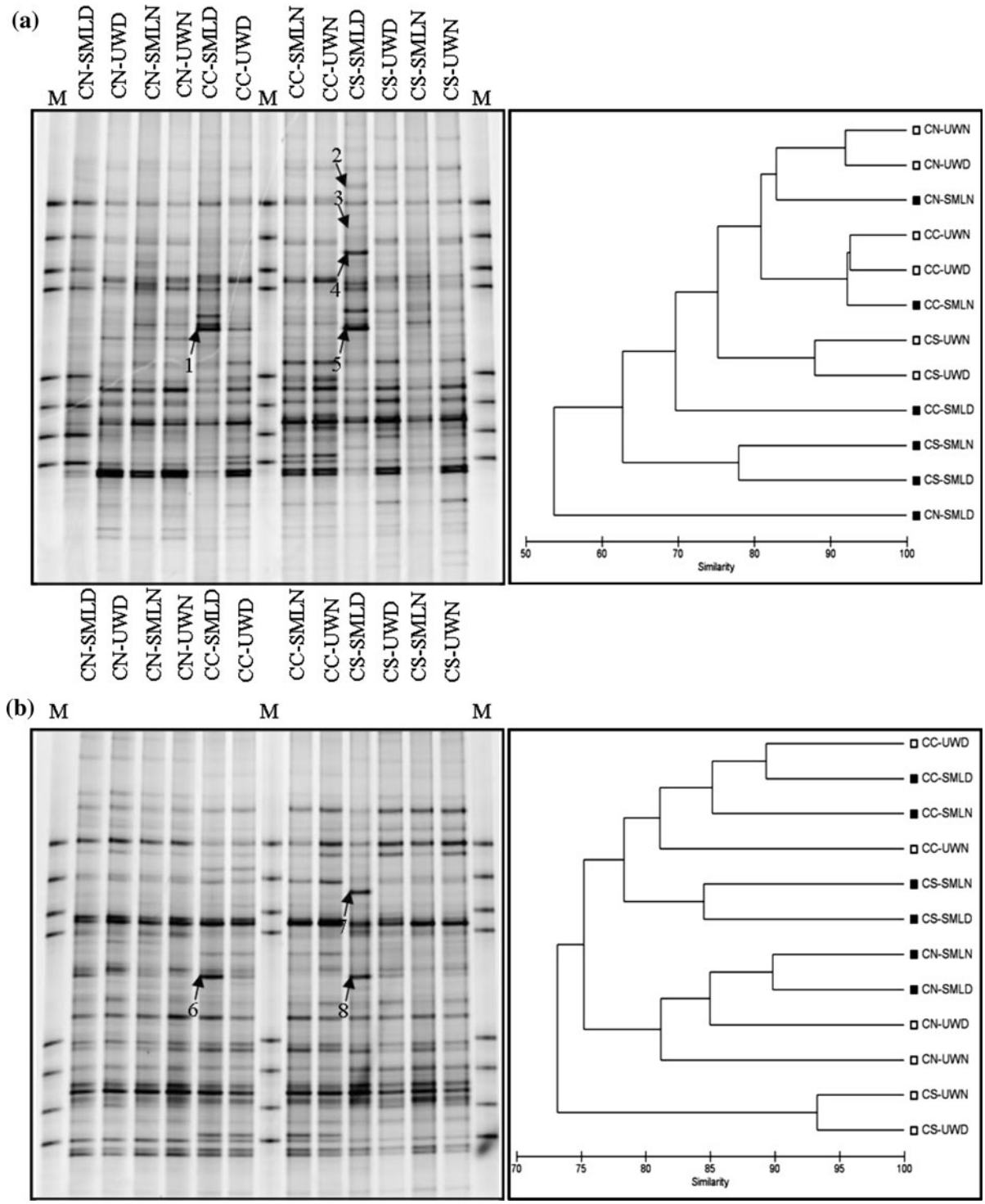


Fig. 5 DGGE analysis of the bacterial diversity in UW and SML samples collected in May (**a**), July (**b**), September (**c**) and October (**d**). *Lane labels* indicate samples collected from sampling sites CN, CC and CS, during day (*D*) and night (*N*). *Arrows* indicate DGGE bands for which the DNA sequence was determined. For each gel a 16S rDNA-based cluster diagram is presented

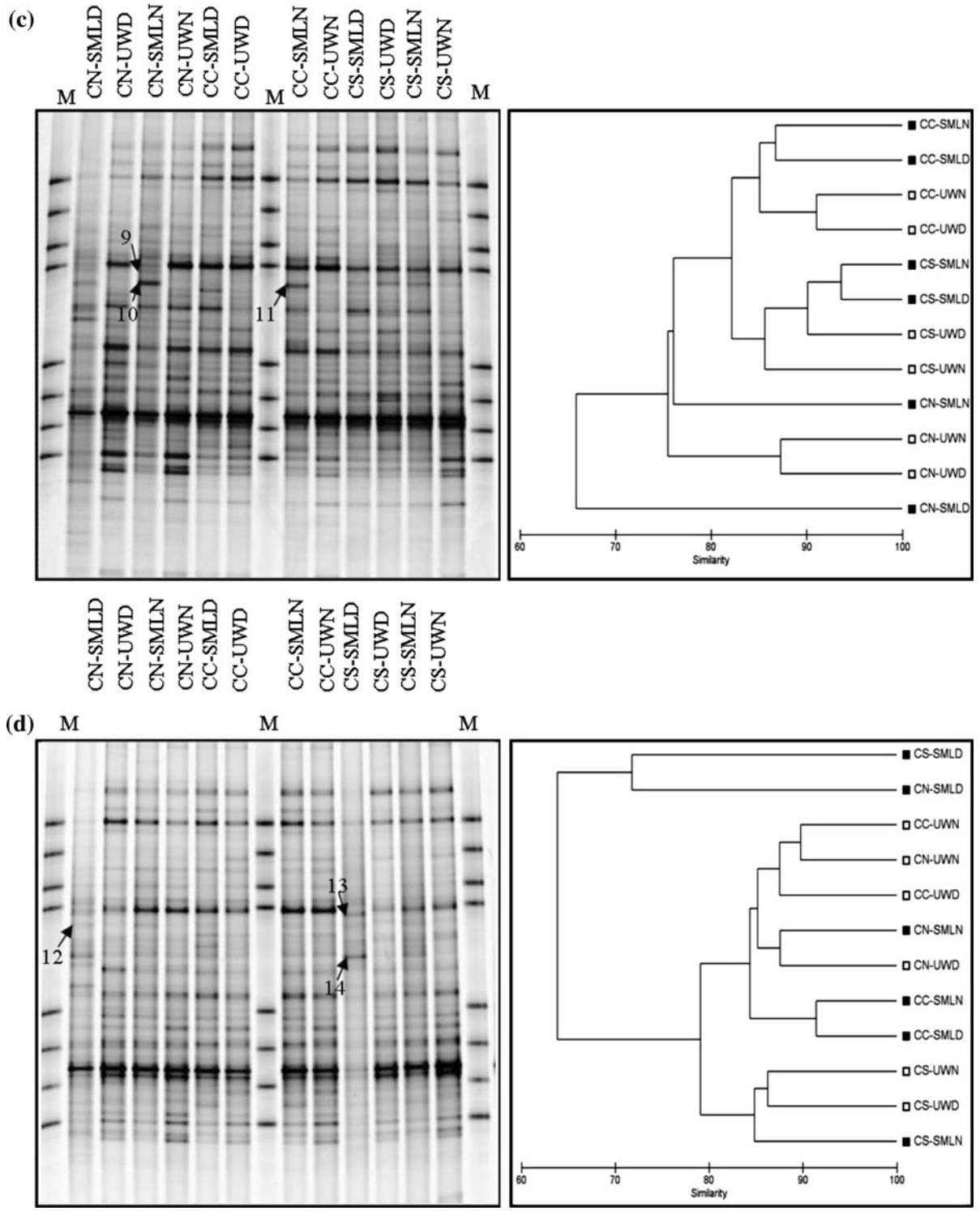


Fig. 5 continued

Discussion

General features of Ria de Aveiro bacterial communities

The analysis of DGGE profiles obtained during this study in terms of number of bands and band positions revealed the presence of complex and highly stable bacterioplankton and bacterioneuston in *Ria de Aveiro*. The observed spatial stability is in accordance with previous studies, which reported the dominance of several widespread phylotypes in this estuary (Henriques et al. 2006). However this apparent stability may be restricted to dominant groups since 16S rDNA DGGE is able to retrieve only sequences that are present in at least 0.5–1% of the total cells in the sample (Muyzer et al. 1993). The culture-based approach led to the successful cultivation of a considerable diversity of heterotrophic bacteria included in five phyla. The analysis of the 16S rRNA gene sequences indicates that potentially new putative species have been cultured since a number of sequences shared <98% identity with any previously cultured isolate. Additionally several sequences affiliated with previously observed but not yet formally described taxa. In general, the dominant bacterial groups found in SML and UW culture collections were similar to those commonly cultivated from estuarine water (Frette et al. 2004; Agogué et al. 2005). The most frequently retrieved genera from both the SML and UW were *Psychrobacter* and *Acinetobacter* followed by *Pseudomonas*, *Aeromonas* and *Shewanella*. Most of the aeromonads were isolated from the selective medium but representatives of the other genera were abundant in estuarine agar indicating that they most likely represent relevant members of the culturable community.

The abundance of *Psychrobacter* was unexpected in view of the present knowledge about this genus: these microorganisms have been isolated mainly from cold environments and were thought to be only marginally successful in other environments (Rodrigues et al. 2009). Contamination with hydrocarbons has been related to *Psychrobacter* enrichment in several previously published studies (Harwati et al. 2007; Prabagaran et al. 2007; Lo Giudice et al. 2010). In our study, *Psychrobacter* isolates were predominantly retrieved from the site CS, mainly impacted by harbor activities and where contamination with hydrocarbons has been previously confirmed (Coelho et al. 2010).

The ability of members of the genus *Acinetobacter* to survive under dry conditions and to be easily transported by air is well known (Hervàs et al. 2009; Reche et al. 2009). Additionally, the presence of airborne *Acinetobacter*-related bacteria in SML has previously been suggested (Hervàs and Casamayor 2009). However, despite being a dominant genus in our samples, differences between SML and UW in terms of abundance or diversity of *Acinetobacter* were not confirmed in this study.

Debates about the shortcomings associated with culture-dependent and culture-independent studies have been frequently carried out and reported. Because most of all the studies that examined differences between SML and UW communities in the last decade used culture-independent methods we decided to combine both approaches. Despite the shortcomings commonly associated with 16S rDNA DGGE (Henriques et al. 2006) this technique has been widely used especially to study spatial and temporal dynamics and to detect relevant compositional differences between communities. The limitations of culture-dependent strategies have also been extensively discussed (Laiz et al. 2003; Tamaki et al. 2005) and we are aware that the cultivated strains may represent only a small and biased fraction of the total diversity. For example the high proportion of *Gammaproteobacteria* obtained in this study can be in part biased, since strains able to rapidly grow on agar media may have been favored by the applied methodology. In fact, culture-independent studies previously conducted reported the dominance of *Alphaproteobacteria* and *Bacteroidetes* on water samples from *Ria de Aveiro* (Henriques et al. 2004, 2006). This last phylum was particularly underrepresented within our culture collections suggesting the occurrence of *Bacteroidetes* refractory to cultivation in this environment.

Bacterioneuston versus Bacterioplankton

Results obtained from both culture-dependent and culture-independent approaches revealed similar (although not identical) bacterial communities inhabiting the SML and UW in *Ria de Aveiro*. These results are in agreement with previous studies conducted in the Mediterranean Sea (Agogué et al. 2005) and in the South Pacific Ocean (Obernosterer et al. 2008). On the other hand, marked differences between both communities were previously observed for other

Table 2 Phylogenetic affiliation of 16S rDNA (1–14) and GyrB (Aer-1 to Aer-9) sequences retrieved from DGGE bands

Band no	Accession no	Sample	Closest relative (accession no)	Origin	Phylogenetic affiliation	Similarity (%)
1	JQ237824	CC-SMLD	Uncultured cyanobacterium (FJ484336.1)	Phreatic sinkhole, Mexico	Cyanobacteria	100
2	JQ237825	CS-SMLD	Uncultured bacterium (EU273177.1)	Taihu lake, China	Bacteria	99
3	JQ237826	CS-SMLD	Uncultured bacterium (FJ390418.1)	Miyun water reservoir, China	Bacteria	94
4	JQ237827	CS-SMLD	Uncultured bacterium (FJ415271.1)	Guanting water reservoir, China	Bacteria	100
5	JQ237828	CS-SMLD	Chloroplast (AY663923.1)	Coastal water, China	Chloroplast	99
6	JQ237829	CC-SMLD	Uncultured cyanobacterium (FJ902638.1)	Phreatic Sinkholes, Mexico	Cyanobacteria	98
7	JQ237830	CS-SMLD	Uncultured bacterium (FJ390418.1)	Miyun water reservoir, China	Bacteria	94
8	JQ237831	CS-SMLD	Uncultured Flavobacteria (AY712378.1)	Salt marsh, Sapelo Island, USA	Bacteroidetes	100
9	JQ237832	CN-SMLN	Uncultured Acidimicrobiaceae (FJ228233.1)	Sediments of acidic mine pit lake Brandenburg, Germany	Actinobacteria	94
10	JQ237833	CN-SMLN	Uncultured cyanobacterium (FJ828481.1)	Eutrophic lake, USA	Cyanobacteria	99
11	JQ237834	CC-SMLN	*Alteromonas* sp (FJ652055.1)	Seawater desalination membrane	γ-Proteobacteria	100
12	JQ237835	CN-SMLD	*Alteromonas* sp. (AB491744.1)	Settlement substrata, Japan	γ-Proteobacteria	100
13	JQ237836	CS-SMLD	*Salinivibrio* sp. (AY839795.1)	Solar salterns, Korea	γ-Proteobacteria	94
14	JQ237837	CS-SMLD	Uncultured Flavobacteriaceae (AM501868.1)	Lagoon anoxic sediments, Italy	Bacteroidetes	100
Aer-1	JQ237838	CN-UWN	*Aeromonas veronii* (AB473092.1)	Sputum	γ-Proteobacteria	98
Aer-2	JQ237839	CS-SMLD	*Aeromonas hydrophila* (AY968042.1)	Activated sludge, China	γ-Proteobacteria	99
Aer-3	JQ237840	CN-SMLN	*Aeromonas allosaccharophila* (FJ238496.1)	Wastewater treatment plant, Portugal	γ-Proteobacteria	96
Aer-4	JQ237841	CC-SMLD	*Aeromonas* sp. ER.1.21 (FJ238503.1)	Wastewater treatment plant, Portugal	γ-Proteobacteria	99
Aer-5	JQ237842	CS-UWD	*Aeromonas veronii* (AB473092.1)	Sputum	γ-Proteobacteria	98
Aer-6	JQ237843	CS-UWN	*Aeromonas bestiarum* (AY987521.1)	Ditch water	γ-Proteobacteria	95
Aer-7	JQ237844	CS-UWN	*Aeromonas eucrenophila* (AY101776.1)	Fresh water fish	γ-Proteobacteria	94
Aer-8	JQ237845	CC-UWD	*Aeromonas caviae* (JF938610.1)	Wastewater treatment plant, Portugal	γ-Proteobacteria	98
Aer-9	JQ237846	CS-UWN	*Aeromonas veronii* (AB473092.1)	Blood	γ-Proteobacteria	98

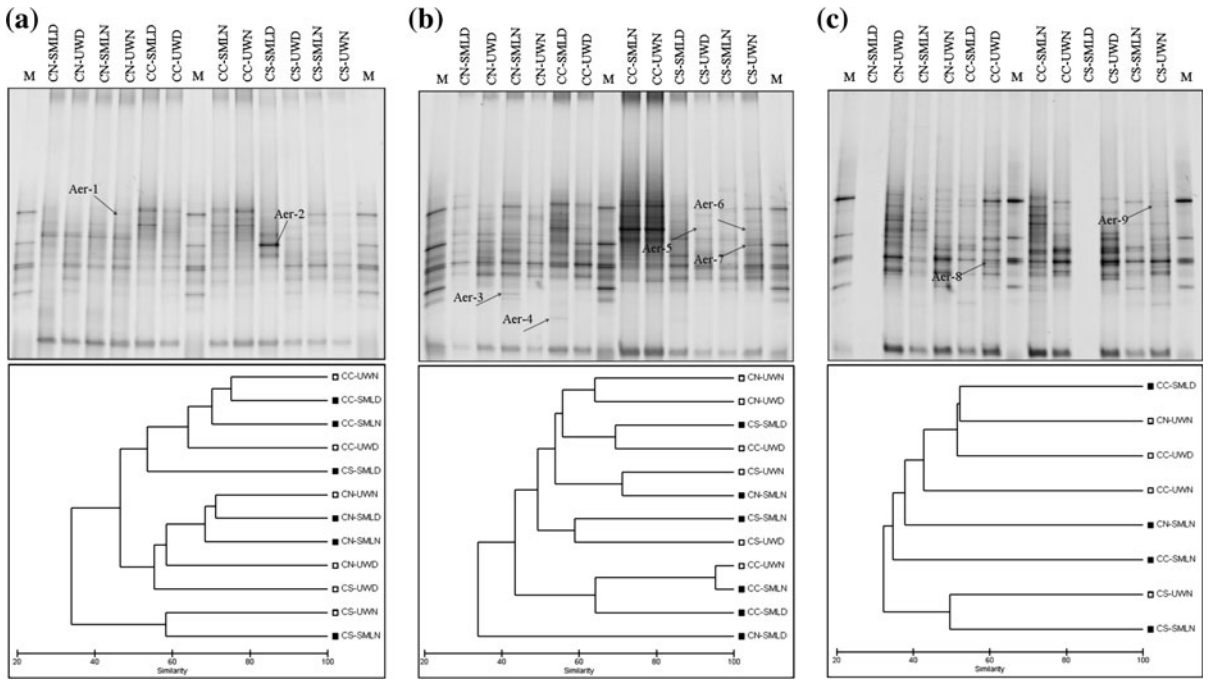


Fig. 6 DGGE analysis of *Aeromonas*-specific *gyrB* amplicons in UW and SML samples collected in May (**a**), July (**b**) and October (**c**). *Lane labels* indicate samples collected from sampling sites CN, CC and CS, during day (*D*) and night (*N*). *Arrows* indicate DGGE bands for which the DNA sequence was determined. For each gel a 16S rDNA-based cluster diagram is presented

geographical locations (e.g. North Sea and the Blyth estuary) but only when SML samples were collected using polycarbonate membranes (Franklin et al. 2005; Cunliffe et al. 2008, 2009). The glass plate method here applied has been previously considered appropriate for sampling culturable and total bacteria from the SML and allegedly avoids biases imposed by selective adsorption properties of membranes (Agogué et al. 2004). However, recently, Cunliffe et al. (2009) argued that samples collected using this method may contain subsurface water in addition to the surface microlayer. Taking this into consideration, divergence between *Ria de Aveiro* SML and UW communities may be higher than noticed during this study and the detected differences are probably more pronounced than suggested by our results.

In fact, some consistent differences were detected. Abundance of heterotrophic bacteria was normally higher in the SML as previously described for other aquatic systems (Sieburth et al. 1976; Hardy 1982). CFU enrichment in the SML has properly been related to higher nutrient concentrations and also to transportation of living cells from underlying waters via electrostatic interactions with rising particles (Joux et al. 2006).

In terms of composition, specific populations, detected as distinct DGGE band positions, were found and sequenced from the SML samples. Previous studies have also noticed structural differences (pronounced or minor) between SML and UW communities when using culture-independent fingerprinting techniques (Agogué et al. 2005; Cunliffe et al. 2008). The fact that most of the SML-specific bands retrieved during this study shared low level similarity with previously reported sequences may suggest the existence of estuarine SML-specific populations. However this aspect certainly needs further detailed investigation.

Regarding culturable heterotrophic bacteria, strong evidences suggesting the occurrence of unusual neustonic phylotypes absent from underlying waters were not gathered. In fact, the foremost difference detected was a higher abundance in UW of the *Actinobacteria* genera *Agrococcus* and *Kocuria*. Also the genus *Vibrio* was mostly retrieved from the UW samples. Our results are in disagreement with previously reported studies which described *Actinobacteria* as more abundant in

the surface microlayer (Agogué et al. 2005) and the genus *Vibrio* as dominant in the bacterioneuston (Franklin et al. 2005). As for other aspects, these contradictory results may be related to different sampling methodologies, geographical locations or even short-term spatial and temporal variabilities.

Differences between bacterioneuston and bacterioplankton in *Ria de Aveiro* were previously reported in what concerns the effects of ultraviolet radiation on both communities (Santos et al. 2011a, b).

Biofilm-forming bacteria

The gelatinous nature of SML may promote the colonization by biofilm-forming bacteria (Cunliffe and Murrell 2009). To our knowledge, this study presents the first attempt to detect differences between bacterioneuston and bacterioplankton focusing on bacteria known to form biofilms. For this we intentionally enriched our culture collection with members of *Pseudomonas* and *Aeromonas* and we also used a culture-independent assay specific for *Aeromonas*. Both genera are ubiquitous in aquatic environments and some species/strains are known to form biofilms in water (Doğruöz et al. 2009). Consistent differences between SML and UW aeromonads communities were not identified neither by the analysis of the culture collections nor by the analysis of DGGE fingerprints. In fact, DGGE fingerprints from both layers shared low similarity but apparently the observed variability cannot be explained by any of the considered variables (layer, site or light regimen). In terms of *Pseudomonas*, an OTU (represented by isolate GCDN9-III in Table 1) was consistently overrepresented within SML samples (74% of the isolates). The ability of strains included in this OTU to form biofilms should be further investigated. Despite preliminary, our results suggest that differences confined to specific phylotypes could be relevant in distinguishing bacterioneuston and bacterioplankton. In accordance, additional efforts should be made to characterize the *Pseudomonas* communities in both layers.

Differences determined by spatial and temporal factors

The properties of SML may vary significantly along time and space (Peltzer et al. 1992; Santos et al. 2009). Therefore we hypothesized that the degree of similarity between adjacent bacterioneuston and bacterioplankton would differ between sampling sites and dates. For that reason, we sampled three geographical locations in four sampling dates and we also included samples collected during day and night. Clear diel patterns were not identified. Even so, our results confirm that differences between both communities frequently vary according to spatial and temporal factors. In terms of culturable bacteria abundance, differences were more pronounced in sampling site CN when compared with the other sampling locations. Also, major differences were detected between samples collected during the third campaign. Water samples were not characterized in terms of organic matter content but site CN is mainly impacted by urban effluents, aquacultures and run-off from agriculture fields (Monteiro et al. 2007). Thus, it is possible that higher organic loads at this site may account for the differences observed.

In terms of composition, the strongest discrimination occurred between DGGE profiles derived from SML and UW samples collected from site CS and most of the SML-specific bands were retrieved from this site. The hydrocarbon contamination at this site (Coelho et al. 2010), which is expected to accumulate at the SML, may favor the establishment of unusual specific phylotypes. Also, the spatial distribution of genera accounting for the most pronounced differences between both communities (*Agrococcus*, *Kocuria* and *Vibrio*) was unequal. *Agrococcus* and *Kocuria* were most frequently retrieved from site CS (71% of the isolates included in these genera) and *Vibrio* was mainly found at site CC (67% of the isolates).

Since temperature values were very stable between sites or sampling dates (Table S1) we can assume that this parameter was not a main driver of bacterioneuston and bacterioplankton compositional shifts. As for salinity, the higher values were observed in campaigns C3 and C4 (corresponding to a dry period). As previously described (Henriques et al. 2006), this factor accounts for a considerable part of the variability in bacterial assemblages in the estuary but if it affects differently bacterioneuston and bacterioplankton remains to be clarified.

Our results suggest that differences between bacterioneuston and bacterioplankton are probably irregular and depend on temporal and spatial factors. This topic has been poorly addressed in previous studies and certainly warrants future investigation.

Acknowledgments This work was financed by the Fundacão para a Ciência e a Tecnologia (FCT) through grants SFRH/BPD/65820/2009 (CSO), SFRH/BD/64057/2009 (JSNA) and SFRH/BPD/21384/2005 (IH) and by the European Union Program of High Level Scholarships for Latin America—Program Alβan—through grant E07D403901BR (JSNA). The authors wish to thank Ângela Cunha and Adelaide Almeida for technical support (sampling device, conductivity meter, etc.). We are also grateful to A. Moura (Department of Biology, University of Aveiro) for reviewing English style and grammar.

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