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Bacterial activity and genetic richness along an estuarine gradient (Rhone River plume, France)

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ABSTRACT: Bacterial diversity and activity were simultaneously investigated by microbial ecological and molecular biological methods along an estuarine gradient from the Rhone River to the Mediterranean Sea. Following a Lagrangian strategy, we sampled plume, frontal and marine layers. The sampled estuarine gradient exhibited large changes both in physico-chemical and in microbiological characteristics. Bacterial abundances and activities showed a more drastic decrease in the low salinity range of the gradient than expected from simple dilution models, indicating that an important fraction of freshwater bacteria disappeared in the mixing area. High specific activities, in particular for leucine, in the marine end-part of the gradient suggested important bacterial protein synthesis, which may be a sign of an active survival strategy for bacterial communities subjected to oligotrophic conditions. Bacterial genetic diversity of the sampled estuarine area, as estimated by the number of DNA-derived denaturing gradient gel electrophoresis (DGGE) bands, was high (13 to 55 bands) compared to that reported in other aquatic ecosystems. This high diversity may be the consequence of the interface position of estuaries. The proportion of active populations was estimated using the ratio of DGGE bands derived from RNA and DNA. This ratio was lower in Rhone water than in marine water, indicating that only a part of the constitutive populations were active, while the activity was distributed within a larger fraction of populations in the marine assemblage. Very few DGGE bands detected in freshwater samples were also detected in the marine end-part of the gradient, suggesting that a very limited number of freshwater bacteria could survive under marine conditions. Detection of these freshwater populations from RNA might indicate that these bacteria were able to synthesize different stress proteins as the result of a survival strategy or that these bacteria were able to maintain metabolic activity under marine conditions. The structure of marine communities was strongly affected by decreasing salinity. However, it seems that the decrease of DNA-derived bands may simply have been the consequence of the mixing of marine and freshwater. No obvious relationship between genetic richness and activity changes was observed. This lack of a relationship may be the consequence of a very short residence time of water in the mixing area studied.

KEY WORDS: Bacteria · Genetic diversity · Activity · Estuary · Rhone River · Mediterranean Sea · PCR-DGGE · Flow cytometry

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INTRODUCTION

In aquatic systems, bacterioplankton plays a major role in the biogeochemical cycles of major elements such as C, O, N and P, and bacterial activity has large

consequences on water quality. However, despite an increasing number of publications on bacterial diversity, little is known about (1) the effect of changes in environmental conditions on the composition and structure of bacterial communities, and about (2) the relationships between bacterial diversity and productivity or activity (Schäfer et al. 2001). This can be explained by the inadequacy of culture-based methods used to record microbial diversity. The application of molecular biological methods theoretically allows a more objective view on microbial diversity. However, so far only a few studies have tried to study bacterial diversity and activity in parallel. Such studies have been limited to very specific environments (e.g. biofilms, Santegoeds et al. 1998) or conducted in mesocosms (Lebaron et al. 1999, Schäfer et al. 2001), mainly because of difficulties in analyzing many samples simultaneously.

The aim of this study was to investigate bacterial diversity and productivity along the estuary of a large river flowing into the Mediterranean Sea (the Rhone River). Estuaries are coastal ecosystems where large changes in environmental conditions occur due to the mixture of fresh and marine waters. Inorganic nutrients and organic matter from watersheds are transported through estuaries and can be processed by different freshwater, brackish and marine populations of microorganisms. Among marine ecosystems, estuaries appear to support high mean bacterial production rates (Ducklow & Carlson 1992). Another striking aspect of estuarine bacterial production and biomass values is their large variations from both spatial and temporal points of view (Ducklow & Shiah 1993, Painchaud et al. 1995, Goosen et al. 1999). Such ecosystems thus appear to be good systems to use in parallel classical microbial ecology methods that result in bulk measurements of the community (i.e. abundance and activity) and molecular biological methods (e.g. polymerase chain reaction [PCR] denaturing gradient gel electrophoresis [DGGE]) for exploring possible links between bacterial activity and diversity. To characterize bacterial community diversity, we used DGGE, which was applied on DNA and RNA extracted from the bacterial community. DGGE results obtained from DNA are considered to reflect the presence of different bacterial populations (Schäfer & Muyzer 2001), while rRNA-derived DGGE results have been recently used to give an indication of the presence of bacterial populations that contribute to the RNA pool, i.e. potentially active populations (Schäfer et al. 2001, Winter et al. 2001).

MATERIALS AND METHODS

Sampling strategy. The Rhone River plume (Gulf of Lion, northwestern Mediterranean Sea) was investigated following a Lagrangian strategy on April 20, 1998 (Fig. 1). For this experiment, a drifter (Naudin et al. 1997) was launched 1 nautical mile (1.8 km) south of the Rhone river mouth (Roustan buoy) to follow the mixing of the river plume with the marine underlying water. The drifter was tracked by the oceanographic RV 'Téthys II', on which continuous conductivity-temperature-depth (CTD) measurements were performed to select stations for sampling of water with different salinity. Five depths (0.5, 1.1, 2.2, 3.4 and 10 m) were sampled along the halocline at Stns B, C, D and E (Fig. 1). Due to the limited water depth at the mouth of the river and the high speed of the drifter, only one surface sample was obtained from this area (A). Samples were collected outside the influence of the ship using a weighted polystyrene floating plate (1 m long, 0.6 m wide) connected to the research vessel with 7 m long Teflon tubes; the system allowed very precise sampling at the desired depth (Naudin et al. 1997). Freshwater samples were collected in the Rhone River upstream from the river mouth at 3 locations (R1, R2 and R3) across the river. For all measured variables, the reported results are means determined at the 3 stations. The marine reference station (Stn S) (43° 15′ N, 4° 44′ E; see Fig. 1) (S) was also sampled at 3 depths (5, 30 and 60 m) except for DGGE analyses, which were performed only for 5 and 30 m depth samples. Samples were processed on board, either stored

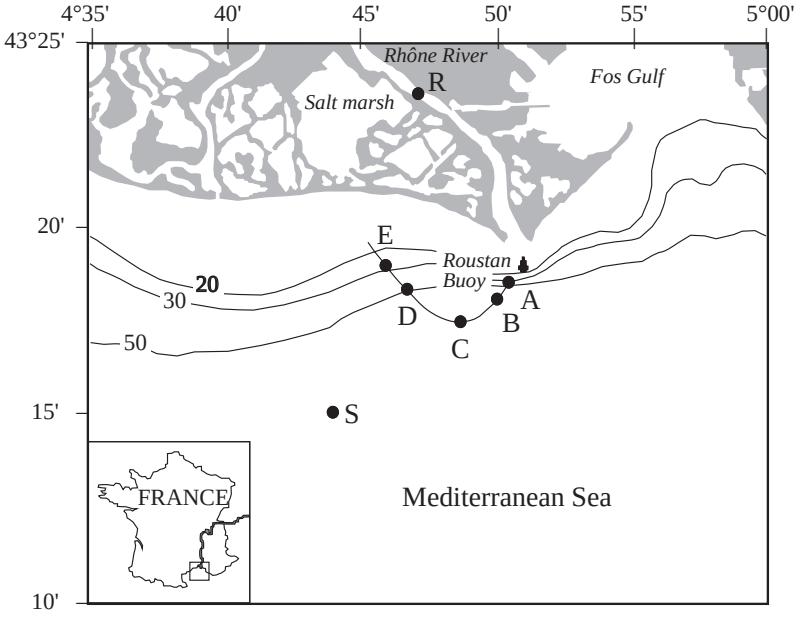

Fig. 1. Sampling locations in the Rhone River plume

without any treatments or fixed (see below) in sterile bottles in the dark at 4°C. To avoid too long a storage, samples were transported from the research vessel to a land-based laboratory (Salins de Giraud) by a high-speed shuttle.

Rough salinities were measured on board with a portable thermo-salinometer for the determination of the sampling stations. More accurate salinity measurements (using the Practical Salinity Scale) were performed at the land-based laboratory using a Guildline 8410 salinometer with an accuracy of ±0.01.

Ammonium concentration. Sub-samples for ammonium analysis were directly collected on board in 100 ml glass bottles. According to Koroleff's (1976) method, the reagents were added immediately and the measurements were performed 24 h after collection by spectrophotometry. The optical density was measured on each original sample to account for turbidity and salt effects in the final concentration. The accuracy of the ammonium measurements was ±0.2 µM.

Phytoplanktonic abundance. Phytoplankton were counted with a FACSCalibur flow cytometer (Becton Dickinson) equipped with an air-cooled argon laser (488 nm, 15 mW). Cells excited at 488 nm were detected and enumerated according to their right-angle light scattering properties (RALS) and their orange (585/42 nm) and red fluorescence (>650 nm) emissions related to phycoerythrin and chlorophyll pigments, respectively. Fluorescent beads (0.94 µm) (Polysciences) were systematically added to each sample. The ratio of mean fluorescence or RALS of a cellular population to that of 0.94 µm beads was used to normalize cell fluorescence emission and scatter values between samples. The precise volume was analyzed and subsequent cell concentrations were estimated by measuring the remaining volume and subtracting it from the initial sub-sample volume (1 ml). Since part of the measured volume was used for flushing the sample line, the outer sleeve of the sample injection port of the flow cytometer was removed.

Different phytoplanktonic groups were discriminated according to their fluorescence and scatter characteristics as described previously (Troussellier et al. 1993, Campbell et al. 1994). For this paper, we have considered only 2 phytoplanktonic groups: prokaryotic cells (*Synechococcus*-like and *Prochlorococcus*) and eukaryotic cells (picoeukaryotic and larger).

Protozoa abundance. The abundance of protozoa was determined by epifluorescence microscopy (Leitz, Laborlux D) after 4′,6-diamidino-2-phenylindole (DAPI) staining. Water samples (20 ml) were preserved with glutaraldehyde (0.5% [v/v] final concentration) and stained with DAPI (10 µg ml^{-1} final concentration) for 15 min. Stained protists were collected by filtration on 0.8 µm Nuclepore black filters. The filters were mounted on microscope slides and stored at 4°C until examination. Pico-sized (<2 µm in diameter) and nano-sized (2 to 20 µm in diameter) micro-organisms were identified, counted and measured at a magnification of 1250× and 625×, respectively, while micro-sized (20 to 200 µm in diameter) micro-organisms were analyzed at a magnification of 125×. A minimum of 100 organisms $filter^{-1}$ was counted. Autotrophic species were distinguished from heterotrophs by the red autofluorescence of chlorophyll *a* observed under blue light excitation. Data presented in this paper concern only heterotrophic protozoa.

Bacterial abundance and cellular characteristics. Bacterial cells were enumerated using SYTO-13 staining and flow cytometry following Troussellier et al. (1999). Briefly, 1 ml formaldehyde-fixed sub-samples were directly incubated with SYTO-13 at a final concentration of 5 µM for 10 to 15 min at room temperature in the dark. For each sub-sample, 3 replicate counts were performed with a FACSCalibur flow cytometer as described above. Stained bacterial cells, excited at 488 nm, were enumerated according to their RALS and green fluorescence (FL1) collected at 530/30 nm. These cell parameters were recorded on a 4-decade logarithmic scale mapped onto 1024 channels. Standardized RALS values (cell RALS divided by 0.94 µm beads RALS) were used as an estimate of the relative size of bacterial cells (Troussellier et al. 1999). Cell abundance was calculated as for phytoplankton (see above). Triplicate measurements were performed for each sample, and mean and standard deviations were computed. Coefficients of variation of the mean abundance were always less than 10% and are not reported in the figures.

Bacterial activity. Two methods based on radioactive incorporation assays were used to estimate bacterial activity: measurement of ^{3}H-thymidine incorporation rate into DNA (Fuhrman & Azam 1982) and ^{3}H-leucine incorporation rate into bacterial proteins (Kirchman et al. 1985). Incorporation of ^{3}H-thymidine (Amersham, 84 Ci $mmol^{-1}$) was measured in each sample at 4 thymidine concentrations (6 to 32 nM). Four 10 ml sub-samples were incubated in the presence of the 4 different concentrations of tritiated thymidine for 1 to 2 h in the dark at *in situ* temperature. After incubation, cold trichloroacetic acid (TCA; final concentration 5% [w/v]) was added, and the samples were filtered over a 0.2 µm pore-size cellulose acetate membrane. Radioactivity associated with the filters was estimated by liquid scintillation. Incorporation rates expressed in pmol l^{-1} h^{-1} at the different thymidine concentrations were calculated and plotted against the added thymidine concentrations. The maximum incorporation rates were estimated by best fitting a hyperbolic function to the experimental data using software based on the least squares criterion (Servais 1995). Data presented

in this paper are maximum incorporation rates. Incorporation of ^{3}H-leucine (Amersham, 151 Ci mmol^{-1}) was measured at 4 leucine concentrations ranging from 2 to 77 nM (2 nM of tritiated leucine in each case with 0 to 75 nM non-radioactive leucine; Servais 1990). Four 10 ml sub-samples were incubated in the presence of the 4 different concentrations of leucine for 1 to 2 h in the dark at *in situ* temperature. After incubation, TCA was added, and the samples were filtered over a 0.2 µm pore-size cellulose acetate membrane. Radioactivity associated with the filters was estimated by liquid scintillation. Maximum incorporation rates were calculated as for thymidine incorporation.

Nucleic acid extraction, PCR and DGGE. DGGE analysis was performed on the DNA and RNA extracted from the different water samples. Nucleic acid was prepared following the hot phenol protocol of Sahm & Berninger (1998). Bead-beating and DNase steps were omitted. Recovery of sequences of Gram-positive bacteria in previous studies has shown that the modified protocol is also suitable to obtain DNA from Gram-positive bacteria (Rossello-Mora et al. 1999). RNA preparation and reverse transcription (RT)-PCR were carried out as described previously (Schäfer & Muyzer 2001). Briefly, RNA was prepared from 5 to 15 µl of nucleic acid extract using 10 U of DNase I (RNase-free; Pharmacia) in 40 mM Tris and 6 mM $MgCl_2$ (pH 7.5), followed by a purification step with phenol-chloroform-isoamyl alcohol (25:24:1 vol). RNA was concentrated by ethanol precipitation. RNA was reverse transcribed into cDNA using 200 U of MMLV-reverse transcriptase (Promega). The purity of RNA preparations from contaminating (undigested) DNA was confirmed by unsuccessful amplifications of 16S rDNA from RNA preparations that had not been reverse transcribed into cDNA.

PCR amplification of 16S rDNA fragments between *Escherichia coli* positions 341 and 926 (Brosius et al. 1981) was carried out as previously described (Muyzer et al. 1998), but reactions contained 1 U of *Taq* polymerase and the buffering conditions recommended by the manufacturer (Pharmacia). Reverse transcription of RNA and amplification of the 16S crDNA was performed as previously described (Schäfer & Muyzer 2001). DGGE was done according to Muyzer et al. (1998) using denaturing gradients from 20 to 80% denaturants (100% denaturants is a mixture of 7 M urea and 40% [v/v] deionized formamide). Electrophoresis was performed at a constant voltage of 200 V for 3.5 h. Gels were stained in ethidium bromide (0.5 µg ml^{-1}), rinsed in Milli-Q water and scanned under UV illumination using the Fluor-S system (Biorad). The acquisition software Multi-analyst (Biorad) was used to invert images and for enhancing the sharpness using the 'sharpen in place' filter.

The ratio between the number of DGGE bands obtained from RNA (R) and from DNA (D) derived PCR products was used as an estimate of the proportion of active sequence types in the bacterial community.

Statistical analyses. To check for the conservative versus non-conservative behaviour of NH_4^+ and bacteria along the salinity gradient, we computed theoretical values of these variables as expected from a simple dilution model of river and seawater. First, fraction of freshwater (F) and marine water ($1 - F$) at each sampling station was estimated from salinity values (S_E) following Officer (1976):

$$F = (S_S - S_E)/S_S$$

where S_S is the salinity at the marine reference station S.

Then, the concentration of the chosen variable for each sampling location (C_x) was computed by the equation

$$C_x = F C_R + (1 - F) C_S$$

where C_R and C_S are the concentrations of the chosen variable in the Rhone River and in the marine reference stations, respectively.

Linear regression models (Statview software, Abacus Concepts) were used to characterize different linear trends detected along the salinity gradient for different variables (protozoa and phytoplankton abundances, DGGE band number).

Similarities between DGGE profiles were estimated by computation of distance values from a binary matrix representing bands that could be unambiguously identified as originating from either the marine or the freshwater bacterial communities using the Jaccard coefficient. The resulting similarity matrix was then submitted to cluster analysis by unweighted pair-group method using arithmetic averages (UPGMA) (Sneath & Sokal 1973). Similarity and clustering analyses were performed with the R software (Legendre & Vaudor 1991)

RESULTS

The environmental gradient

During the sampling cruise the Rhone River flow was 2500 m^3 s^{-1} (river discharge measured at Beaucaire, 65 km upstream from the river mouth). The wind velocity during the preceding days was high (10 m s^{-1}). As a consequence, the plume was not very well established. The time taken by the drifter to go from the beginning (Roustan buoy, Fig. 1) to the end of the sampling transect (Stn D, Fig. 1) was 5 h and 30 min. Nevertheless, the estuarine area showed a 3-layer system as depicted by the salinity pattern (Fig. 2). A

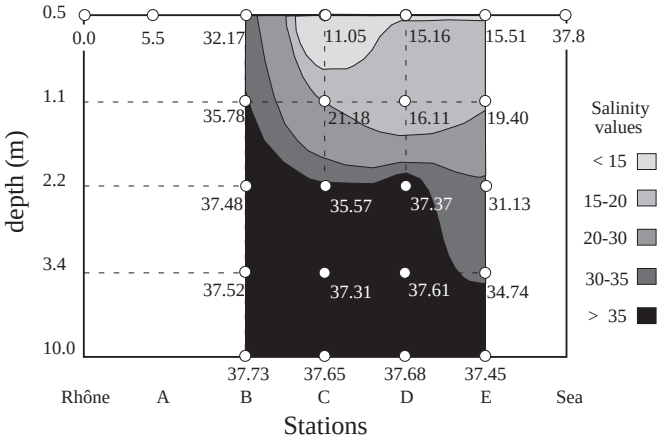

Fig. 2. Salinity distribution during the sampling campaign

hydrological discontinuity could be seen in the plume at Stn B, which may have been due to upwelling of deep water.

Plots of NH_4^+ concentration against salinity did not show significant deviations from the straight line linking river and seawater reference stations (Fig. 3a). This result indicates that the concentration of NH_4^+ followed the linear mixing model.

Phytoplanktonic cell numbers increased with increasing salinity (Fig. 3b). Regression analysis indicated that this increase was more important for prokaryotic than for eukaryotic cells, leading to an increase of the ratio of prokaryotes to eukaryotes. Heterotrophic protozoa counts also increased along the salinity gradient (Fig. 3c); a 2.7-fold increase was observed between the river and the first station along the gradient.

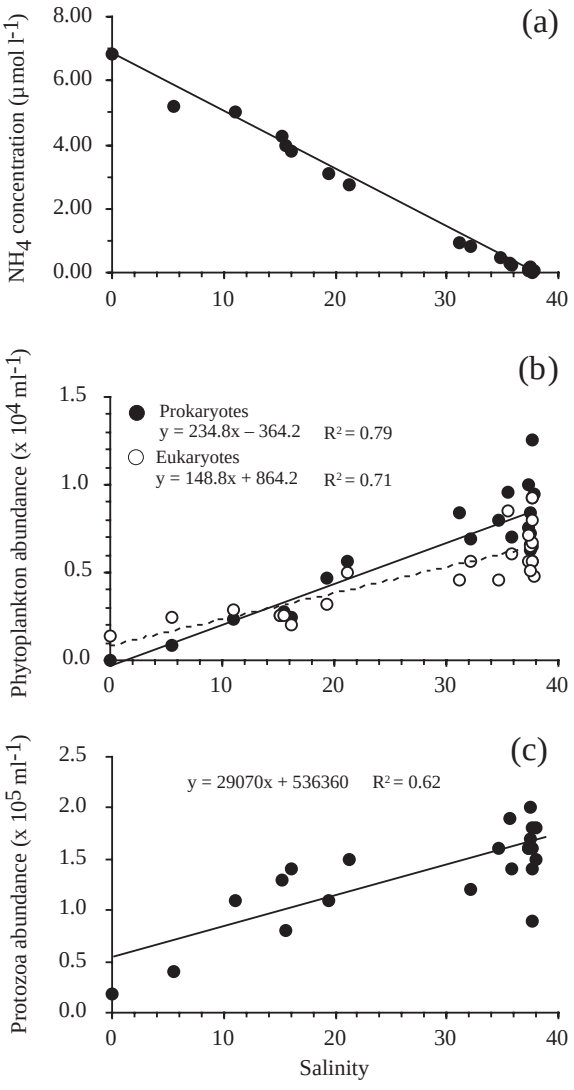

Fig. 3. (a) NH_4 concentration, (b) phytoplankton and (c) protozoa abundance versus salinity values. Line in (a) corresponds to theoretical values for NH_4 if only dilution process occurred; lines in (b) and (c) are regression lines obtained from linear regression models computed between abundance and salinity

Bacterial abundance and activity

Contrary to concentrations of NH_4^+, bacterial abundance showed a non-conservative pattern along the salinity gradient resulting in a decrease from the river to the mixing area (Fig. 4). Thymidine and leucine incorporation rates showed a similar decreasing pattern. Abundance, leucine and thymidine incorporation rates decreased by a factor of 1.6, 3.0 and 4.5, respectively, between the first 2 stations of the gradient.

However, when thymidine and leucine specific activities were computed, a strong decrease in thymidine specific activity was observed in the first part of the salinity gradient followed by an increase at the end-part of the gradient (Fig. 5a). Leucine specific activity also decreased from the Rhone River water to the beginning of the mixture area, but then showed a strong increase from the beginning to the end of the salinity gradient (Fig. 5b).

Apparent mean sizes of bacterial cells decreased all along the salinity gradient (Fig. 5c).

Genetic composition and richness

The DNA-derived DGGE (D-DGGE) fingerprints of the bacterial communities from the river reference (Stn R) and from 2 depths (Sea 1: 5 m; Sea 2: 30 m) of the marine reference station were obviously different (Fig. 6a)

The dendrogram illustrating the clustering of different D-DGGE patterns confirmed that river and marine communities were distant communities (Fig. 7). It also

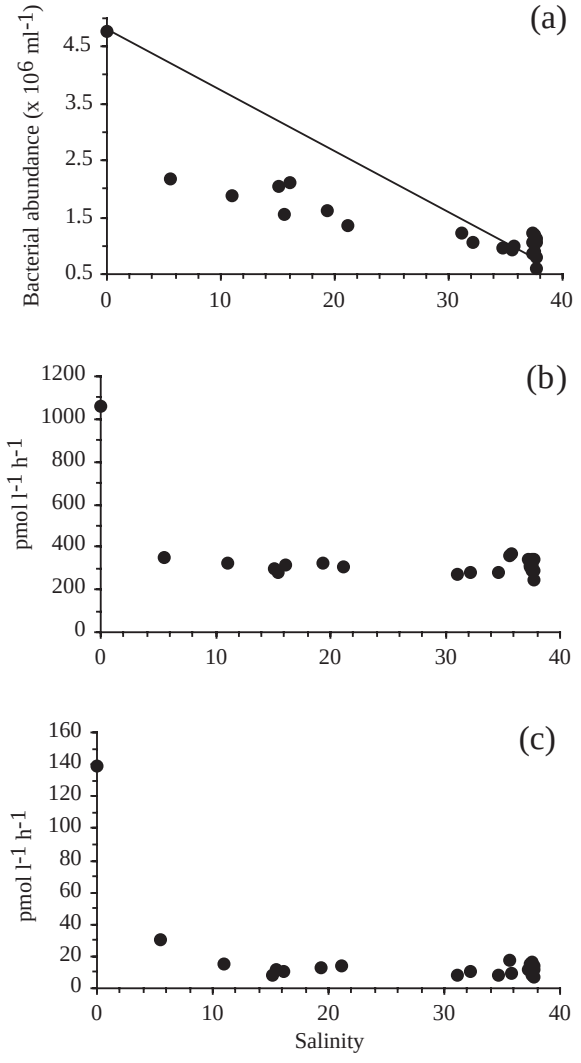

Fig. 4. (a) Bacterial total counts, (b) leucine and (c) thymidine incorporation rates versus salinity values. Line in (a) corresponds to theoretical values for bacterial abundance if only dilution process occurred

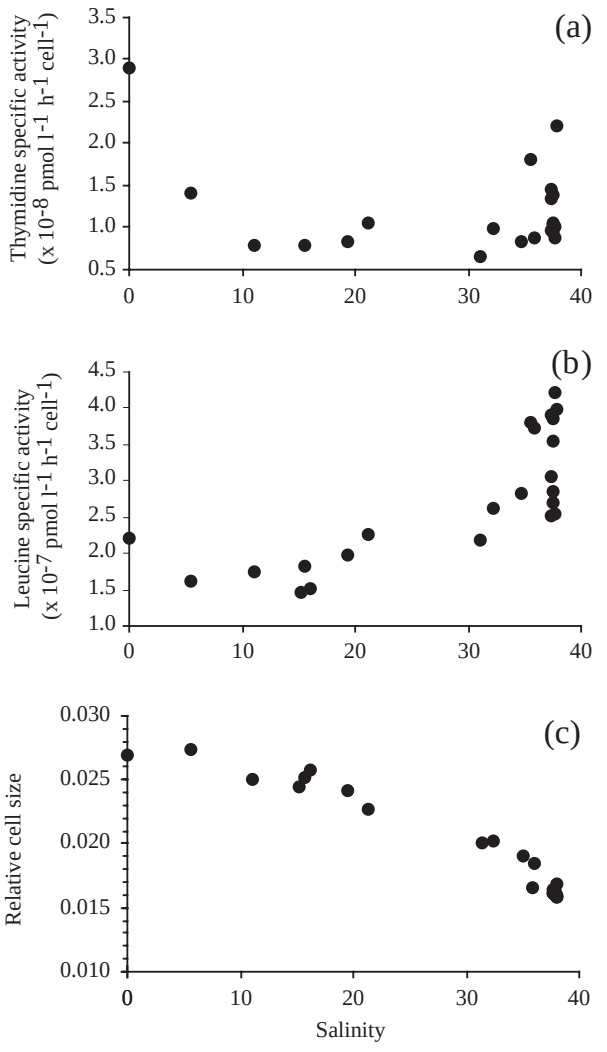

Fig. 5. (a) Thymidine, (b) leucine specific activities, and (c) relative cell size of bacteria, versus salinity

showed that 2 groups can be clearly discriminated. The separation of these 2 groups on the basis of their genetic composition can be related to the hydrological structure of sampled water masses illustrated in Fig. 2. The 2 groups appeared to belong to 2 distinct water layers: the first group (Group I in Fig. 7) was composed of samples coming from upper-layer waters with a salinity ranging from 0 to ~35; the second group (Group II in Fig. 7) was formed by samples with a salinity >35, i.e. originating from deeper layers and the seawater reference station.

The community fingerprint of the river sample showed a total of 47 discernible bands, while the fingerprint of the marine reference station displayed 35 and 34 bands for the water samples from 5 m and 30 m depths, respectively. These estimates, however, have to be regarded as minimum values, as dissimilar sequence types may co-migrate to the same position in the gel, thus leading to a potential underestimation of the number of different sequence types. Assuming that the truly Mediterranean and Rhone bacterial communities shared no common bacterial populations, a total genetic richness, defined as the number of apparent DGGE bands across systems, accumulated to 82 different 16S rRNA sequence types. However, in a number of cases, bands from river and seawater samples were often at a similar position in the gel and could not always be unambiguously identified as river or marine bands in the patterns obtained from samples of the zone where Mediterranean and Rhone water masses were mixing. Therefore, the number of discernible DGGE bands across systems (i.e. Rhone and Mediterranean) that could unambiguously be identified as originating from marine or river communities is 21 and

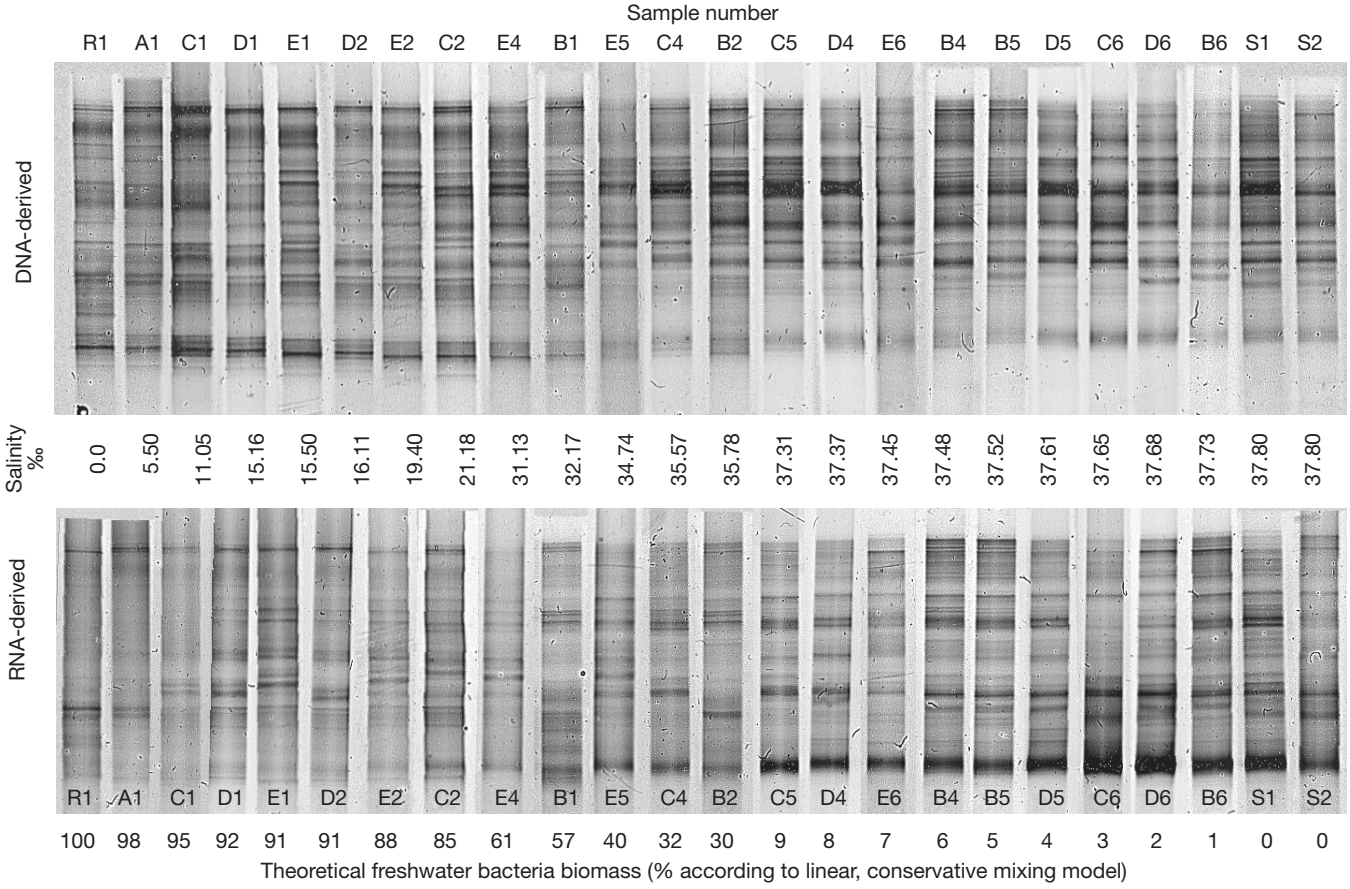

Fig. 6. Composite figure showing the denaturing gradient gel electrophoresis (DGGE) profiles derived from either DNA (top panel) or RNA (bottom panel) of samples aligned according to salinity (increasing from left to right; values indicated between the 2 gel figures). Individual samples were run on 3 gels, so some samples are imperfectly aligned with respect to co-migrating DGGE bands (confirmed by gel electrophoresis). The theoretical contribution of freshwater bacteria biomass to the total bacterial biomass has been calculated according to the linear mixing model and the biomasses of the river and sea reference samples

27, respectively. The following analyses were based only on unambiguous profiles.

When the numbers of D-DGGE bands of the Rhone River (i.e. DF bands from freshwater [F] samples) were plotted against the salinity values, they exhibited a linear decrease (Fig. 8a), suggesting the disappearance of freshwater species.

DM bands (D-DGGE bands from marine [M] samples) showed the opposite pattern to the DF profile (Fig. 8b), although in the marine part of the gradient (salinity between 40 and 30) there was an exponential decrease of the number of bands with salinity decrease.

The proportion of active sequence types in sampled bacterial communities was estimated by the ratio between the number of DGGE bands obtained from R- and D-derived PCR products (Fig. 6).

In the Rhone waters this ratio was 0.45, while in seawater it was 0.75. This ratio was computed for freshwater (F, Fig. 9a) and for marine (M, Fig. 9b) communities at each sampling station. Marine and freshwater communities showed quite different patterns. For the bands corresponding to the freshwater pattern (Fig. 9a), the proportion of R-derived bands remained low in the surface layer where river and marine waters were progressively mixed (0 to 20) and first decreased then increased at higher salinity (>20). However, the highest ratio (1.00) corresponded to a very limited number of DGGE bands (<5), suggesting that only a few populations from the freshwater community remained detectable from both DNA and RNA patterns. The proportion of R-derived bands from the marine community was high and less variable in the marine part of the salinity gradient but decreased strongly at salinity below 15 (Fig. 9b).

No statistically significant relationship was observed between genetic richness (i.e. the number of DGGE bands obtained either from DNA or RNA) and bacterial activity expressed by thymidine or leucine incorporation rates.

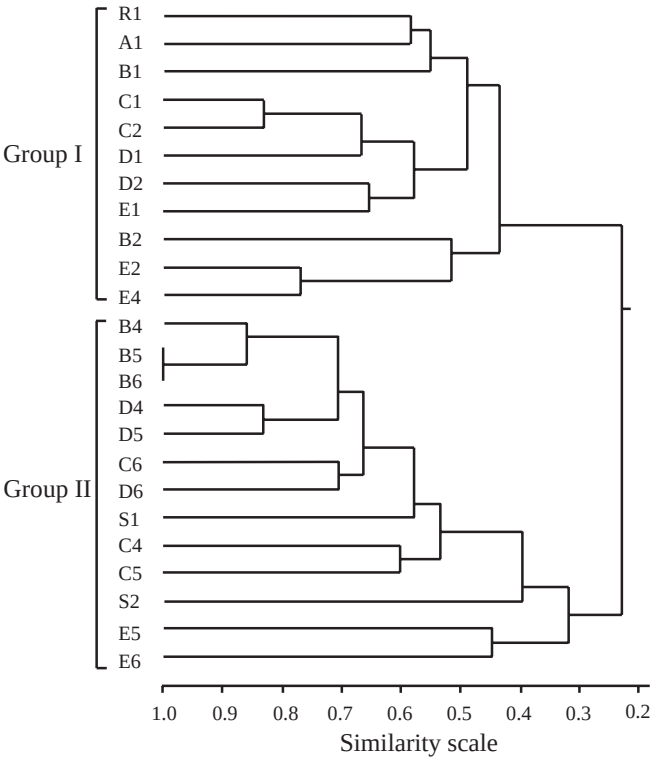

Fig. 7. Dendrogram showing the similarity between DNA-derived (D) DGGE patterns obtained for each sample and the 2 main groups obtained from unweighted pair-group method using arithmetic averages (UPGMA) clustering

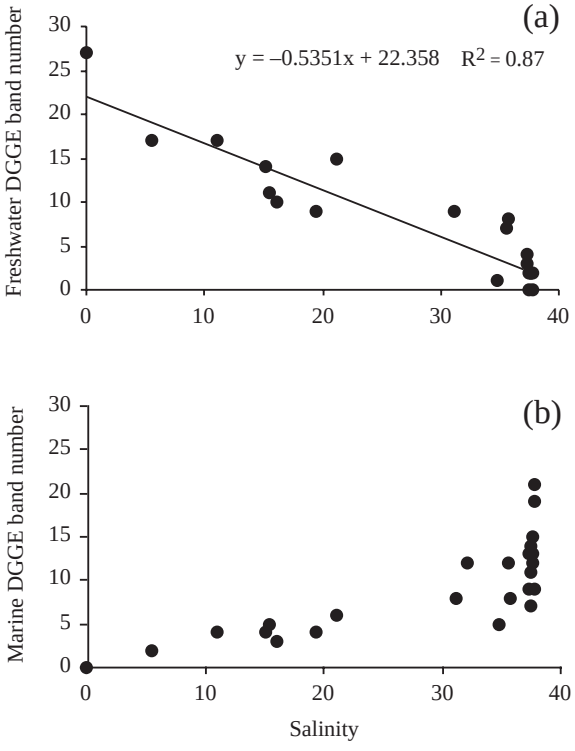

Fig. 8. Number of (a) freshwater (DF) and (b) seawater (DM) DNA derived DGGE bands versus salinity

DISCUSSION

The main objective of this study was to determine whether a relationship exists between activity and diversity of bacterial communities in the estuarine area of the Rhone River in the south of France.

As expected, important changes in some environmental variables, which may directly or indirectly affect the bacterial assemblages, occurred along the Rhone estuary. According to the salinity variations, the sampled area was characterized by a 3-layer system (i.e. plume, frontal and marine layers) as described by Soto et al. (1993). Changes in NH_4^+ concentrations followed a simple dilution process of Rhone waters in Mediterranean seawater without significant gain or loss from biogeochemical processes. A similar trend was reported by Lochet & Leveau (1990).

The increase in abundance of both phytoplanktonic cells and heterotrophic protozoa along the salinity gradient has been reported at least for the first part (i.e. the low salinity range) of the estuarine gradient (Soto et al. 1993). The ratio of prokaryotes to eukaryotes increased from freshwater to seawater, indicating a decrease in the water trophism, because prokaryotes predominate in oligotrophic waters as a consequence

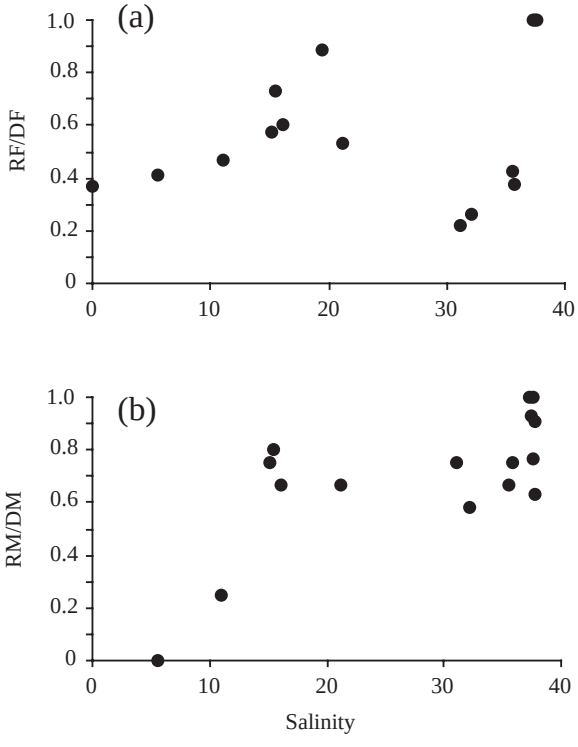

Fig. 9. Ratios between the number of RNA (R) and DNA (D) derived bands for (a) freshwater (F) and (b) marine (M) communities versus salinity

of their better efficiency in using scarce nutrients than eukaryotes (Weisse 1993).

Observed bacterial abundance and incorporation rates of tritiated thymidine and leucine in water samples taken along the transect were in the same range as those previously reported in the same area (Kirchman et al. 1989, Martinez et al. 1991, Bianchi et al. 1994). The decrease of bacterial abundance observed in the low salinity range of the gradient was more important than expected if the simple mixing model occurred between freshwater and marine bacterial communities. This indicated a net loss of freshwater bacterial cells when they encountered marine water. This trend is commonly found in estuarine systems (see Painchaud et al. 1995 and references therein). This decrease of bacterial numbers was concomitant with a more important loss of activity as determined from thymidine and leucine incorporation rates. This suggests either that the activity of freshwater cells decreased strongly in the first part of the gradient or that an important fraction of active cells disappeared.

The decrease of activity for individual cells can be the consequence of environmental changes, such as the decrease of nutrient concentration and the increasing salinity. Disappearance of active cells can be the result of a lytic process due to an osmotic stress. However, at the beginning of the gradient only a limited change in salinity was observed. From the literature dedicated to the survival of allochthonous bacteria in seawater (e.g. Gauthier 2000), it seems that such a limited salinity change does not allow a significant decrease in total bacterial counts. Another explanation may be that active cells were preferentially grazed by protozoa (Del Giorgio et al. 1996, Bernard et al. 2000). This is congruent with the increasing number of heterotrophic protozoa from the beginning to the end of the salinity gradient. Furthermore, the decreasing cell size of bacteria from the river to the sea could be a consequence of this increasing grazing pressure because heterotrophic protozoa more intensively grazed large cells than small cells.

At the marine end-part of the gradient, where more oligotrophic conditions predominate, overall thymidine and leucine incorporation rates were lower than in freshwater. However, thymidine and leucine specific activities showed that cells in the marine part of the gradient exhibited a relatively high specific activity. Leucine specific activities increased more than thymidine specific activities, suggesting an important bacterial protein synthesis, which, in temperate estuaries, has been considered to be the sign of a survival strategy of bacterial communities when they are submitted to oligotrophic conditions (Shiah & Ducklow 1997).

There was a clear correspondence between the hydrological structure of the study area and the spatial distribution of the 2 main groups identified by UPGMA clustering of the samples on the basis of their DGGE profile similarities. The location where the 2 groups were discriminated corresponded to the area where salinity values exhibited the largest changes (i.e. the halocline). This result showed that the physical structure of the Rhone estuary precluded a strong mixing of river and marine bacterial communities.

The genetic richness of the Rhone River and Mediterranean Sea bacterial assemblages expressed by the number of bands in a DGGE profile was high compared to that reported in other aquatic ecosystems (Table 1). Diversity estimates from our study based on the analysis of 24 samples ranged from 13 to 55 DGGE bands for D-derived PCR products. It has to be kept in mind, though, that very few studies reported in Table 1 have applied an appropriate sampling strategy to estimate genetic richness at the ecosystem level. Such a high genetic diversity may be the result of the interface position of estuaries where continental and marine bacterial communities are mixed. A link between diversity and activity was further investigated by comparing both D- and R-DGGE profiles. Many studies in molecular microbial ecology have used RNA as a marker of (recent) cellular metabolic activity, since active cells usually have a higher ribosomal RNA concentration (MacGregor 1999, see same paper for examples). In a recent study Schäfer et al. (2001) used RT-PCR and DGGE to detect active bacterial populations

Table 1. Number of denaturing gradient gel electrophoresis (DGGE) bands (bacterial genetic richness) reported for different aquatic ecosystems (studies are ranked following decreasing maximum genetic richness)

Ecosystem	No. of bands	Source
Algae-detritus	54	Van Hannen et al. (1999)
Rhone estuary	47	This study
Mediterranean mesocosms	24–40	Schäfer et al. (2001)
Activated-sludge biofilm	15–37	Santegoeds et al. (1998)
Mediterranean Sea	34–35	This study
Aegean Sea	28–36	Moeseneder et al. (1999)
San Francisco Bay	22–31	Murray et al. (1996)
Mediterranean mesocosm	19–29	Schäfer et al. (2000)
Antarctic coastal waters	8–29	Murray et al. (1998)
Tomales Bay	15–24	Murray et al. (1996)
Lakes	8–23	Konopka et al. (1999)
Biofilm of bivalve shell	9–21	Gillan et al. (1998)
Meromictic lake (oxic zone)	15–20	Øvreas et al. (1997)
Boreal forest lake	6–15	Lindström (1998)
Hot spring cyanobacterial mats	14	Santegoeds et al. (1996)
Shallow eutrophic lake	6–10	Jaspers & Overmann (1997)

and follow changes in the genetic diversity of active bacterial populations during a mesocosm experiment. Interestingly, the number of R-DGGE bands increased during phases with increasing bacterial productivity (estimated by leucine incorporation); thus, the changes in number of R-derived bands were a useful indicator of changes in general bacterial activity.

For freshwater samples, the number of bands found in DNA and RNA profiles was different and the ratio of RNA to DNA was only 0.45. Assuming that bands are representative of different species, this suggest that only a part of the constitutive populations was active at the time of sampling. In contrast, the RNA to DNA ratio was 0.75 for marine communities, showing that activity was distributed within a large diversity of populations.

The linear decrease in the number of D-derived bands found in the profile of freshwater communities along the salinity gradient indicated that continental bacterial populations were not able to significantly affect the structure and composition of marine communities. However, a few bands initially found in the freshwater profile ($n < 5$) were detected in both R- and D-DGGE patterns determined for samples from the end of the gradient. The corresponding increase of the RNA/DNA number of bands may be considered to be the result of adaptive processes to survive under adverse conditions, which may necessitate the synthesis of different stress proteins and thus previous expression of different genes. If so, this process seems to be operated by only a limited number of species because there was an overall decrease in the total number of cells. The fact that these freshwater populations were also detected in the RNA profile may also suggest that they not only survive but even maintain metabolic activity under marine conditions. Unfortunately, our results do not allow for discrimination between the 2 explanations.

The high ratio found between the number of R- and D-derived bands in the marine community profiles suggests that most populations were able to grow in oligotrophic waters. To be able to survive and eventually to grow (e.g. to be capable of an efficient uptake of scarce substrates), it may be important that these bacteria are able to maintain elevated levels of mRNA and tRNA. This hypothesis has early been suggested by Koch (1971) and verified *in vitro* by Morita (1982).

The structure of marine communities was strongly affected by decreasing salinity. One might hypothesize that the decrease of D-derived bands found in the seawater profile with decreasing salinity originated in a high sensitivity of some marine species to small changes in salinity. However, most probably, this pattern was a consequence of the different bacterial abundances in river versus marine samples. If the mean cell counts obtained from marine waters (6.15×10^5 ml^{-1}) versus freshwater (4.6×10^6 ml^{-1}) are considered, it can be easily demonstrated that mixing marine with river waters would establish an exponential decrease of the ratio between theoretical abundance of marine versus freshwater bacteria. Consequently, marine bacteria are quickly outnumbered and might not be detectable as distinct bands in DGGE patterns.

In the case of this study, the lack of a relationship between bacterial diversity and activity measurements can be at least partly explained by hydrodynamic conditions. In fact, along the salinity gradient, we have sampled bacterial assemblages that either appeared very different (such as the true Rhone and Mediterranean Sea communities) and adapted to their specific environment or were extracted from a very dynamic area (plume) in which bacterial assemblages that were formed by the mixing of river and seawater bacteria probably do not have enough time to adapt (the residence time of the plume was estimated to be <6 h). Crump et al. (1999) found a residence time of 1 to 2 d in the case of the free-living bacterial communities in the Columbia River estuary to be insufficient for the development of an adapted estuarine assemblage. On the other hand, the aggregate-attached bacterial community of the Columbia River estuary seemed to be adapted to the distinct estuarine conditions. Adaptation of the community is possible due to the prolonged residence time of particle-attached bacterial communities in the estuary, which was estimated to be 2 to 4 wk. We hypothesize that only in estuarine systems where the residence time of water from the mixing area is long enough can we observe an adapted bacterial community that can support a relationship between activity and diversity.

To our knowledge, this is the first study that combined bacterial diversity and activity parameters in an attempt to get insight in the behavior of bacterial populations along a salinity gradient. Further understanding the role of bacterial populations in biogeochemical processes occurring along environmental gradients requires provision of information on the structure of bacterial communities (i.e. relative abundance of populations) and not only on richness. This is essential to really investigate the behavior of targeted species in these environments.

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