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Bacterial diversity of epilithic biofilm assemblages of an anthropised river section, assessed by DGGE analysis of a 16S rDNA fragment

Emilie Lyautey[1], Samuel Teissier[1], Jean-Yves Charcosset[2], Jean-Luc Rols[1], Frédéric Garabétian[1,*]

[1]Laboratoire d'Ecologie des Hydrosystèmes, UMR 5177 CNRS–Université Paul Sabatier, 118 route de Narbonne, 31062 Toulouse cedex 04, France

[2]Laboratoire 'Dynamique de la Biodiversité', UMR 5172 CNRS–Université Paul Sabatier, 29 rue J. Marvig, 31055 Toulouse cedex 04, France

ABSTRACT: PCR-DGGE analysis of a 16S rDNA fragment was used to determine spatial patterns of epilithic bacteria diversity over a 100 km river section. Epilithic biofilms were collected on natural substrata in a river reach (mid-slope of the river Garonne) where changes in epilithic bacterial activities and densities were expected due to the presence of a major urban centre (Toulouse city). Nitrogen and phosphorus forms increased 2- to 10-fold downstream of this urban centre. Close values of biomass (18 to 27 g m^{-2} in 2000 and 10 to 16 g m^{-2} in 2001), bacterial densities (3 to 11 $\times$ 10^{12} cells m^{-2} for total bacteria) and activities (3.6 mg N m^{-2} h^{-1} for nitrification and 0.3 to 1.1 mg N m^{-2} h^{-1} for denitrification) were recorded at the different sampling sites. Conversely, in regard to community composition, up- and downstream samples were discriminated between according to their banding patterns: >60% similarity within clusters versus <45% similarity between clusters as calculated with the Jaccard similarity index. Up- and downstream samples shared 50 of the 74 detected bands, the maximum number of bands being 45 for 1 sample. Derived from relative intensities, Simpson (ca. 0.034) and Shannon (ca. 5.06) diversity indices indicated diversified communities. For all of the tested samples, specific richness and diversity indices exhibited relatively homogeneous values, suggesting that there may be a similar level of diversity in biofilms from contrasting sources.

KEY WORDS: Biofilms · Bacterial diversity · DGGE fingerprinting · River Garonne

INTRODUCTION

To assess bacterial community dynamics in aquatic environments, most studies of global bacterial diversity have focused on planktonic communities (Murray et al. 1996, Øvreås et al. 1997, Casamayor et al. 2000, Schauer et al. 2000). In running waters, other typical microbial assemblages are biofilms colonising pebbles in so-called gravel bed rivers (Biggs 1995).

Such river epilithic biofilms are complex microbial communities composed of algae, bacteria and other micro-organisms (Lock 1993). Algae account for more than 30% of total biomass (Peterson 1996) and were shown to control either biomass (Sobczak 1996) or diversity (Jackson et al. 2001) of bacteria by providing organic nutrients and physical habitats.

For benthic algal communities, it was observed that a source of trophic effluents causes a modification of community structure, without affecting richness and diversity or even biomass as measured by chlorophyll *a* (chl *a*) levels (Stelzer & Lamberti 2001, Griffith et al. 2002). On the other hand, Montuelle et al. (1996) have reported changes in *Nitrobacter* serotypes associated with wastewater treatment plant discharge, suggesting that such external factors may also affect epilithic bacterial communities. Community patterns of natural

*Corresponding author. Email: frederic.garabetian@cict.fr

epilithic bacteria may thus result both from autogenic succession and from external factors affecting predominantly spatial variation.

Only 3 studies on epilithic bacterial diversity in freshwater systems have been carried out: (1) Brümmer et al. (2000) have detailed the main phylogenetic groups of epilithic communities in 2 rivers of different trophic levels using fluorescence *in situ* hybridisation (FISH); (2) Jackson et al. (2001) have focused on the mechanisms of succession during colonisation using the PCR-DGGE; and (3) Araya et al. (2003) have detailed the bacterial composition of epilithic communities using both methods. Applied to the study of bacterial diversity, PCR-DGGE is a cultivation-independent technique based on the study of a 16S rDNA fragment (Muyzer et al. 1993), which provides rapid assessment along with relevant comparison of diversity patterns.

In the present work, we used the latter fingerprinting method to study spatial patterns of epilithic bacterial communities over a 100 km river section. Concerning bacterial diversity, a realistic view of the actual diversity was expected from sampling natural substrata (pebbles) as artificial substrata are known to affect algal diversity (Cattaneo & Amireault 1992). In regard to spatial patterns at the river reach scale, we intended to analyse the similarity of bacterial assemblages in a system including identified sources of spatial changes in communities (Teissier et al. 2002).

MATERIALS AND METHODS

Study sites. Sites are located on the mid-slope of the river Garonne (mean flow: 600 m^3 s^{-1}), which corresponds to a 6th-order shallow river. During low-water periods, current velocities on the river bottom (<1 m s^{-1}) and mean depths (<1 m) enable the development of an important epilithic biomass. Six sites were studied up- and downstream of Toulouse city, whose distances from the main wastewater treatment plant (550 000 equivalent inhabitants) are respectively: Aouach (U1): –36 km; Pinsaguel (U2): –12 km; Fenouillet (D1): 6 km; Gagnac (D2): 12 km; Bourret (D3): 37 km; and Verdun (D4): 55 km. U1 and U2 are the upstream reference sites, D1 is located at the complete mixing point of the river waters with Toulouse main wastewater treatment plant discharge and D2 is located at the suburbs boundaries. Note that the years of sampling are indicated in subscript, e.g. $D2_{00}$ indicates a sample collected at the second site downstream, Gagnac, in 2000.

Water chemistry. Water samples were collected at the different sites in order to assess nitrogen and phosphorus concentrations. Nitrogen forms were measured according to Rodier (1996) and phosphorus forms were quantified according to Motomizu et al. (1983).

Sampling procedures. Sampling was performed in September 2000 and 2001. For each sampling date, all sites were sampled, except in 2000, where no sample was taken from U1. Nine pebbles (mean diameter: 12 cm) from 3 distinct depths of a transect of the riffle (30, 50 and 70 cm) were sampled and kept in sterile bags at 4°C. Within 6 h following the sampling procedure, biofilm was removed from the upper surface with a toothbrush (treated with NaOH 1 N to remove all trace of DNA) and suspended in filtered water (0.2 µm). The scrubbed surface of the pebbles was traced on tracing paper and its area (m^2) calculated from the mass of the tracings. Except for biomass and activities, all measurements are performed using a mixed suspension from the 9 pebbles biofilms.

Measurement of biomass. For each depth, a 50 ml aliquot of biofilm suspension (about 5 g dry mass) was centrifuged (2300 × *g*, 20 min, SIGMA-202). Dry mass was determined by weighing the dry pellet (80°C, overnight). This pellet was then combusted (500°C, overnight) for the determination of the ash-free dry mass (AFDM).

Nitrification and denitrification rates. The activity measurements were performed *in situ* in benthic chambers for 2001 samples from U1, D2 and D3 according to Teissier & Torre (2002).

Numeration of nitrifying bacteria. Microvolume most probable number (MPN) numerations of NH_4^+-oxidising bacteria for each site were performed according to Teissier et al. (2002) using the medium recommended by Schmidt & Belser (1994).

Faecal coliforms. Appropriate dilutions of homogenised biofilm suspension were inoculated on TTC-tergitol 7 medium (Biokar Diagnostics) and incubated between 18 and 24 h at 44°C. Typical colonies (yellow or orange colonies surrounded by a yellow halo) were counted as characteristics of coliforms and expressed as colony-forming units (CFU).

Bacterial densities. In 2000, the total bacterial densities were measured according to Porter & Feig (1980) using DAPI staining. In 2001, bacteria were counted after staining with a LIVE/DEAD *Bac*Light bacterial viability kit (Molecular Probes) according to Boulos et al. (1999). Counting was carried out on a Leitz Dialux 22 microscope (1250× magnification) fitted for epifluorescence: HBO (Halogen Photo Optic, Osram) 100 W mercury light source, with an excitation filter for 270 to 380 and 450 to 490 nm, and a barrier filter of 410 to 580 nm and a 515 nm cut-off filter for DAPI and LIVE/DEAD *Bac*Light, respectively.

A linear relationship ($y = 1.045x$) was found between LIVE/DEAD *Bac*Light and DAPI staining ($R^2 = 0.997$; $n = 16$).

DNA extraction and purification. DNA extraction was carried out according to Dumestre et al. (2001). Briefly, 10 ml of the biofilm suspension were centrifuged (1500 × *g*, 45 min, 4°C, Jouan K-63F) and the supernatant was discarded. Then 1 ml of lysis buffer (40 mM EDTA, 50 mM Tris-HCl pH 8.3, 0.75 M sucrose) was added to the pellet for storage at −70°C. Biofilm material was incubated with lysozyme (1 mg l^{-1}) for 45 min at 37°C and then with Proteinase K (0.2 mg l^{-1}) and SDS (1%) for 1 h at 55°C. DNA was extracted with phenol:chloroform:isoamyl-alcohol (25:24:1) and chloroform:isoamyl-alcohol (24:1). The aqueous phase was concentrated using a microconcentrator (Amicon, Centricon 100, 100 000 Da cut-off) to remove humic acids. After extraction, DNA concentration was determined by fluorimetry using the DNA Quantitation Kit Fluorescence Assay (Sigma, DNA-QF).

PCR. The variable regions V3 to V5 were amplified using the following primers (Genset): 341F (5′-CCT ACG GGA GGC AGC AG-3′) with a 40 bp GC sequence clamped to its 5′ end (5′-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G-3′) and 907R (5′-CCG TCA ATT CMT TTG AGT TT-3′). This set of primers was designed to be specific for most bacteria (Muyzer et al. 1997). The reaction mixture (50 µl) contained 50 ng microbial DNA, 200 µM of each deoxynucleoside triphosphate, 0.48 µM of each of the primers, 2.5 mM $MgCl_2$, 75 mM Tris-HCl (pH 8.0), 3 mg ml^{-1} BSA and 2.5 U DNA polymerase (Goldstar). The PCR was performed with a T3 thermocycler (Biometra) using a programme described elsewhere (Schauer et al. 2000). PCR products were quantified by agarose gel electrophoresis using Molecular Mass Ruler (BioRad).

DGGE. Vertical DGGE was performed using a D-Code Universal Mutation Detection System (BioRad) as described by Muyzer et al. (1997). The gel contained a gradient of denaturant ranging from 35 to 70% (100% denaturant is 7 M urea and 40% deionised formamide). DGGE was run at 100 V for 18 h at 60°C. After electrophoresis, the gel was stained with 2× SYBR Green I (Sigma) and visualised by UV transillumination. The gel image was captured using a CCD camera and Biocapt software (Vilber Lourmat). Image analysis was done using Bio-1D++ software (Vilber Lourmat), which allows fragment detection and quantification. For each sample, a densitometric profile was generated to determine the relative contribution of each band to the total signal in the sample lane.

Similitude and dendrogram construction. Amplified bands were scored as present or absent. A triangular matrix was constructed from the Jaccard similarity index $J = [c/(a + b - c)] \times 100$, where a is the number of bands of Sample A, b the number of bands of Sample B and c the number of bands that are in common to Samples A and B (Jaccard 1908). A dendrogram was constructed using the unweighted pair-group method of arithmetic averages (UPGMA) (Michener & Sokal 1957).

Diversity indexes. The values of relative intensity of each band were used to calculate the Shannon (H') and Simpson (D) diversity indices:

$$H' = -\sum_{i=1}^{i=n} pi \ln pi \qquad D = \sum_{i=1}^{i=n} pi^2$$

where n is the number of bands in the sample and pi the relative intensity of the ith band (Simpson 1949, Shannon & Weaver 1963).

RESULTS

Water chemistry and quality

Differences in water quality were recorded up- and downstream of Toulouse. In 2001, the mean concentrations, taken over 12 wk before biofilm sampling, of the different forms of nitrogen and phosphorus were about 2- to 10-fold higher at D2 than at U1 (Table 1).

Parameters of the biofilms

In both 2000 and 2001, biofilms exhibited similar values of AFDM ranging from 18 to 27 g m^{-2} in 2000

Table 1. Concentrations (mg l^{-1} ; mean ± SE) of nitrogen and phosphorus forms at 2 sites (U1 and D2) in the river Garonne during the 12 wk before biofilm sampling in 2001

	U1	D2
NH_4^+-N	0.029 ± 0.008	0.252 ± 0.079
NO_2^--N	0.020 ± 0.005	0.119 ± 0.034
NO_3^--N	0.737 ± 0.024	1.642 ± 0.097
PO_4^{3-}-P	0.009 ± 0.001	0.093 ± 0.008
Total P	0.030 ± 0.003	0.123 ± 0.007

Table 2. Ash-free dry mass (AFDM ± SE) and bacterial densities of epilithic biofilms collected in 2000

Site	AFDM (g m^{-2})	Bacterial densities (10^{12} cells m^{-2})
$U2_{00}$	27 ± 3	3
$D1_{00}$	21 ± 20	5
$D2_{00}$	18 ± 7	10
$D3_{00}$	23 ± 7	4
$D4_{00}$	18 ± 3	8

Table 3. Ash-free dry mass (AFDM ± SE), bacterial densities, NH_4^+-oxidising and faecal coliforms recorded on epilithic biofilms collected in 2001. The recorded values of nitrification (mean of light and dark values) and denitrification activities are given for the samples $U1_{01}$, $D2_{01}$ and $D3_{01}$. na: not available

Site	AFDM ($g\ m^{-2}$)	Bacterial densities (10^{12} cells m^{-2})	NH_4^+-oxidising bacteria (10^9 MPN m^{-2})	Faecal coliforms (10^6 CFU m^{-2})	Nitrification rates (mg N $m^{-2}h^{-1}$)	Denitrification rates (mg N m^{-2} h^{-1})
$U1_{01}$	16 ± 2	5	418	2	3.6	0.3
$U2_{01}$	15 ± 4	11	184	9	na	na
$D1_{01}$	12 ± 3	4	145	6	na	na
$D2_{01}$	10 ± 2	4	58	8	3.9	1.1
$D3_{01}$	13 ± 10	3	6	1	3.1	0.4
$D4_{01}$	10 ± 3	3	8	6	na	na

(Table 2) and from 10 to 16 g m^{-2} in 2001 (Table 3). Bacterial densities were similar, ranging from 3 to 11 × 10^{12} cells m^{-2} for both years.

In 2001 (Table 3), high MPN counts of NH_4^+-oxidising bacteria were found (>8 × 10^9 MPN m^{-2}), and faecal coliforms densities remained rather stable (between 1 and 9 × 10^6 CFU m^{-2}) along the longitudinal transect.

The values measured for the nitrification rates in light and dark conditions (about 3.6 mg N m^{-2} h^{-1}) were similar for all of the samples. Denitrification rates in dark conditions ranged from 0.3 to 1.1 mg N m^{-2} h^{-1} (Table 3).

DGGE analysis

Reproducibility

Reproducibility between the different phases of the process was assessed (extraction, amplification and DGGE) and gave respectively similitude values of 97, 97 and 90%. Reproducibility of the sampling phase was assessed by comparing 3 replicates of sampling for 3 samples. For the 3 tested samples, similar patterns were obtained for the sampling replicates (Fig. 1). The percentage of similitude for these replicates, calculated from the binary matrix using the distance of Jaccard, ranged from 84 to 92%.

Application of DGGE on natural samples

Seventy-four different bands were detected for the 11 samples collected in 2000 and 2001 (Fig. 2), and the number of bands per samples varied from 34 to 45 in each year. For the samples taken in 2000, 61 bands were detected; 19 of them were present in all of the 5 samples, and 9 in only 1 sample, thus being specific of these samples. In 2001, 62 different bands were detected; 17 were present in the 6 samples, and 9 in only 1 sample.

In all cases (419), the bands exhibited relative intensities lower than 10%; 28 bands had intensities lower than 1% and most bands (255/419) showed intensities ranging from 1 to 3%. However, for each sample, 21 to 28 bands (corresponding to 63% of the detected bands) accounted for about 80% of the total intensity of a lane.

The relative intensities of the bands were used to calculate diversity indices (Fig. 2). Similar values were found between up- and downstream samples; the mean values are about 0.034 and 5.06 for the Simpson and Shannon indices respectively.

The range of similitude values was very similar between years 2000 and 2001 (45 to 82 and 37 to 81% respectively).

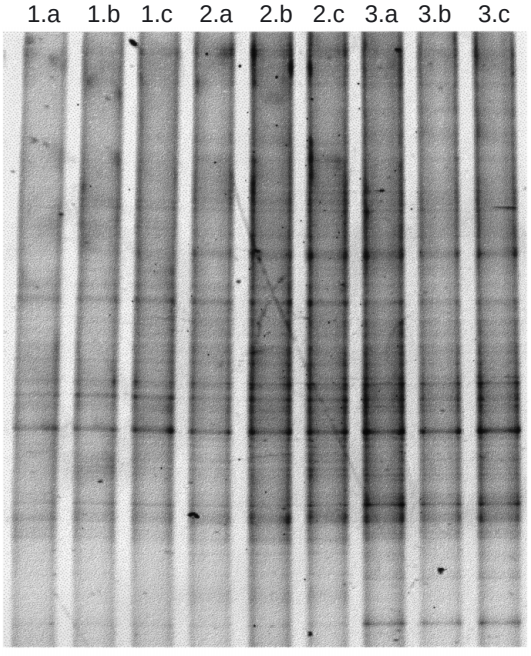
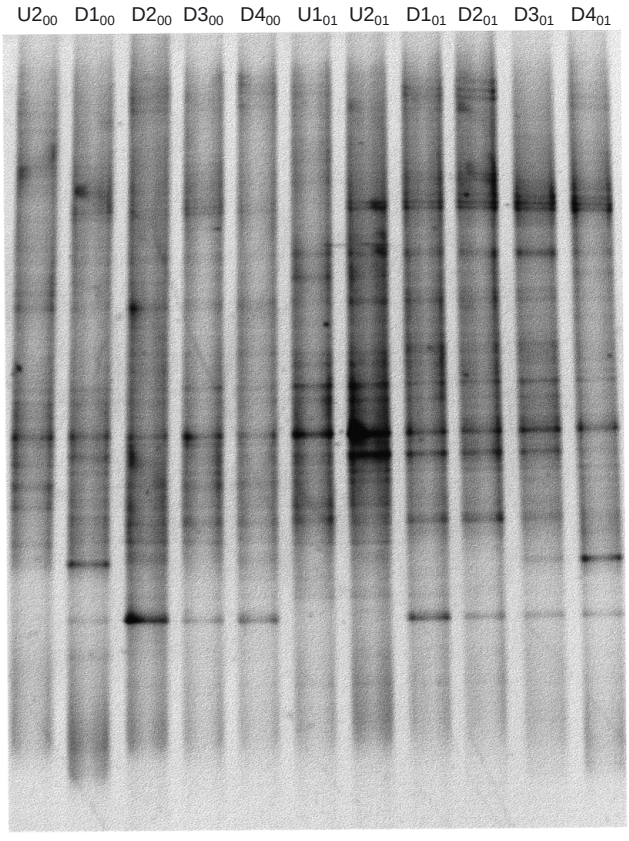


Fig. 1. Negative image of the DGGE gel obtained for the triplicates (a, b and c) of sampling. The 3 samples (1, 2 and 3) were collected at Site D2 in July 2000



Nb of bands	36	37	45	40	40	38	43	41	34	32	33
Simpson	0.034	0.033	0.030	0.031	0.033	0.034	0.032	0.032	0.035	0.038	0.037
Shannon	5.01	5.04	5.27	5.16	5.10	5.05	5.18	5.16	4.95	4.84	4.90

Fig. 2. Negative image of the DGGE gel for biofilm samples taken in 2000 and 2001 at the different sites of the river Garonne. Sample labels are explained in 'Materials and methods'

Taking into account that no samples were collected from Site U1 in 2000, the 2 dendrograms are very similar (Fig. 3a,b). In 2000, U2$_{00}$ can be separated from a cluster of the downstream samples (47% similarity), while up- and downstream sample clusters can be separated in 2001 (41% similarity). Moreover, a similar pattern in downstream sample clustering is observed in both 2000 and 2001.

DISCUSSION

Methodological details

In the present work, a relationship between recorded trends in epilithic bacterial responses and bacterial diversity was checked using PCR-DGGE. Applied to the study of unknown natural communities, fingerprints of a 16S rDNA region provide information on the dominant ribotypes of the community (Muyzer & Smalla 1998), while the top-to-bottom approach generally developed with FISH probing (Amann et al. 1995) often targets higher taxonomic levels. A more accurate description of the community structure is thus expected by fingerprinting (Araya et al. 2003) and in our case, a better discrimination between up- and downstream communities was aimed for.

PCR-DGGE is a method that has been widely used to study complex bacterial assemblages in natural environments (Muyzer 1999). However, sampling reproducibility appeared to be a factor that should be checked prior to monitoring the influence of point source anthropic discharges on spatial variability of epilithic bacterial communities. The samples that we intended to compare were processed at the same time (PCR mix and amplification) and were loaded in the same gel because the reproducibility between DGGE gels has been questioned (Moeseneder et al. 1999). As compared to similitude values calculated for sampling replicates (84 to 92%), the range of similitude values obtained for samples from different sites (82 to 37%) allows us to conclude that the differences observed between banding patterns can be related to sample characteristics.

We found that up- and downstream community patterns differentiate, while total bacterial densities, NH_4^+-oxidising bacteria and faecal coliforms densities, nitrification and denitrification rates were relatively homogeneous over the river reach. The same

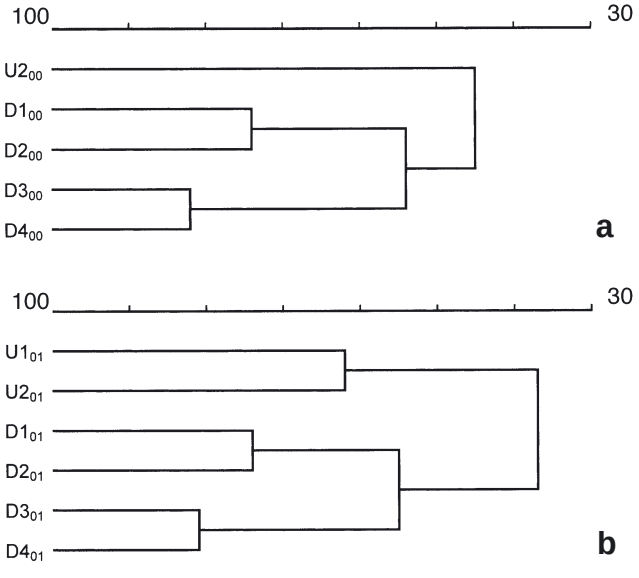


Fig. 3. UPGMA Jaccard distances dendrogram generated from the DGGE profiles of the biofilm samples collected (a) in 2000 and (b) in 2001 in the river Garonne

kind of results has been found with benthic algal communities where water nutrient concentrations did not modify quantitative community descriptors, but affected the algal community structure instead (Stelzer & Lamberti 2001, Griffith et al. 2002). Thus, it can be suspected that changes detected in the DGGE fingerprinting would be caused by changes in the algal community since the PCR primers used are known to amplify plastidial DNA (Schauer et al. 2000). However, when analysing bacterial diversity of epilithic biofilms by the cloning technique, O' Sullivan et al. (2002) did not detect the presence of any sequences belonging to plastidial DNA using primers that proved to amplify plastidial 16S rDNA (Marchesi et al. 1998). The DGGE patterns obtained should thus be considered to reflect the bacterial community.

Global diversity analysis

As an estimate of bacterial richness, a higher number of bands or operational taxonomic units (OTUs) was found (38 bands on average per sample) compared to those reported for similar assemblages using the same fingerprinting method: 7 to 24 OTUs were found for epilithic biofilms grown in mesocosms (Jackson et al. 2001) and about 20 OTUs for epilithic biofilm grown on artificial substrata in a river (Araya et al. 2003). However, the highest richness recorded in this study may come from the origin of samples, i.e. pebbles in a natural system, suggesting that the actual epilithic bacterial diversity may be distorted by using artificial substrata as stated for algae by Cattaneo & Amireault (1992).

The use of band relative intensity to calculate diversity indices is controversial, but Nübel et al. (1999) provided some evidence that comparable diversity indices were found between values derived from PCR-DGGE and morphotypes. In the studied set, calculated diversity indices (Shannon and Simpson) compared with values reported for diversified and balanced bacterial communities (Nübel et al. 1999, Schauer et al. 2000). Moreover, comparable values calculated for up- and downstream sites highlight bacterial communities' ability to remain diversified even in the presence of anthropic disturbances.

Influence of anthropic discharges

In the present study, epilithic biomass descriptors show equivalent values over the studied reach conflicting with previous findings (Teissier et al. 2002) where enhanced epilithic biomass was recorded downstream of a great centre. Obviously, these differences may depend on whether sampling occurs closely after a high-flow period (Teissier et al. 2002) or after a long period of low and stable flow (this study). During the accrual phase that follows the flood, enhanced epilithic biomass may occur downstream of anthropic discharges due to the stimulation of the accrual rate by trophic inputs (Biggs 1996). Conversely, after a long low-flow period, biomass may have reached stability (stationary phase) over the whole river section. Epilithic biomass is known to control bacterial densities (Sobczak 1996), activities (Teissier et al. 2002) and diversity (Jackson et al. 2001). Therefore, biomass stability over the studied section might explain why no differences were found between up- and downstream bacterial densities (total and NH_4^+-oxidising bacteria), nitrification and denitrification rates.

If epilithic biomass roughly describes biofilm maturation stage (Lock 1993), then the accordance between homogeneous biomass and the important number of ubiquitous taxa (50 taxa among 74) agrees with the diversity model proposed by Jackson et al. (2001).

Ubiquity of bacterial populations has already been mentioned in the literature for microbial assemblages (Gillan et al. 1998, Brümmer et al. 2000, Schauer et al. 2000) and is supposed to be responsible for the maintenance of stability in systems (Lindström 1998). For instance, in our case, the maintenance of activity rates such as nitrification and denitrification may be due to this stability. This may be emphasised in biofilm assemblages that represents an individualised community partly preserved from conditions prevailing in the overlying water (Costerton 2000).

On the other hand, for both years, indicating the same trends from one year to the next, $\frac{1}{3}$ ($\frac{24}{74}$) of the detected taxa discriminates between (<45% similarity) up- and downstream biofilms. As known for most animal and vegetal communities, bands discriminating between up- and downstream communities may behave as bio-indicative taxa of changes in water quality up- and downstream of the urban centre. This suggests that epilithic bacterial communities are not completely governed by interactions between organisms and resources within the assemblage, but also react to environmental fluctuations.

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