

## Autogenic versus environmental control during development of river biofilm

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**Abstract** – In the natural environment, microbial community structure of river biofilm is controlled by biotic and abiotic factors. This study explored the capacity to manipulate the structure of microbial communities by modifying environmental conditions during the course of biofilm development. River epilithic biofilm was cultivated *in situ* on artificial substrates placed parallel to river water flow. Substrates were incubated for 3 and 5.5 weeks in river to allow natural biofilm development, at two sites with contrasting physico-chemical characteristics. The first site (Auradé, Gers, France) was located in an agricultural watershed basin and the second site (Larroque, Haute-Garonne, France) was located in a forested watershed basin. After 3 weeks of biofilm development, a subset of substrates was collected from one site and transplanted to the second site where they remained for 2.5 further weeks. Epilithic bacterial community structure (at 3 weeks from each site and at 5.5 weeks from biofilms with and without transplantation) was assessed using PCR-DGGE of 16S rDNA fragment. Biofilm biomass was estimated using ash free dry mass (AFDM). After 3 weeks of development, biofilms from the two sites exhibited comparable AFDM values (average of  $1.4 \pm 0.2 \text{ g.m}^{-2}$ ). A difference between the two sites was observed after 5.5 weeks of development: AFDM decreased for biofilms from the agricultural watershed basin (from 1.4 to  $0.18 \text{ g.m}^{-2}$ ) as a consequence of grazing pressure (*Bithynia*), and increased for biofilms from the forested agricultural watershed (from 1.4 to  $2.6 \text{ g.m}^{-2}$ ). Microbial community analyses revealed a differentiated community structure between biofilms from the different sites and exhibited a change of microbial community structure after 5.5 weeks of biofilm development. These observations confirm a process of ecological succession in microbial communities. Changing the incubation site during biofilm development modified the trajectory of these ecological successions, suggesting that site characteristics mainly conditioned the structure of these microbial communities.

**Key words:** Colonization experiment / community structure / grazing / microbial ecology / succession

### Introduction

Epilithic biofilms are microbial aggregates constituted by heterotrophic micro- and meio-organisms (*e.g.*, bacteria, protozoan) and phototrophic micro-organisms (*e.g.*, diatoms, cyanobacteria), embedded in a exopolymeric substances matrix secreted by the microorganisms (Lock *et al.*, 1984). Epilithic biofilm development occurs at the interface between river bed substrates (pebbles) and the water column where hydrodynamics, light and geomorphological characteristics favor the development of a sessile biomass (Wetzel, 1983). Typically, epilithic biomass is dominated by the algal component of the community (Peterson, 1996). In lotic systems, epilithic biofilms

represent a major compartment involved in primary production (Dodds, 2006), mineralization and element recycling processes (*e.g.*, dissolved organic carbon dynamics, Romani *et al.*, 2004) and participate in autoepuration (Sabater *et al.*, 2002; Teissier *et al.*, 2007) and biodegradation of pollutants in aquatic environments (Lawrence *et al.*, 2001; Sabater *et al.*, 2007).

Epilithic biofilm functioning is conditioned by the community structure and diversity. Overall, epilithic biofilm structure can be influenced by (i) abiotic parameters such as water temperature (DeNicola, 1996), nutrient availability (Bothwell, 1993; Della Bella *et al.*, 2007), substrate types (Murdock and Dodds, 2007), hydrodynamics (Biggs, 1996; Battin *et al.*, 2003), light (Wetzel, 1983; Boston and Hill, 1991; Hill, 1996) or pollutants (Lawrence *et al.*, 2005; Tornes *et al.*, 2007; Morin *et al.*,

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2008; Tlili *et al.*, 2008); and by (ii) biotic interactions such as predation and competition (Bourassa and Cattaneo, 1998; Jackson *et al.*, 2001).

Epilithic biofilm development was demonstrated to be associated with population succession processes, both for the algal and the bacterial compartments. Colonization of bare substrates is completed by early-colonizing diatoms which will modify the microenvironment and favour the settlement and colonization of other communities (Stevenson, 1983). However, some environmental conditions can favour initial colonization of green algae (high light intensities) or of heterotrophic bacteria (low light intensities) (Roeselers *et al.*, 2007). After initial colonization, succession of diatom populations associated with the biofilm development was recorded (Eulin and Le Cohu, 1998). Succession processes were also demonstrated for epilithic bacterial communities and were associated to biofilm maturation (Manz *et al.*, 1999; Jackson *et al.*, 2001; Araya *et al.*, 2003). Biofilm maturation can ultimately promote the occurrence of micro-niches within the aggregate that will favour the development of populations involved processes such as denitrification (Lyautey *et al.*, 2005a). According to a succession model, late development stages are associated with a decrease of the exchanges between the water column and the biofilm, and with an increase of species interaction within the aggregate (Jackson, 2003). The main interaction is competition, which results in a decrease of the number of new species added to the community in late development stages (Santegoeds *et al.*, 1998; Jackson, 2003; Lyautey *et al.*, 2005a).

Because of its ubiquitous character, short generation time, sessile nature, and rapid response to changes in environmental conditions, epilithic biofilms are widely used as bioindicators of water quality in lotic systems (Burns and Ryder, 2001). The algal compartment, and more especially diatoms, is used to assess water quality using the Diatoms Biological Index (Prygiel and Coste, 1993). Bacteria are also sensitive to environmental or human-induced perturbations. A spatial heterogeneity of bacterial communities was recently reported along an upland river gradient, and was related to temperature and pH variations (Anderson-Glenna *et al.*, 2008). Seasonal variations of environmental parameters proved to cause recurrent changes in community structure (Hullar *et al.*, 2006). Anthropogenic perturbations also proved to influence bacterial community structures (Lyautey *et al.*, 2003; Brümmer *et al.*, 2004).

In the present work, we conducted a 5.5 weeks colonization experiment: epilithic biofilm development was carried out on artificial substrates at two sites presenting contrasting environmental conditions. After 3 weeks of development, epilithic biofilms were either left for 2.5 more weeks at the same site, or were transplanted to the other study site where they were left for 2.5 more weeks of development. Bacterial community composition was assessed using 16S rDNA based PCR-DGGE on 3 and 5.5 weeks old biofilms from transplanted and unmoved communities. The objectives of this study were to (i) assess

bacterial community structure changes during biofilm development; (ii) compare bacterial community structures at two contrasting sites; and (iii) evaluate the influence of a transplantation during biofilm development on bacterial communities. The transplantation process was intended to assess the relative influence of autogenic (succession) and allogenic (environmental conditions) parameters on epilithic bacterial communities.

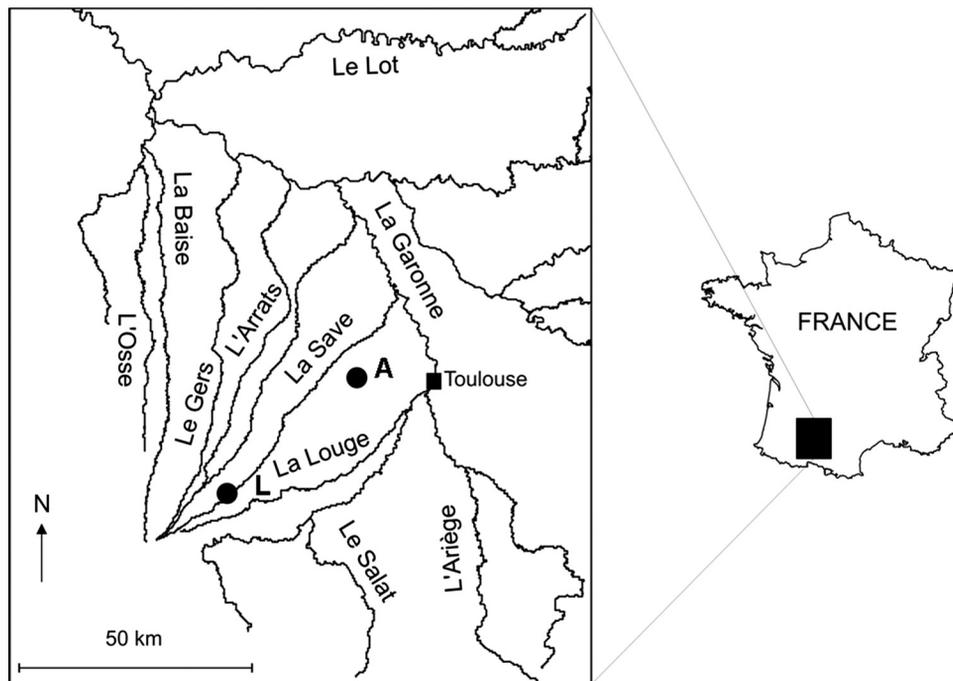
## Materials and methods

### Study sites

Two sites (sites L and A) exhibiting different physico-chemical parameters were chosen to carry out *in situ* epilithic biofilm cultures (Fig. 1). Site L on the Save River is located in a forested watershed basin. The study site is located upstream of Save Gorges and at 2 km of the city of Larroque (France) (43°11'43.77"N/00°36'28.59"E). The basin is dominated by forested and pasturage landscapes. The river bed is very rocky and wide 6.7 m. During biofilm development, the average water depth was of 50 cm and the flow velocity (flow velocity meter, Flo-mate model 2000) was about 1 m.s<sup>-1</sup> during low water periods and 3 m.s<sup>-1</sup> during high water periods. Site A on the Montoussé stream is located in an experimental agricultural watershed basin outlet (328 ha) of Auradé city (France) (43°33'55.06"N/1°3'30.92"E). The area is dominated by an agricultural landscape and reported as highly contaminated by pesticides and nitrate. The main cultivated crops are wheat and sunflower. The river bed is sedimentary, confined (1.5 m wide), and woody. Water depths (50 cm) and flow velocity (0.045 m.s<sup>-1</sup>) were constant during the biofilm development period.

### Experimental protocol and sample collection

Biofilm *in situ* colonization was carried out between March 22 and April 30, 2007 to cover the pesticide application period in the experimental agricultural watershed basin. Artificial substrates were immersed in river water to allow *in situ* epilithic biofilm colonization and development. The use of artificial substrates permitted (i) to reduce the subjectivity of sample collection; (ii) to ease biofilm scraping; and (iii) to compare two stations with different physico-chemical characteristics (Cattaneo and Amireault, 1992). Moreover, no natural substrate was available at site A. Cleaned and smooth glass slides (244 × 78 mm) were chosen as uniform artificial substrates. Glass slides were supported vertically within plastic trays (400 × 300 × 100 mm) and recovered by a 5-mm mesh wire fence, as described by Morin *et al.* (2008). Every tray was made up of four glass slides which represented four replicates. Plastic trays were submerged about 20 cm below the water surface, and placed parallel to water flow, in order to avoid sedimentary deposit on glass slides and to promote micro-organisms fixation. Biofilm colonization



**Fig. 1.** Location of the two sites (black dots) chosen for *in situ* epilithic biofilm cultures. Site L is located in a forested watershed and site A is located in an agricultural watershed.

was performed as follows: three glass slide tray tracks were placed at each of the two sites. For each site, four glass slides were sampled randomly after 3 weeks of biofilm development, and four glass slides were sampled after 5.5 weeks of biofilm development. The last set of four glass slides for which biofilm has initially developed for 3 weeks at one site were removed from their site of origin and transplanted in the other site where biofilm was left for 2.5 further weeks of development. In summary, experimental conditions included two study sites with different environmental conditions, two incubation period lengths (3 and 5.5 weeks, A and AA for site A, and L and LL for site L, respectively) and a transplantation of the biofilms during development (LA: from site L to site A, and AL: from site A to site L). After collection, glass slides were transported to the laboratory in cool bottles within moisturized garbage bags. Biofilm was removed from glass slides by scraping with a toothbrush, previously treated with NaOH 1 N in order to avoid all trace of DNA and by scraping with a microscope blade. Biofilm was suspended in 90 mL of river water previously filtered through a 0.2  $\mu\text{m}$  pore size filter (cellulose acetate membrane, Whatman) and homogenised (13 500 rpm, Ultra Turrax, T25). Biofilm suspension was aliquoted for further analyses.

### Physico-chemical characteristics analysis

Water physico-chemical characteristics of each study site were estimated during the biofilm development period. Temperature, conductivity, dissolved oxygen concentration and pH were measured *in situ* using specific electrodes. Conductivity and pH values were measured with a

conductimeter Hanna HI 99 1300 and a pH meter 320 WTV (electrodes Sentix41), respectively. Dissolved oxygen concentration values were determined with an Oxi323 oxymeter (electrodes oxical-S) and temperature values were represented by the mean values that were determined by pH meter and oxymeter. In parallel, water samples were collected to assess the water quality using the following parameters: nitrate ( $\text{NO}_3^-$ ), total phosphorus and ammonium ( $\text{N-NH}_4^+$ ) concentrations. These parameters were measured by classic colorimetric methods (Secoman, Uvi Light, XT5) according to standard methods (APHA, 1992). Ammonium concentrations were determined within 10 h following sampling. Dissolved organic carbon (DOC) concentrations were determined in water filtered through a 0.45  $\mu\text{m}$  pore size filter (cellulose acetate membrane, 25 mm diameter, Whatman) and analyzed using a platinum catalyzer at 680  $^\circ\text{C}$  (Shimadzu, Model TOC 5000). All measures and sampling were performed in the morning to allow homogeneity between samples and once or twice a week. Water samples were refrigerated during transport to the laboratory. Twenty three pesticides, commonly used in the southwest of France, among which 17 herbicides (Aclonifen, Atrazine, Atrazine desethyl, Chlorotoluron, Cyanazine, Hexazinone, Imazathabenz-methyl, Isoproturon, Linuron, Metazachlor, Metolachlor, Metoxuron, Monolinuron, Sebuthylazine, Simazine, Terbutylazine, Trifluralin) and six fungicides (Cyproconazol, Epoxiconazol, Fenpropimorph, Flusilazol, Pendimethalin, Tebuconazol) were quantified at three times during biofilm development: T0 corresponds to the time of artificial substrates positioning, T3 and T5 correspond to 3 and 5.5 weeks of colonization, respectively. Three liters of water were collected in closed glasses bottles previously

cleaned and sterilized by autoclaving. For every sample, 10 mL of dichloromethane were added to 1.5 L of water. Then, samples were kept in a cold chamber at 4 °C. Pesticides were extracted from water using dichloromethane, and pesticides levels were quantified by Gas Chromatography Mass Spectrometer (GC-MS, Thermo Fisher, Model Trace DSQ), according to [Devault \*et al.\* \(2007\)](#).

### Biomass determination

Dry mass (DM) was measured by weighing the dried pellet (24 h at 80 °C) from an aliquot of 10 mL of biofilm suspension (centrifuged at 3500× *g* for 25 min, Heraeus Function Line). The pellet was subsequently combusted (8 h at 550 °C) to provide the ash free dry mass (AFDM). Another 10-mL aliquot of the biofilm suspension was centrifuged (12000× *g*, 20 min, 4 °C). After removing the supernatant, Chlorophyll *a* content of the pellet was determined following an extraction in 90% acetone by spectrophotometry according to [SCOR-Unesco \(1966\)](#). Autotrophic index (AI) was defined as the ratio between AFDM and Chlorophyll *a* ([APHA, 1992](#); [Steinman and Lamberti, 1996](#)), and indicates the relative importance of autotrophic organisms *versus* heterotrophic organisms and detritus. For each sample, the ratio between AFDM and DM was determined to indicate the relative importance of organic fraction in the biofilm.

### Microbial community structure

After centrifugation (12000× *g* at 4 °C for 20 min, Heraeus Multifuge) of an aliquot of 10 mL of the initial biofilm suspension, the pellet was stored at –80 °C until further analysis. Genomic DNA extraction was performed on the pellet using Ultra Clean TM Soil DNA Isolation kit according to the manufacturer's protocol (Mobio Laboratories). The extracted DNA concentration was quantified by fluorimetry (Fluoroscan Ascent, Labsystem) using SYBR green (Sigma Aldrich). The 16S rDNA variable regions V3 to V5 were amplified using primers (Proligos) described as universal within the Bacteria domain: 341F (5'-CCTACGGGAGGCAGCAG-3') with a 40 bp GC sequence clamped at its 5' end (5'-CGCCCGCCGCGC-CCCGCGCCCGTCCCGCCGCCCGCCCG-3') and 907F (5'-CCGTCAATTCMTTGTGAGTTT-3') ([Muyzer and Smalla, 1998](#)). Amplification was carried out using an Eppendorf Mastercycler following a protocol described elsewhere ([Lyautey \*et al.\*, 2005b](#)) using 20 ng of extracted DNA as template for the PCR. Three replicate amplifications were performed for each sample. Amplified product concentrations were quantified on a 1.65% agarose gel (Eurogentec) using precision Molecular Mass Ruler (BioRad) as described previously ([Lyautey \*et al.\*, 2005b](#)). DGGE was carried out using D-Code Universal Mutation Detection System (BioRad). An amount of 700 ng (approximately 234 ng from each of the three sample replicates) of PCR products were loaded onto an

acrilamide gel containing a gradient of denaturant ranging from 35 to 70% (100% denaturant is 7 M urea and 40% deionized formamide). For practical purposes (only 20 samples can be processed on each DGGE gel), the four replicates of the six samples were processed on two different DGGE gels (two replicates of each sample on each DGGE gel). The replicate 2 from 5.5 week biofilm sample of site L (LL2) exhibited in the DGGE analysis a number of bands drastically lower (< 10 OTUs) than the three other replicates suggesting that a methodological problem happened with this sample, either during DNA extraction or DNA amplification. Data from this sample were thus not included in the analysis. Electrophoresis was performed at 100 V for 18 h at 60 °C. The gels were stained with SYBR Green (Sigma Aldrich) for 30 min. The gel image was captured using a CCD camera and Biocapt Software (Vilbert Lourmat) and analyzed using BioNumerics 5.1 software (Applied Maths, Sint-Martens-Latem, Belgium).

### Data analysis

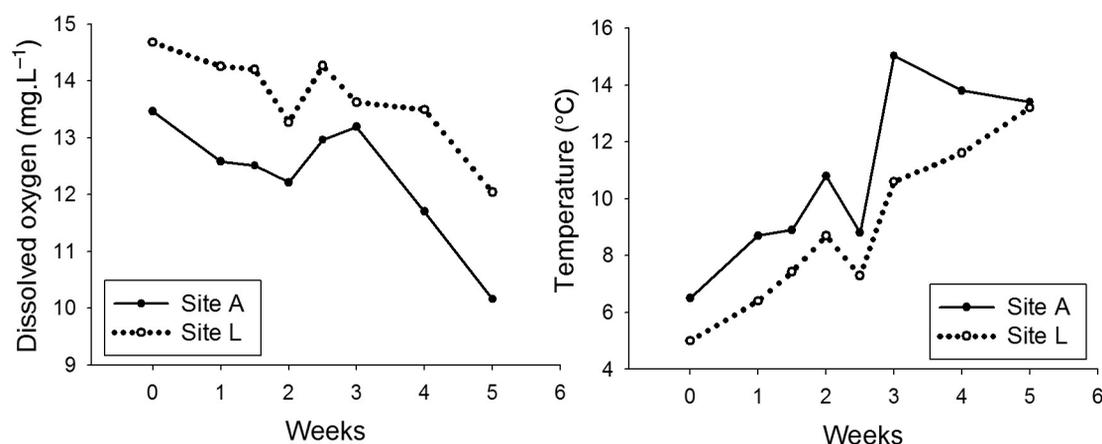
In order to avoid methodological biases due to DGGE intergel variability, each gel were analyzed separately. DGGE bands (defined as operational taxonomic units (OTUs)) were scored as present or absent from DGGE gel analysis. A matrix was constructed from the Jaccard similarity index ( $J = c/(a + b + c)$  where *a*, the number of bands found only in sample A, *b*, the number of bands found only in sample B and *c*, the number of bands shared between samples A and B). To assess changes in the microbial community structure during biofilm development, DGGE patterns were analyzed by non-metric multidimensional scaling (NMDS) analysis as described elsewhere (Van [Hannen \*et al.\*, 1999](#)). NMDS analysis is a mathematical technique which provides a graphical representation of every band pattern (sample) as one plot where relative changes in community structure can be interpreted as distances between the plots. The closer the plots are to each other, the more similar are the DGGE banding patterns. NMDS was carried out using SPSS 13.0 software for Windows (SPSS Inc., Chicago, IL, USA) using a stress value < 0.1.

The difference in physical chemical characteristics between stations and the difference in DM, AFDM, Chlorophyll *a*, number of bands on DGGE banding patterns between biofilm samples were assessed with the Kruskal Wallis test using SPSS 13.0 software for Windows. Differences were considered statistically different at  $P < 0.05$ .

## Results

### Water physico-chemical parameters

For each site, water physico-chemical parameters values presented below represent average value



**Fig. 2.** Weekly evolution of dissolved oxygen concentrations and temperatures for site A (black dots) and site L (white dots) during the period of *in situ* biofilm colonization. “Week = 0” indicates the date where artificial substrates were placed *in situ* (March 22, 2007).

**Table 1.** Cumulated pesticide concentrations measured in river water from study sites A and L for three dates (T0, T3, and T5). T0 corresponds to the time of artificial substrates positioning, T3 and T5 correspond to 3 and 5.5 weeks of colonization, respectively. All concentrations are expressed in  $\mu\text{g.L}^{-1}$ .

	T0	T3	T5
A	1.02	3.03	4.78
L	3.59	3.75	2.17

( $\pm$  standard deviation) from the eight water samples collected during biofilm development (Fig. 2). Water temperatures were comparable between site A ( $10.9 \pm 3^\circ\text{C}$ ) and site L ( $8.8 \pm 2.8^\circ\text{C}$ ) ( $P = 0.128$ ). Dissolved oxygen concentrations measured at site A ( $12.3 \pm 1 \text{ mg.L}^{-1}$ ) were significantly lower than at site L ( $13.7 \pm 0.8 \text{ mg.L}^{-1}$ ) ( $P < 0.01$ ). For both sites, temperature increased and dissolved oxygen concentrations decreased during the experimental period, in accordance to seasonal variations. pH and conductivity values remained constant for both sites over the sampling period, but were significantly different between sites. pH values were above 7 for site A ( $7.6 \pm 0.1$ ) and were lower for site L ( $6.2 \pm 0.4$ ) ( $P < 0.001$ ). Conductivity was higher at site A ( $725 \pm 8 \mu\text{S.cm}^{-1}$ ) than at site L ( $128 \pm 4 \mu\text{S.cm}^{-1}$ ) ( $P < 0.001$ ). Site A is located in a calcareous watershed basin which explained conductivity and pH values. Nitrate concentrations remained constant over time for both sites with higher  $\text{NO}_3^-$  values recorded for site A ( $41 \pm 1.2 \text{ mg.L}^{-1}$ ) than for site L ( $7 \pm 0.6 \text{ mg.L}^{-1}$ ) ( $P < 0.001$ ). No difference was observed for  $\text{N-NH}_4^+$  concentration values between site A ( $16 \pm 4 \mu\text{g.L}^{-1}$ ) and site L ( $25 \pm 12 \mu\text{g.L}^{-1}$ ) ( $P = 0.382$ ). Total phosphorus concentrations were significantly lower for site A ( $17 \pm 5 \mu\text{g.L}^{-1}$ ) than for site L ( $63 \pm 34 \mu\text{g.L}^{-1}$ ) ( $P < 0.001$ ). Two flood events at site L were recorded during the colonization period. DOC concentrations available were obtained for site A in 2008 (from March to April,  $n = 10$ ) and for site L in 2004 (from February to May,  $n = 6$ ). DOC concentrations were higher at site A ( $2.25 \pm 0.07 \text{ mg.L}^{-1}$ ) than at site L ( $1.32 \pm 0.08 \text{ mg.L}^{-1}$ ) ( $P < 0.05$ ).

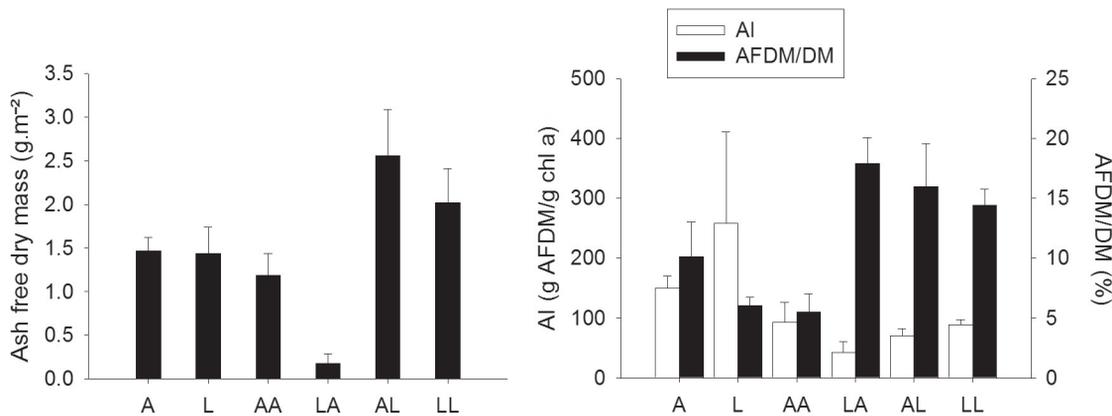
Thirteen of the 23 pesticides tested were detected with a concentration above  $0.1 \mu\text{g.L}^{-1}$  at least once during the study period. Cumulated pesticide concentrations are presented in Table 1. Contamination levels by pesticides on both sites were from 1 to  $5 \mu\text{g.L}^{-1}$  during biofilm development. Concentrations were in the same order of magnitude for both sites at each period.

### Biomass descriptors

AFDM values ranged from  $0.18$  to  $2.6 \text{ g.m}^{-2}$  (Fig. 3). After 3 weeks of development, biofilms from sites L and A exhibited comparable AFDM values around  $1.4 \text{ g.m}^{-2}$  ( $P = 0.773$ ). After 5.5 weeks of development, biofilms from site L (AL and LL) (up to  $2.6 \text{ g.m}^{-2}$ ) exhibited higher AFDM levels than 5.5-weeks biofilms from site A (LA and AA) (down to  $0.18 \text{ g.m}^{-2}$ ) ( $P < 0.001$ ). At site L, important biomass were consistent with the growth process and biofilm accretion, whereas the low biomass recorded at site A were consistent with the occurrence of grazing as revealed by the presence of several *Bithynia* molluscs (*Prosobranchia*, *Bithyniidae*) on glass slides. AFDM/DM ratio values ranged from 6.0 to 17.8%. Low values reveal a biofilm rich in detritus and sedimentary particles. These sedimentary particles are likely to have been imported by the two flood events observed in site L and by the presence of sediments on river bed in site A. Important standard deviation values were caused by the presence of filamentous algae which decreased the homogeneity of biofilm samples. The autotrophic index (AI) values ranged between 42 and 258. Three weeks old biofilms exhibited higher AI levels than 5.5 weeks old biofilms ( $P < 0.001$ ), indicating that late stages were essentially constituted by autotrophic organisms.

### Microbial community structure

The number of bands (OTUs) obtained for each experimental conditions are presented in Table 2. Analysis



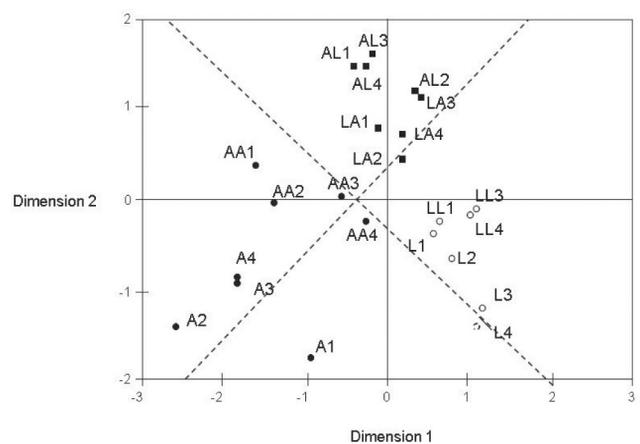
**Fig. 3.** Average  $\pm$  SD value plots of ash free dry mass (AFDM), autotrophic index (AI, white bars) and AFDM/DM ratio (black bars) for biofilm samples according to the different experimental conditions: A, L, AA, LA, AL and LL (refer to materials and methods for the letters meaning).

**Table 2.** Number of bands (OTUs) obtained from DGGE pattern analysis for different experimental conditions (A, L, AA, LL, AL and LA, refer to materials and methods for the letters meaning). Four replicates (numbers 1 to 4) were analyzed for each condition.

	A	L	AA	LL	AL	LA
1	58	52	57	61	54	54
2	56	60	51	–	54	51
3	36	33	39	40	48	37
4	34	31	42	39	48	38

of DGGE banding patterns revealed a total of 66 and 94 OTUs for gel 1 (replicates numbers 3 and 4 of each samples) and gel 2 (replicates 1 and 2), respectively. The average number of OTUs per sample varied from 34 and 56 for communities from 3-week biofilm to 40 and 55 for 5.5-week biofilm communities, for gel 1 and gel 2, respectively, indicating a constant richness along with biofilm maturation ( $P=0.371$ ) and the transplanted experiment ( $P=0.752$ ). Along with biofilm development new species appeared in the communities. An average of 15 OTUs appeared at site A between 3 week old and 5.5 week old communities and an average of 12.5 OTUs appeared at site L. At the same time, the disappearance of OTUs along with the aggregate development was observed, with an average number of 9 OTUs disappearing for both sites. The transplanted experiment also induced the appearance of news OTUs: 12.5 at site A and 10 at site L. Mature biofilms both from the transplanted communities and from the untransplanted biofilms only shared six common OTUs.

NMDS analysis was carried out on the presence-absence matrix separately for each DGGE gel following by a superposition of both dimension representation plots (Fig. 4). NMDS takes the community-level similarity of the samples. The stress values of final configuration were 0.123 and 0.105 and the proportions of variance explained were 0.93 and 0.94. The proximity between replicates on the plot revealed an important similarity of the DGGE banding patterns. Three and 5.5 week old biofilm



**Fig. 4.** Two dimension representation plot of non-metric multidimensional scaling (NMDS) analysis of DGGE banding patterns from the two gels analyzed in the present work for bacterial communities from 3 and 5.5 week biofilms from site L (white dots) and from site A (black dots) and from transplanted biofilms (dark squares). Plots issued from the first DGGE gel were rotated ( $45^\circ$  counterclockwise – axis represented by dashed lines) to allow superposition of plots from gels 1 and 2. The numbers 1, 2, 3 and 4 indicate replicates from one same experimental condition.

communities from sites A and L exhibited differentiated structures according mainly to the different incubation sites and then to the state of maturation of the aggregate. Bacterial communities from the transplanted samples differed according to their site of origin (three first weeks of development) and of the site they were transplanted to. NMDS analysis showed that differentiated bacterial community structures were mainly mediated by the environmental conditions.

## Discussion

The primary objectives of this work were to evaluate the relative importance of development and of environmental

conditions on epilithic biofilm bacterial community dynamics.

The choice of the two study sites proved to be relevant for the purpose of this work since different environmental conditions were recorded during the study period. Hydrodynamics, which discriminated the two sites, generally causes substrate instability, and can generate a loss of biomass during flood events (Biggs, 1996). To minimize the importance of this parameter and to homogenize growth between our two study sites, biofilms were cultivated on artificial substrates. Artificial substrates prevent the occurrence of biases due to intra- and inter-site variations during sampling. Although it has been suggested that substrate properties such as surface roughness or hydrophobicity can influence bacterial community structure (Anderson-Glenna *et al.*, 2008), it was shown that artificial substrate support the development of algal community composition representative of natural communities (Eulin and Le Cohu, 1998; Barbiero, 2000; Lane *et al.*, 2003). However, artificial substrate ability to reproduce natural communities remains controversial (Cattaneo and Amireault, 1992). The water physico-chemical parameters of the two study sites were very contrasted. Site A, located in an agricultural watershed basin, was characterized by high levels of pH, conductivity, nitrate and DOC concentrations. Site L, located in a forested watershed basin, was mainly characterized by its higher total phosphorus concentrations. Concerning pesticides, the two sites presented similar levels of contamination. On the basis of environmental factors, sites A and L are clearly discriminated by their trophic states.

The choice of the incubation period length was based on the dynamic of epilithic biofilm growth occurring in large rivers (Lyautey *et al.*, 2005a; Boulêtreau *et al.*, 2006). Epilithic biofilm growth is defined by an accretion phase related to colonization and growth processes and characterized by an increase of AFDM resulting in a biomass peak. Observed AFDM variations were consistent with previous experiments: at site L, epilithic biofilms presented a biomass increase over time (colonization and growth) whereas at site A, a biomass decrease was observed after three weeks. The presence of aquatic *Bithynia* molluscs (*Prosobranchia*, *Bithyniidae*) on glass slides suggests that the disturbance was due to a grazing event. The biomass removal was more important for biofilms transplanted from site L to site A suggesting that they might have been more attractive for the invertebrates. The maximum biomasses reached in the present work were slightly below those previously recorded in the literature (up to 12 g AFDM.m<sup>-2</sup> in Lyautey *et al.* (2003) and between 15 and 25.6 g AFDM.m<sup>-2</sup> in Biggs (1996)). These lower biomasses could be explained by (i) a shorter growth period in the present work (5.5 weeks) as opposed to up to 11 weeks in Biggs (1996); (ii) a limited adhesion of cells due to nature of the substrates used; or (iii) a limited nutrient availability (Dodds *et al.*, 1997). The low AFDM/DM values recorded in the present work suggest that biofilms were rich in detritus and sedimentary particles despite artificial supports being parallel to flow. Sedimentary

particles were likely imported by the two flood events observed in site L and by the presence of sediments on river bed in site A. The autotrophic index indicates the relative importance of autotrophic *versus* heterotrophic organisms and detritus. With values between 42 and 258, epilithic biofilms described here were stable and constituted by equivalent proportions of photoautotrophic and heterotrophic microorganisms (Bourassa and Cattaneo, 1998).

Bacterial community composition was assessed using 16S rDNA based PCR-DGGE. Methodological biases (chimera and heteroduplex formation, template annealing, detection of the dominant populations) are well known and have been widely discussed in the literature (Muyzer *et al.*, 1993). Applied to epilithic biofilms, the use of Eubacteria-specific primers was proved to over-estimate bacterial richness by allowing the amplification of cyanobacteria and plastid DNA (Lyautey *et al.*, 2005b). With averages of 40 (replicates 3 and 4) to 55 (replicates 1 and 2) OTUs per sample of 5.5-week biofilm, bacterial richness was higher than the richness observed for epilithic biofilm grown on artificial substrates (Cody *et al.*, 2000; Araya *et al.*, 2003; Dorigo *et al.*, 2007), natural substrates (Lyautey *et al.*, 2005a), and bacterial communities from phytoplankton in tropical water bodies (Dumestre *et al.*, 2002) or in marine environment (Schauer *et al.*, 2000). This could reflect limited niches and resource availability or much harsher environment caused by continual grazing and sloughing processes (Anderson-Glenna *et al.*, 2008). The use of artificial substrates did not seem to affect bacterial community richness.

Dynamics of biofilm bacterial community were assessed at two sampling dates (at 3 and 5.5-week development) which reveals constant richness values during biofilm maturation but the appearance or the disappearance of species suggest changing bacterial communities and then a species succession consistent with theoretical models of biofilms succession (Jackson, 2003). Observed succession is thus mostly driven by autogenic processes. Allogenic factors also influence bacterial community composition, and can explain the differences observed between the two sampling sites. In freshwater environments, it was suggested that pH, temperature, and nutrient availability were correlated with variations of structure (Lyautey *et al.*, 2003; Hullah *et al.*, 2006). Turbulent flow was also demonstrated to influence bacterial community composition during the biofilm initial growth phase, by selecting the pioneer algal species that will create the biofilm microenvironment (Besemer *et al.*, 2007).

The transplant experiment was intended to assess the relative influence of autogenic (succession) and allogenic (environmental conditions) parameters on epilithic bacterial communities. Epilithic biofilm community development initially occurred in two separate stream environments characterized by their proper trophic states, and then some of the biofilms were transplanted to the second environment for further development. It appeared that transplantation contributed to modifications of the bacterial communities, and modified the trajectory of

the epilithic biofilm succession. However, the fact that some OTUs were commons between 5.5 week biofilms with and without transplantation indicates that succession was also involved in the temporal differentiation process by allowing common populations, either already present at time 3 weeks but below detection level or inoculated from surface waters from both sites, to develop in the aggregate. This implicates an influence of allogenic factors over autogenic ones. Our results suggest that the three initial weeks of biofilm development were too long to already determine the bacterial community composition and the 2.5 weeks of development following transplantation allowed to modify the community. Hypothetically, grazing might favour the addition of new species by decreasing the number of competitive species or by increasing vacant microhabitats. However, such a phenomenon was not observed, probably because the period of time between grazing and sample collection was too short to allow the initiation of a secondary ecological succession.

The present work confirmed that bacterial community structure was initially controlled by allogenic factors and then followed a succession pattern dominated by autogenic factors. Transplantation modified the structure and dynamics of epilithic bacterial communities that revealed the importance of allogenic factors on bacterial community successions. It could have been of interest to link the functional diversity with bacterial community structure during epilithic biofilm development and following transplantation, by identifying the populations that appeared in the community. A minimal number of species is responsible for stability and function in ecosystem processes, and all changes in this biodiversity could cause processes changes (Chapin *et al.*, 2000; Loreau *et al.*, 2001). According to the experimental design used, it could be hypothesized that bacterial community structure changes would reflect variations of functional diversity. Epilithic biofilm could thus represent a powerful model to study relationships between structure and function in aquatic ecosystem.

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