RESEARCH ARTICLE

Spatial and temporal variability in epilithic biofilm bacterial communities along an upland river gradient

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Abstract

Riverine biofilms remain one of the least-studied habitats despite the significant increase in the examination of aquatic microbial communities in recent years. In this study, the dynamics of epilithic biofilm communities native on rocks from a low-order upland stream were examined over a period of 3 years. Spatial and temporal variations in bacterial communities were assessed using terminal restriction fragment length polymorphism, based on analysis of the 16S rRNA gene. In total, 108 epilithic biofilm samples were analysed and 170 different ribotypes were detected. A strong temporal gradient in ribotype composition was noticed, especially between samples collected in 2001 and those collected in 2002 and 2003, most likely reflecting interannual differences in weather conditions, such as temperature. A spatial gradient in ribotype composition, from upstream sites to the low-lying sites, was also evident and interpreted as an environmental variation gradient along the river course. Distinct biofilm communities consistently occurred at the first site along the river, which was significantly correlated to low pH. Temporal factors explained the highest degree of variation within the epilithic biofilms. Recurrent blooms of certain bacteria were noted within the system. Phylogenetic relationships of bacteria at one point in the river were determined using a cloning and sequencing approach, with *Alphaproteobacteria* dominating the community, followed by *Cyanobacteria*, *Bacteroidetes* and *Betaproteobacteria*.

Introduction

Rivers are characterized by their unidirectional flow along a continuum from source to entry to the sea or any other large water body. This continuum from fast-flowing headwater streams to the slow-moving lowland stretches is influenced by changing physical and chemical properties as well as by its tight linkage to the terrestrial ecosystem. Factors that vary along the gradient, such as sediment load, nutrient status, temperature, pH and water velocity, determine the diversity and productivity of stream biota (Moss, 1998). Autochthonous and allochthonous sources of carbon vary in importance along the river continuum. For example, in wooded reaches, input from falling leaves and litter ensure that allochthonous processes dominate the carbon input. However, in open river stretches where photosynthesis can occur uninhibited, carbon production within the stream itself is dominant. Longitudinal variations in riverine communities have been correlated with temperature, nutrients and carbon source (Koetsier *et al*., 1997; Battin *et al*., 2001; Winter *et al*., 2007).

Bottom processes dominate open rivers, occurring within biofilms that form on submerged substrata such as rocks. This mode of existence is advantageous for microorganisms, particularly in constantly flowing environments providing substrata for attachment, increased nutrient supply and retention and mutalistic opportunities. The high density of biota and their complex nature ensure that the multiphyla epilithon is the most active component in river systems, making them important sites for biogeochemical cycling, particularly carbon and nitrogen, as well as bases of foodwebs. Riverine biofilms are dynamic, with new microorganisms constantly seeded from the overlying river water and recruited into the biofilm matrix, while cell death, sloughing of biofilms and invertebrate grazing ensure constant turnover (Kaplan & Bott, 1989).

In addition to variations of epilithic communities from different sites located along the river continuum, spatial variation can occur between substrata at the same site (intrasite variation). In riverine biofilms, microscale variation in biofilm architecture, both structurally and chemically, may result in spatial variations (Costerton *et al.*, 1994). For example, on a rock surface where a thick biofilm has developed, an anoxic layer may exist at the base of the biofilm and consequently be occupied by anaerobic bacteria (Schramm *et al.*, 1999). This microspatial variation contributes to intrasite differences observed in microbial communities.

Changing environmental conditions over the course of a year result in seasonal effects on the dynamics of aquatic communities (Leff *et al.*, 1998; Lindström *et al.*, 2005; Hullar *et al.*, 2006). Temporal variation within riverine biofilm communities has been observed and related to factors such as temperature and dissolved organic carbon (Brümmer *et al.*, 2000, 2004; Hullar *et al.*, 2006). Additional temporally varying factors such as rainfall and light levels can influence aquatic communities by their effects on nutrient levels, organic carbon input and flow rates as well as indirectly by effects on the surrounding terrestrial environment (Kaplan & Bott, 1989).

The majority of studies on freshwater planktonic microbial communities have focused on lakes (Lindström, 1998, 2000; Pernthaler *et al.*, 1998; Glöckner *et al.*, 1999, 2000; Casamayor *et al.*, 2000; Pearce, 2005) and larger rivers (Crump *et al.*, 1999; Cébron *et al.*, 2004; Besemer *et al.*, 2005) and has led to the conclusion that there exist globally distributed clades of freshwater bacteria (Zwart *et al.*, 1998). Recently, increased attention has been paid to the benthic communities of rivers in sediments and as biofilms (Brümmer *et al.*, 2000; Jackson *et al.*, 2001; Araya *et al.*, 2003; Lyautey *et al.*, 2003; Hullar *et al.*, 2006), with benthic communities of smaller rivers being more influenced by terrestrial ecosystems (Feris *et al.*, 2003). Development of the study of epilithic communities has also seen the divergence away from the use of biofilm reactors in the laboratory (Neu & Lawrence, 1997; Manz *et al.*, 1999) or of artificial substrata *in situ* such as glass or tiles (Brümmer *et al.*, 2000, 2004; Romani & Sabater, 2000; Jackson *et al.*, 2001) to the study of native communities on naturally occurring substrata within rivers (O'Sullivan *et al.*, 2002, 2006; Lyautey *et al.*, 2005; Fukuda *et al.*, 2006; Hullar *et al.*, 2006).

The preservation of biodiversity is important especially in the era of environmental change spurred by global warming. Consequently, knowledge of natural bacterial diversity contained within river biofilms at various scales is necessary in assessing the overall diversity, variation and stability of natural communities. Pristine head water streams, such as the one examined in this study, present an opportunity to study uncompromised community dynamics over time and at various spatial scales.

In this study, spatial heterogeneity and temporal dynamics of bacterial communities in native rock biofilms have been examined using terminal restriction fragment length polymorphism (TRFLP), a sensitive molecular community fingerprinting methodology (Marsh, 1999; Moeseneder *et al.*, 1999; Lukow *et al.*, 2000; Osborn *et al.*, 2000), at both intrasite (centimetre scale) and intersite (kilometre scale) levels along a stretch of a pristine upland river. Additionally, taxonomic affiliations of dominant bacterial members of the biofilm community were determined by cloning and sequencing of the 16S rRNA gene.

Materials and methods

Field site

Biofilm and river water samples were collected from first- and second-order segments of the Cloghoge River, County Wicklow, Ireland. The source of the Cloghoge River, Crokan pond, is located 560 m above sea level. The river drains a pristine catchment in its upper reaches, consisting of peat bog land dominated by *Sphagnum* spp. and *Calluna vulgaris* and developing into acidic grassland with sparse wooded vegetation further downstream. The catchment geology is predominantly granite. Annual rainfall averages between 2000 and 2800 mm and daily air temperatures between 4 and 15 °C (Met Eireann, http://www.met.ie). Sampling sites were located 0.5, 3.4, 5.2, 8.5, 9.7 and 10.7 km downstream of the source of the river. Sampling positions are listed in a 'keyhole markup language' (klm) file for use with 'Google Earth' software, which is available in the supplementary material.

Sample collection

Samples were collected from the river in July and September 2001, March and May 2002 and March, May and August 2003. River water samples for chemical analysis were only collected in 2001 and 2002. Three biofilm samples were collected from rocks in riffle areas at each of six sample sites. Owing to flood or other factors, on occasion, samples were not collected from all sample sites. Rocks averaged 20 cm in diameter and were collected between 5 and 30 cm below the surface of the river. Biofilm samples were scraped off a 7.5 cm^2 area of rock surface with a sterile toothbrush into 10 mL of sterile STE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA and 100 mM NaCl) and transported on ice. On return to the laboratory, 1.5 mL of biofilm suspension was pelleted at 19 600 ***g*** (IEC Micromax RF, Thermo Electron Corporation, MA) for 5 min, resuspended in 630 μL of sterile TE buffer (10 mM Tris-HCl pH 8.0 and 1 mM EDTA) and stored at −20 °C until nucleic acid extraction.

Water chemistry and chlorophyll *a* determination

Before ion analysis, river water samples were filtered through 0.45 μm filters. Cations were analysed using a Varian Liberty 200 Inductively Coupled Plasma atomic emission spectrometer (Varian Incorporated, Victoria, Australia). Anions were analysed using a Dionex DX-120 ion chromatography system (Dionex Corporation, Sunnyvale, CA). Total organic carbon (TOC) concentrations were determined the combustion/nondispersive infrared gas analysis method using a Shimadzu TOC-5050A instrument (Shimadzu Corporation, Kyoto, Japan). Chlorophyll *a* in the biofilm was determined spectrophotometrically (Holm-Hansen, 1978).

Community DNA extraction

Bacterial DNA was extracted from biofilm samples as follows: 70 μL of 10% (w/v) sodium dodecyl sulphate was added to 630 μL of a biofilm suspension and incubated at 65 °C for 10 min. The lysate was extracted with an equal volume of phenol : chloroform : isoamyl alcohol (25 : 24 : 1) and subsequently with an equal volume of chloroform : isoamyl alcohol (24 : 1). The aqueous phase (600 μL) was transferred to a new tube and nucleic acids were precipitated with 1.2 mL of ethanol following addition of 60 μL 3 M sodium acetate (pH 5.2) by centrifugation at 19 600 ***g*** for 20 min. The pellet was washed with 70% (v/v) ethanol and dissolved in 100 μL of TE buffer. DNA was further purified using a High Pure PCR product clean-up kit (Roche Diagnostics GmbH, Penzberg, Germany) according to the manufacturer's instructions. DNA was eluted in 100 μL of elution buffer and stored at − 20 °C until further analysis.

Bacterial community analysis by TRFLP

Bacterial 16S rRNA genes were amplified using F27 (5′-AGAGTTTGATCMTGGCTCAG-3′) and R1492 (5′-TACGGYTACCTTGTTACGACT-3′) for TRFLP analysis (Moeseneder *et al*., 1999). The 5′-end of the forward primer (F27) was labelled with Beckman Coulter fluorescent dye D4 (Invitrogen, Paisley, Scotland). Each 100 μL PCR mixture contained 2 μL of extracted DNA, 10 μL of 10 × PCR buffer (Promega, Southampton, UK), 1.25 mM $MgCl_2$, 15 pmol of each primer, 200 μM of each dNTP, 50 μg of nonacetylated bovine serum albumin and 2.5 U of *Taq* DNA polymerase (Promega). DNA amplification was performed in a Perkin Elmer Thermal Gene Amp PCR system (Perkin Elmer Ltd, Beaconsfield, UK). The reaction mixture was incubated at 94 °C for 3 min, followed by 26 cycles of 94 °C for 1 min, 53 °C for 2 min and 72 °C for 2 min with a final extension of 5 min at 72 °C. PCR products were purified using a High Pure PCR product purification kit (Roche Diagnostics, Penzberg, Germany) and eluted in 100 μL of elution buffer according to the manufacturer's instructions. The purified PCR product (50 ng) was digested with HaeIII (5 U) at 37 °C for 3 h according to the manufacture's instruction. Digests (1.5 μL aliquots) were desalted by ethanol precipitation in the presence of glycogen (5 μg) and subsequently resuspended in 38.25 μL of deionized formamide and 0.25 μL of Beckman Coulter size standard CEQ 600 (Beckman Coulter, High Wycome, UK). Samples were electrophoresed on a CEQ 8000XL DNA Analysis System at 4.8 KV for 60 min. Terminal restriction fragment (TRF) lengths were determined using the Beckmann Coulter Fragment Analysis Package 8000, version 8.0.52. Fragment sizes were determined automatically by comparison against the internal size standard CEQ 600. A quartic polynomial model was applied during the fragment analysis. Ribotypes that differed in size by 0.5 bp or less were considered to be identical. A binary matrix was created based on the presence or the absence of ribotypes within the samples.

Ordination

The variation in bacterial ribotype composition at the different sites and time periods was analysed by detrended correspondence analysis (DCA) (Hill, 1979; Hill & Gauch, 1980). DCA calculations were performed in the program CANOCO for Windows version 4.5 (ter Braak, 1987; ter Braak & Smilauer, 2002). As some infrequent bacterial ribotypes appeared as outliers in ordinations, ribotypes with a frequency lower than the median in proportion to their frequency were down-weighted as recommended by Eilertsen & Pedersen (1989).

Correlation analyses were performed between environmental parameters and DCA-ordination values to achieve a better interpretation of the different gradients along the ordination axis. In addition to the environmental parameters, time [computed as the month of sampling (1–12)] and space (distance from the source of the river) were also included in the analysis. As a measure of correlation, the Kendall τ was used (Conover, 1980). Kendall τ is a nonparametric measure (it only takes the ranks of variables into account), and it is recommended by Fenstad *et al*. (1977) whenever the underlying distribution is unknown. The calculation of Kendall τ and the corresponding statistical testing of this were performed in SPSS v11.0 (SPSS Inc., Chicago, IL).

Tests of similarity of ribotype composition

Spatial and temporal similarity (and dissimilarity) in ribotype composition was tested by canonical correspondence analysis (CCA, ter Braak, 1987) using site and time as factor variables with six levels (six different sites) and seven levels (seven different time points) using the Vegan package

(Oksanen *et al.*, 2006) in R software Version 2.4.1 (R Development Core Team, 2006). Tests of similarity (and dissimilarity) between one site and the rest of the sites (i.e. the unique contribution of ribotype composition in one site compared with the rest of the sites) were performed by CCA using sites as dummy variables. The significance of the constrained variables (site factor, time factor and individual sites) was assessed by Monte Carlo permutation tests with 499 permutations.

Clone library construction

Clone libraries were constructed from biofilm samples collected from Site B on the river in September 2001 using unlabelled universal bacterial primer set F27 and R1492. PCR products were ligated into the pGEM-T-easy cloning vector (Promega) and transformed into *Escherichia coli* DH5α [*sup*E44Δ*lac*U169 (ϕ80*lac*ZΔM15) *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1]. Transformants were selected on Luria–Bertani (LB) agar plates containing ampicillin (100 μg mL^{-1}), X-gal (20 μg mL^{-1}) and IPTG (0.5 mM). Randomly chosen white colonies were grown in LB medium; the insert in each clone was subsequently analysed by PCR using the vector-specific primers SP6 (5′ATTTAGGTGACACTATAG-3′) and T7 (5′-TAATAC-GACTCACTATAGGG-3′), which targeted flanking regions of the insert. Clones containing full-length inserts (*c.* 1400 bp) were analysed by RFLP analysis by digesting with the restriction endonuclease HaeIII (Roche Diagnostics GMBH, Penzberg, Germany). High-resolution agarose gels were compared manually and identical clones were grouped together into operational taxonomic units (OTUs). A representative from each of the OTUs was selected for sequencing.

Sequencing and phylogenetic analysis

The Dye Terminator Cycle Sequencing (DTCS) Quick Start Kit (Promega) was used to sequence the 16S rRNA genes. Sequence reaction products were electrophoresed on a CEQ 8000XL DNA Dye Terminator Cycle Sequencer at 4.8 kV for 60 min. Sequences were processed and assembled using the GAP4 program of the Staden Package (Staden *et al.*, 2003). The Ribosomal Database Project (RDP) Classifier hosted by the Ribosomal Database Project-II release 9 (Version 9.55) was used to assign taxonomic affiliation to selected clone sequences. The naive Bayesian rRNA classifier (Classifier Version 2.0) classifies bacterial 16S rRNA gene sequences into the higher order taxonomy as proposed by Garrity *et al.* (2004). The allocation of sequences to a specific phylotype is accompanied by a confidence estimate that is calculated based on the number of times that that phylotype was selected out of 100 bootstrap trials (Wang *et al.*, 2007).

The closest matching sequences to the clones were determined using the Sequence Match Tool (SEQMATCH) of the RDP II. A k-nearest-neighbour (k-NN) classification method is used by the SEQMATCH tool to assign taxon. It has been shown that the SEQMATCH is more accurate at identifying the most similar rRNA gene sequence than the BLAST tool of the National Center for Biotechnology Information (NCBI) (Cole *et al.*, 2005). The data were filtered to include all 16S rRNA gene sequence data, both full length and shorter length good-quality sequences.

Nucleotide sequence accession numbers

Sequences were submitted to GenBank under the accession numbers EF032652 to EF032668.

Results

TRFLP analysis

Based on analysis of 108 epilithic biofilm samples from the six sites along the river by TRFLP, 170 different ribotypes were identified. The number of different ribotypes per site sampled varied between 4 and 55 and averaged 30. The number of ribotypes recovered from an individual rock biofilm varied between 1 and 51 with average numbers ranging between 17 and 26 for each of the six sample sites. Biofilms recovered from Site D in March 2002 were dominated almost exclusively by one ribotype (TRF_{379}), accounting for *c.* 96% relative abundance. This ribotype also dominated biofilm samples at Site A in May 2003, where it had a relative abundance of 57% on average; the community diversity was low, with only nine other ribotypes being detected. Site A displayed the most unique community, with 34% of all ribotypes detected at Site A occurring only at that site. 32% of ribotypes detected were present only at one sample time point while 3.5% occurred at all seven time points. TRF_{379} was one of two most frequently occurring ribotypes, both occurring in 78 of the 108 samples analysed. A further six ribotypes were present in more than 50% of the samples. In the majority of samples, a small number of ribotypes accounted for 50% or more of the relative abundance of the microbial communities. Dominant ribotypes varied with time, with only one occurring within the top five most abundant ribotypes at each of the seven time points. TRF_{379}, for example, dominated a number of sites in March 2002 (up to 96%) and March and May 2003 but occurred with a low abundance at other times (<3%). In general, intrasite similarity between epilithic biofilm communities was higher than for intersite similarities, ranging from 33% to 100% and averaging 62.5%.

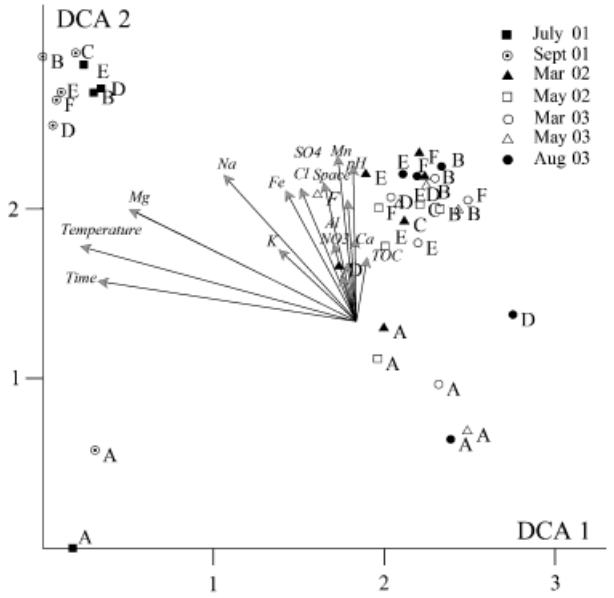

Fig. 1. DCA ordination of 36 samples consisting of six different sites in different time points [DCA axes 1 (horizontal) and 2 (vertical)]. Site is indicated by letter and sampling period by symbol as detailed in the key. Eigenvectors representing environmental parameters are shown as vectors. Additionally, eigenvectors representing time (month of sampling) and space (distance from the source of the river) are included. Scaling of axes is in standard deviation (SD) units. The two primary DCA axes show gradient lengths of 2.75 and 2.92 S.D. units. The amount of explained cumulative variation for the first two ordination axes was 36.7%.

Variation in bacterial biofilm communities

Multivariate statistics were used to help elucidate and display the patterns within the biofilm communities. The DCA ordination separated sites into three different clusters for the first two axes shown in the ordination diagram (Fig. 1). One cluster, consisting of two sample plots, Sept01A and July01A, had low DCA 1 values (>0.5 SD units) and low DCA 2 values. The next cluster, consisting of all other September and July 2001 samples, was located at the left-hand side of the diagram at high DCA 2 values pulling out the second axis. The last cluster, which also had the highest number of sample plots (most of the 2002 and 2003 samples), was centred to the right of the diagram and spread evenly along most DCA 2 values. The July and September 2001 sites had much lower DCA 1 values than any of the 2002 or 2003 sites, which suggests that changes in ribotype composition along this axis are caused by temporal processes. Along DCA 2, the A sites separated from all the other sites by having all the lowest scores, suggesting a spatial explanation for community differences along this axis.

The mean values for physical and chemical parameters measured in river water samples in 2001 and 2002 are presented in Table 1. A noteable recurrent pattern was the low pH at Site A, near the source of the river, which was much lower than at any other site. Time, temperature and Mg ($|\tau| > 0.400$) were correlated with the first DCA axis (Table 2). Five additional variables were correlated with DCA 2 at the $P < 0.05$ significance level, with pH as the most significant variable. It is interesting to note that spatial location, distance from the source of the river, was also correlated with DCA 2. Hence, of the variables analysed, temperature, decreasing towards higher DCA 1 values, may be an important factor explaining variation along this axis, and pH, increasing towards higher DCA 2 values, may be the most important variable for separation of sites in time along the second axis.

Tests of similarity of species composition in space and time

Intersite variation (as measured by the constrained axes) accounted for 16.6 % ($P < 0.001$, Monte Carlo permutation test) of the total variation in the ordination. The time factor accounted for 28.3% of the total variation ($P < 0.001$). This is also indicated by the DCA ordination where temporal and spatial variation variables were closely correlated with DCA 1 and DCA 2, respectively.

The unique contribution to ribotype composition in any site was only significant at Site A, which alone explained 7.5% of all variation ($P < 0.001$). The other sites contributed only between 1.9% (Site C) and 3.2% (Site D) in unique variation.

Phylogeny

Epilithic biofilm clones identified from analysis of a composite biofilm sample are listed in Table 3, together with their taxonomy, closest matches and percent similarity as determined by RDP Classifier and SEQMATCH (RDP II). Also listed in the table is the habitat where the closest unnamed matches from the database were recovered. The epilithic community was dominated by the *Alphaproteobacteria*, which accounted for just over half of the clones analysed by restriction analysis. *Cyanobacteria* were the next most abundant group; interestingly, a number of these clones were most closely related to clones recovered in the Antarctic. Clones were distributed across phyla as follows: *Alphaproteobacteria* 51%, *Cyanobacteria* 27%, *Betaproteobacteria* 9% and *Bacteroidetes* 13%.

Two Alphaproteobacterial clones were classified with confidence (80%) to the genus level: one as a *Bosea* spp. (*Bradyrhizobiaceae*) and one as a *Hyphomicrobium* spp. (*Hyphomicrobiaceae*). All other Alphaproteobacterial clones could not be classified beyond the Order *Rhizobiales*. Cyanobacterial clones remained either unclassified *Cyanobacteria* or were classified into the Family 4.1 (two clones). Betaproteobacterial clones were designated as unclassified *Betaproteobacteria*. The *Bacteroidetes* clone was classified to

Table 1. Physico-chemical characteristics measured along the Cloghoge River from July 2001 to May 2002. Mean values are reported, with the range shown in parentheses

Site	Potassium (K)	Sodium (Na)	Aluminium (Al)	Calcium (Ca)	Manganese (Mn)	Iron (Fe)	Magnesium (Mg)	TOC	Chloride (Cl)	Nitrate (NO_3)	Sulphate (SO_4)	pH	Temperature	Chl a*
A	0.15	2.91	0.14	0.59	0.00	0.15	0.42	41.03	4.13	0.21	0.65	3.72	8.55	2.67
	(0.09–0.30)	(2.04–3.93)	(0.09–0.20)	(0.43–0.84)	(0.00–0.01)	(0.08–0.19)	(0.23–0.62)	(31.43–49.84)	(2.39–6.84)	(0.17–0.29)	(0.50–0.97)	(3.64–3.85)	(5.90–11.10)	(0.44–6.88)
B	0.49	5.35	0.13	1.20	0.04	0.39	0.62	34.28	5.02	0.14	1.20	5.44	10.35	3.63
	(0.30–0.73)	(4.23–6.94)	(0.03–0.19)	(1.06–1.50)	(0.02–0.06)	(0.11–0.55)	(0.53–0.76)	(11.05–60.76)	(3.95–6.34)	(0.00–0.19)	(0.97–1.52)	(4.40–6.10)	(7.10–13.80)	(0.94–10.55)
C	0.36	4.64	0.10	2.02	0.03	0.21	0.74	26.37	5.11	0.12	1.73	6.14	12.37	4.04
	(0.15–0.69)	(3.45–6.21)	(0.03–0.22)	(1.12–2.93)	(0.01–0.08)	(0.07–0.45)	(0.45–1.13)	(22.11–31.28)	(4.97–5.38)	(0.11–0.13)	(0.99–2.89)	(5.50–6.70)	(8.30–15.00)	(1.05–11.57)
D	0.44	4.83	0.07	1.44	0.04	0.22	0.61	22.33	7.29	0.51	2.14	5.72	12.60	2.32
	(0.36–0.51)	(4.02–6.60)	(0.03–0.11)	(0.92–2.68)	(0.01–0.05)	(0.17–0.36)	(0.52–0.73)	(15.93–29.13)	(6.26–8.69)	(0.43–0.61)	(1.99–2.21)	(5.45–5.92)	(7.00–16.00)	(0.32–5.91)
E	0.43	4.18	0.09	1.07	0.03	0.17	0.62	32.61	6.15	0.51	2.07	5.86	13.03	11.35
	(0.22–0.56)	(2.97–5.30)	(0.07–0.12)	(0.73–1.25)	(0.01–0.06)	(0.15–0.20)	(0.46–0.81)	(12.71–61.30)	(5.18–6.68)	(0.44–0.60)	(1.76–2.51)	(5.43–6.40)	(8.70–16.50)	(3.28–19.33)
F	0.37	4.45	0.09	1.04	0.03	0.16	0.58	24.91	6.26	0.48	2.17	5.56	13.08	4.39
	(0.19–0.68)	(3.43–6.32)	(0.05–0.13)	(0.70–1.55)	(0.01–0.05)	(0.11–0.22)	(0.47–0.67)	(16.29–37.72)	(5.91–6.67)	(0.39–0.66)	(1.70–2.60)	(5.18–5.60)	(9.30–16.70)	(1.34–10.88)

*Within the biofilm.
All values are recorded in mg L^{-1} with the exception of pH and temperature which are recorded in pH units and °C, respectively.
TOC, total organic carbon.

Table 2. Kendall's rank correlations coefficients τ between site scores along DCA ordination axes and 16 variables

	DCA1		DCA2	
Variables	τ	*P*	τ	*P*
pH	0.0190476	0.9287	**0.4571429**	**0.0033**
Temp	**−0.468905**	**0.0031**	**0.4880439**	**0.0021**
K	0.0294123	0.861	0.2411806	0.151
Na	−0.216374	0.2109	**0.380117**	**0.0236**
Al	0.1737025	0.3071	−0.221621	0.1926
Ca	−0.02924	0.8903	**0.3333333**	**0.0491**
Mn	0.0904025	0.5967	0.0180805	0.9157
Fe	0.0529421	0.7526	0.064707	0.7
Mg	**−0.410559**	**0.0143**	**0.3870984**	**0.0209**
TOC	0.122807	0.4893	**−0.333333**	**0.0491**
Cl	−0.163399	0.3686	0.124183	0.5009
NO_3	−0.154699	0.4427	0.309397	0.1247
SO_4	−0.091804	0.5956	0.301641	0.0812
Species richness	0.058263	0.7159	−0.058263	0.7159
Time	**−0.6358438**	**0.0002**	0.2047633	0.2329
Space	0.06631445	0.6896	**0.3825834**	**0.0212**

Correlation coefficients and their significance probabilities are specified. Strong correlations ($P < 0.001$) are in bold face.

the genus *Terrimonas* (*Crenotrichaceae*) with 80% confidence by the RDP classifier.

Although 70% of the bacterial clones were related with a high percent similarity (greater than 97% similarity to clones within the database), only three clones were related with a high percent similarity to named entries. A further three were possibly related at the genus level (assuming this is demarcated by 95% or greater similarity). The high relatedness to unnamed clones within the database illustrates that these bacteria may be part of globally distributed clades occurring within aquatic and terrestrial environments but that are not yet represented by isolated and described bacteria. Bacterial clades typical of freshwater environments have been proposed in previous studies (Glöckner *et al.*, 2000; Zwart *et al.*, 2002; Eiler & Bertilsson, 2004). A number of clones from this study shared similarity with some of these clades. One Betaproteobacterial clone was closely affiliated with a clone recovered from Cyanobacterial blooms in Swedish lakes (LiUU-11-174.2 cluster) (Eiler & Bertilsson, 2004). An Alphaproteobacterial clone was related to lake clones within the Alpha1 cluster suggested by Glöckner *et al.* (2000), with *Beijerinckia indica* as the closest named relative, while another was included within the CR-FL11 cluster suggested by Zwart *et al.* (2002).

Discussion

In this study, the dominant bacterial ribotypes within epilithic biofilm communities along a pristine upland river

Table 3. Taxonomic affiliation of epilithic biofilm clones from the Cloghoge River

Accession no. assigned to clone	Division	Closest unnamed match		Closest named match			
		Accession no.	Habitat	16S rRNA gene similarity (%)	Accession no.	Name	16S rRNA gene similarity (%)
EF032667	*Alphaproteobacteria*	AY289489	Soil	97.4	AJ250796	*Bosea thiooxidans*	93.5
EF032657	*Alphaproteobacteria*	EF667544	River	94.4	AB222020	*Hyphomicrobium facile*	93.5
EF032655	*Alphaproteobacteria*	EF438236	River	99.7	AY158812	*Methylobacterium* sp.	94.6
EF032654	*Alphaproteobacteria*	DQ828857	Soil	94.2	AM411913	*Hyphomicrobium* sp.	93.7
EF032656	*Alphaproteobacteria*	EF438254	River	99.9	AF484596	*Mesorhizobium* sp.	91.5
EF032661	*Alphaproteobacteria*	AJ518406	Freshwater	97.8	AF370880	*Nordella oligomobilis*	93.7
EF032652	*Alphaproteobacteria*	AY289489	Soil	96.7	AY488508	*Bosea thiooxidans*	94.0
EF032658	*Alphaproteobacteria*	AJ290010	Lake	98.1	AJ563930	*Beijerinckia indica*	95.0
EF032665	*Alphaproteobacteria*	AB205810	Sludge	96.6	AB299683	*Afipia* sp.	97.4
EF032662	*Bacteroidetes*	DQ414821	Water	97.8	AB267477	*Flavosolibacter ginsengiterrae*	91.5
EF032653	*Betaproteobacteria*	AY509460	Lake	97.9	AJ575087	*Collimonas fungivorans*	90.5
EF032664	*Betaproteobacteria*	EU034406	River	99.0	AM392325	*Burkholderia glathei*	91.2
EF032660	*Cyanobacteria*	EF438280	River	98.3	EF512279	*Leptolyngbya* sp.	92.8
EF032668	*Cyanobacteria*	AF076163	Wetland	96.9	AY170472	*Chamaesiphon subglobosus*	97.1
EF032666	*Cyanobacteria*	AM773416	Rock	99.5	AY170472	*Chamaesiphon subglobosus*	95.8
EF032659	*Cyanobacteria*	AY124357	Glacial ice	97.1	AY170472	*Chamaesiphon subglobosus*	96.8
EF032663	*Cyanobacteria*	AM773416	Rock	99.5	AY170472	*Chamaesiphon subglobosus*	97.7

continuum were discriminated using a molecular-based community fingerprinting approach.

Our results showed that the numbers of different ribotypes within epilithic biofilms varied between four and 55 at the site level, which is fairly consistent with findings in the other limited numbers of studies. On occasion, low numbers of different ribotypes were observed at selected times related to blooms of a single individual that dominated during that time period. Differences in methodological approaches between this study and the previous studies on river biofim bacterial communities prevent direct comparisons of ribotype richness (as a proxy for species richness). Bearing in mind that different methodological approaches may provide differences in the levels of phylogenetic resolution, other molecular-based riverine biofilm studies have reported between 19 and 45 different OTUs from riverine biofim samples (Cody *et al.*, 2000; Araya *et al.*, 2003; Lyautey *et al.*, 2003).

It has been suggested previously that natural biofilms may present a more diverse community than those grown on artificial substrata (Lyautey *et al.*, 2003). In contrast to most other molecular characterizations of epilithic biofilms, this study uses TRFLP, which has been advocated as a sensitive analysis method possibly allowing the efficient detection of less abundant ribotypes (Marsh, 1999). Characterization of epilithic bacterial communities on artificial substrata using this method is lacking and so it is not possible to say as yet whether or not these contrasting communities vary in ribotype number. It is further interesting to note that bacterial communities in river biofilms are much less diverse than those of soil habitats, which may point to limited niche or resource availability or may indeed just indicate that epilithic biofilms present a much harsher environment exemplified by continual sloughing and grazing.

Variations occurred in microbial community structure at the patch (intrasite) and local (intersite) scales along the river as well as temporally. The intrasite variation could result from microspatial variations in surface properties and has implications for the sampling strategies used in diversity studies. Surface roughness, charge, hydrophobicity, critical surface tension, wetability, microtopography and mineral content have all been implicated as factors contributing to variations in biofilm communities on surfaces (Brading *et al.*, 1995; Busscher & Van Der Mei, 1995; Bos *et al.*, 1999). Microniches can be created within a developing biofilm when factors such as oxygen availability and redox potential alter as biofilm structures develop (Schramm *et al.*, 1999). Additionally, shear forces exerted by stream water flow may influence biofilm structure (Battin *et al.*, 2003), as may the order of attachment of microorganisms (Kolenbrander *et al.*, 2002). Previous studies have observed lower levels of intrasite variation based on denaturing gradient gel electrophoresis estimates of bacterial richness (e.g. Ferris & Ward, 1997; Cody *et al.*, 2000). This may reflect the variability in the detection ability of more rare ribotypes characteristic of aquatic ecosystems (Kemp & Aller, 2004) or indeed natural variability among rivers. In this study, dominant ribotypes were observed to be replaced by less dominant community members over time and with varying environmental conditions. Less abundant 'species' have been attributed to increase community stability in the face of changing environmental factors as suggested by the diversity-stability and biological insurance hypotheses (Boles *et al.*, 2004).

Physical and chemical gradients along the river continuum created a range of microenvironments that accounted for the observed intersite variation. pH and temperature in particular were correlated with variations in the microbial communities. Accordingly, temperature, pH, Ca, Mg and Na increased downstream, creating an ecocline (a combined change in both environmental and ribotype composition) gradient from high to low elevation. The river is sourced within acidic peat bogland, which may contribute to acidic run-off and inputs of allochthonous carbon (Dedysh *et al.*, 1998), resulting in a substantially lower pH than that observed at downstream sites. Consequently, a unique population of bacteria was observed at this upstream location. This site, A, was the only site that had a significantly unique contribution to the ribotype composition compared with all other sites. The uniqueness of the bacterial community at this site may possibly be explained by the occurrence of specialist bacteria adapted to more extreme pH conditions and/or to high concentrations of humic acids and C1 carbon compounds. In other freshwater studies, pH has been strongly correlated with variations in microbial communities (Lindström *et al.*, 2005; Yannarell & Triplett, 2005). Along the river-elevation gradient, biofilms are also subject to environmental variables such as turbulence, flow, sediment load and variations in light availability, which may contribute to the observed variation in ribotype composition. Previously, differences in chemical properties such as availability of nutrients and carbon, and the quality of the carbon source, have been shown to determine the presence or the absence of certain bacterial species (Koetsier *et al.*, 1997).

In contrast to the specialist bacteria recorded upstream, a small group of bacteria were detected occurring across all time points and were frequently detected along the river continuum. These bacteria may be generalists with broader niche capabilities, which allow them to survive and grow under a range of environmental conditions. This may be an advantage to biofilm community members as sloughing of biofilms often occurs in flowing habitats. Species with more general resource requirements can then reattach downstream and successfully reintegrate into biofilm communities. Persistent generalist communities in aquatic systems have been noted previously (Crump *et al.*, 2003).

Our results showed that temporal variation in ribotype composition could explain a substantial component of the total variation within the biofilms. Accordingly, fluctuations in environmental conditions in temperate ecosystems over the course of a year result in seasonal effects on the dynamics of communities. In previous studies, temperature has been correlated with changes in aquatic microbial communities (Lindström *et al.*, 2005; Hullar *et al.*, 2006; Winter *et al.*, 2007). Likewise, in our results, temperature was the variable (among those measured) that was most significantly correlated with the observed temporal variation in ribotype composition (DCA 1). It appears that the cold temperatures in March and May had an effect on the ribotype composition (all high DCA 1 values) while the composition in 2002 and 2003 all stabilized on higher DCA 1 values. Seasonal variations in populations collected on glass slides in a large European river have been observed previously with a dominance of *Betaproteobacteria* and variations in maxima of *Planctomycetes* and *Cytophaga-Flavobacteria* (Brümmer *et al.*, 2000). Blooms within aquatic microbial communities have also been noted (Pernthaler *et al.*, 1998). Blooms by a single ribotype on various occasions were noted along the Cloghoge River, particularly in Spring. A small number of ribotypes were observed to dominate communities, with dominant TRFs changing with time, implying a pool of bacteria able to respond to fluctuating conditions. Although temporally variable factors commonly exert control over riverine communities, observations that communities from certain sites clustered together would imply that temporally independent factors were also important in the structuring of bacterial communities.

Biofilms in this study were dominated by *Alphaproteobacteria* but also comprised members of the *Cyanobacteria*, *Betaproteobacteria* and *Bacteroidetes*. A number of bacterial divisions are frequently encountered in freshwater studies including the *Proteobacteria* (Alpha, Beta and Gamma), *Bacteriodetes*, *Cyanobacteria*, *Actinobacteria*, *Verrucomicrobia* and the *Planctomycetes* (Glöckner *et al.*, 2000; Zwart *et al.*, 2002; Eiler & Bertilsson, 2004). As for all PCR-based analyses, these data are subject to potential biases and so these results should be interpreted in the light of this knowledge (von Wintzingerode *et al.*, 1997). It is quite unusual that these freshwater biofilms were dominated by the *Alphaproteobacteria* as these are most often found to dominate marine and coastal environments (Rappé *et al.*, 1997; Cottrell & Kirchman, 2000) including biofilms (Dang & Lovell, 2002). Nonetheless, *Alphaproteobacteria* have been found to be more frequent within bacterial clone libraries from certain lakes (Glöckner *et al.*, 2000; Zwart *et al.*, 2002). *Alphaproteobacteria* have been noted to grow rapidly in situations with reduced resource competition and grazing pressure (Fuchs *et al.*, 1997). In the upper reaches of the study river a diminished grazing community exists as a result of high aluminium concentrations (M. Kelly-Quinn, pers. commun.). Additionally, *Alphaproteobacteria* appear to thrive in more oligotrophic environments (Pinhassi & Hagström, 2000; Morris *et al.*, 2002) and have been detected in high abundance in acidic peat bogs (Dedysh *et al.*, 2006). In the majority of aquatic studies, *Betaproteobacteria* have been found to dominate in both lake and riverine systems (Glöckner *et al.*, 1999; Brümmer *et al.,* 2000; Zwart *et al.*, 2002, 2003).

According to 16S rDNA gene sequence similarities, a number of clones recovered from the Cloghoge River

biofilms were affiliated with acidophilic nitrogen-fixing or methylotrophic bacteria from the division *Alphaproteobacteria*. Members of the *Bradyrizobiaceae* are aerobic or anaerobic, are metabolically diverse but include nitrogen-fixing bacteria as well as chemolithoheterotrophic bacteria-oxidizing reduced sulphur compounds such as *Bosea* spp. (Garrity *et al*., 2004). Described species of the *Hyphomicrobiaceae* are methylotrophs and are often found in freshwater systems (Garrity *et al*., 2004). Peat bogs are known to be both limited in available nitrogen and to be a rich source of C1 compounds, such as methane and methanol (Dedysh *et al*., 2004). Clones were possibly related at the genus level (assuming 95% similarity as the cutoff point) to *Beijerinckia* spp. (most closely to *B. indica*) and *Afipia* spp. *B. indica* is methylotrophic, can fix nitrogen and is commonly found in acidic soils (Garrity *et al*., 2004). *Afipia* spp. has been detected previously in freshwater biofilms and lake samples (Glöckner *et al*., 1999; Rickard *et al*., 2002). Methylotrophic *Afipia* spp. have also been isolated from Antarctica and a Portuguese river (Azra Moosvi *et al*., 2005). Although it is impossible to deduce physiology from phylogeny alone, the presence of either nitrogen-fixing or methylotrophic bacteria would be consistent with the location of the study river within a *Sphagnum* peat bog environment.

The dominant *Cyanobacteria* were closely related to *Chamaesiphon subglobosus*. This *Cyanobacteria* commonly occur as a member of epilthic communities in streams (Müllner & Schagerl, 2003). Members of the *Bacteroidetes* are known to be degraders of high-molecular-weight polymers and are often recovered from humic waters such as the study river (Kirchman, 2002).

Variations within epilithic biofilm ribotype composition were observed in this study at three different scales. Communities varied within the same site (intrasite variation), along a river gradient (gradient in spatial variation) and between time periods (temporal variation). Taxonomically, biofilm members were related to both globally distributed aquatic bacterial clades as well as to terrestrial bacteria. Ribotype composition appeared to be determined by similar controlling factors as for macroorganisms such as temperature and pH. However, more detailed studies are required examining the link of additional physiochemical variables to community dynamics. Changes in bacterial diversity may be accompanied by variations in functional capabilities; exploration of this possibility would increase our understanding of the effects of environmental change on system processes.

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Supplementary material

The following material is available for this article online:

Fig. S1. Location of sampling sites along the Cleghoge River, County Wicklow, Ireland.

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1574-6941.2008.00480.x (This link will take you to the article abstract).

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