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Longitudinal changes in bacterial community composition in river epilithic biofilms: influence of nutrients and organic matter

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ABSTRACT: Epilithic bacterial communities play a major role in biogeochemical cycles of rivers; however, distributional patterns and controls of epilithic communities remain unclear. The objective of the present study was to examine possible environmental factors that affect longitudinal distributional patterns of epilithic bacterial communities in 2 rivers (Yasu and Ado Rivers) draining the Lake Biwa basin, Japan. Phylogenetic analyses were conducted using denaturing gradient gel electrophoresis (DGGE) of 16S rRNA genes and fluorescence *in situ* hybridization (FISH) with oligonucleotide probes targeted to small subunit rRNA. Non-metric multidimensional scaling and canonical correspondence analysis of the DGGE profile indicated that a clear shift in community composition occurred at the middle reach of the Yasu River. This shift was most closely related to variation in the nitrogen stable isotope ratio ($\delta^{15}N$) of epilithic materials (BIOENV analysis, $\rho_w = 0.512$), which is an indicator of the extent of anthropogenic nitrogen loading. The concentration of dissolved organic carbon (DOC) also affected community composition ($\rho_w = 0.510$ for the combination of $\delta^{15}N$ and DOC). The longitudinal pattern of the DGGE profile was less evident in the Ado River. In both rivers, FISH results indicated that *Alphaproteobacteria* and *Betaproteobacteria* generally accounted for a large fraction (37 to 71%) of total bacterial abundance, indicating that species and clones affiliated with a limited number of the major phylogenetic groups occupy diverse epilithic habitats. Our results are consistent with the hypothesis that nutrients and organic matter of anthropogenic origin are major determinants of epilithic bacterial community structure in rivers.

KEY WORDS: Bacterial community · Epilithic biofilm · River · Stable isotopes · Nutrients · Dissolved organic carbon · Denaturing gradient gel electrophoresis · Fluorescence *in situ* hybridization

INTRODUCTION

Epilithic biofilms covering the surface of stones and cobbles in river beds consist of diverse microbes (e.g. bacteria, archaea, heterotrophic protists, and photosynthetic algae) that play a major role in the regulation of food webs and biogeochemical cycles in rivers (Mulholland et al. 1995, Hall & Meyer 1998). Evaluating distributional patterns and controls of epilithic bacterial communities is fundamentally important, because such information may help to improve our ability to predict the responses of river ecosystems to distur-

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bances and environmental changes that alter bacterial community structure. Rivers generally exhibit environmental gradients in physical variables along a longitudinal axis. These gradients often coincide with shifts in organic carbon sources and quality, eliciting a longitudinal transition in ecosystem properties and biotic communities (Vannote et al. 1980). However, whether lotic bacterial communities change in a predictable manner along general environmental gradients in rivers remains unclear (Leff 2000).

Anthropogenic inputs of organic matter and inorganic nutrients from urban and agricultural watersheds have increased in rivers worldwide during recent decades (Mulholland et al. 2008), leading to the enhancement of algal production (eutrophication) and changes in redox conditions (Wetzel 2001). Depending on the extent of the pollution and hydrological conditions, anthropogenic disturbances may create spatial patterns in the longitudinal distribution of dominant bacterial taxa in rivers. For example, Winter et al. (2007) demonstrated that the community composition of planktonic bacteria in the Danube River shifts over river stretches in response to changes in indices of anthropogenic eutrophication such as concentrations of nutrients and chlorophyll *a* (chl *a*). Much less information is available about longitudinal variation in epilithic bacterial communities. Rubin & Leff (2007) suggested that human-induced changes in concentrations and elemental ratios of nutrients in river water affect epilithic bacterial community structure in the Mahoning River (USA). Other studies have reported that epilithic bacterial community composition varies across reaches (Lyautey et al. 2003), seasons (Brümmer et al. 2000, 2003, Araya et al. 2003), or rivers (Brümmer et al. 2000, 2003, Fazi et al. 2005), presumably in response to nutrient inputs and the alteration of the biogeochemical state of the rivers. In addition to chemical factors, Anderson-Glenna et al. (2008) reported that seasonal and interannual changes in temperature significantly affect bacterial community composition. However, the geographical extent of data obtained in previous studies of epilithic communities is quite limited, which hampers thorough examinations of the patterns and controls of spatial variation in epilithic bacterial community composition along entire river stretches.

The objective of the present study was to examine potential environmental factors affecting the longitudinal distributional patterns of epilithic bacterial communities in 2 rivers (Yasu and Ado Rivers) draining the Lake Biwa basin, Japan. Phylogenetic analyses were conducted using denaturing gradient gel electrophoresis (DGGE) of 16S rRNA genes and fluorescence *in situ* hybridization (FISH) with oligonucleotide probes targeted to small subunit rRNA.

MATERIALS AND METHODS

Study sites and sampling of epilithic materials. On 28 and 29 May 2004, we collected samples in the Yasu River, which drains the basin of Lake Biwa, Japan (basin area 3848 km^2) (Fig. 1). Among the 19 stations deployed in the main channel, 2 were close (<400 m) to the discharge site of major dams, including the Yasu (Stn Y4) and Ohzuchi dams (Stn Y6) (see Appendix 1, Table A1; Fig. 1). Four additional stations were located in major tributaries, near the points of confluence. The Yasu River drains a watershed (387 km^2) in which the proportions of agricultural fields (0 to 31%) and residential areas (0 to 10%) tend to increase from the headstream to the river mouth and which houses an average population density of 320 people per km^2 (land use and demographic analyses of the watersheds were conducted according to Kohzu et al. (2008a). On 27 and 28 September 2004, we collected samples in the Ado River (see Appendix 1, Table A2; Fig. 1). The Ado and its watershed (306 km^2) are similar in size to the Yasu but are much less densely populated (25 people per km^2) and more extensively covered by forest (96% of the entire watershed). Eleven stations were located in the main channel, and 3 additional stations were deployed in major tributaries. For both rivers, samples were collected during the period of baseline flow, i.e. the water level during sampling was within ±0.3 m of the annual average water level (Ministry of Land, Infrastructure and Transportation, Japan; www1.river.go.jp/).

At each station, 4 cobbles with linear dimensions of 10 to 20 cm were sampled in the middle of the main channel (depth = 10 to 30 cm) with a relatively high flow rate (i.e. in a riffle). Although we did not examine types and chemistry of cobbles, the geological chart of the Lake Biwa basin (Geological Survey, Japan; riodb02.ibase.aist.go.jp) indicates that basal rocks of the Yasu River watershed mainly consist of sedimentary rocks throughout the main channel, although a granitic zone exists in the southern part of the watershed. In the Ado River, the dominant type of basal rocks is the accretionary complex (mainly sedimentary rocks) at the upstream site, whereas it is sedimentary at the downstream sites. Epilithic material from a 5 × 5 cm area was scraped off the top surface of each cobble using a toothbrush and then suspended in autoclaved Milli-Q water. The samples collected from all 4 cobbles were combined into a sterile, high-density polyethylene tube (Falcon) and kept on ice and in the dark for later analysis in the laboratory.

On-site determination of physical and chemical variables. Water temperature, dissolved oxygen (DO), and specific conductivity (EC) were measured using a Model 85 SCOOT meter (YSI/Nanotech). Current velocity was measured by using flow meters: a Pocket Tachometer

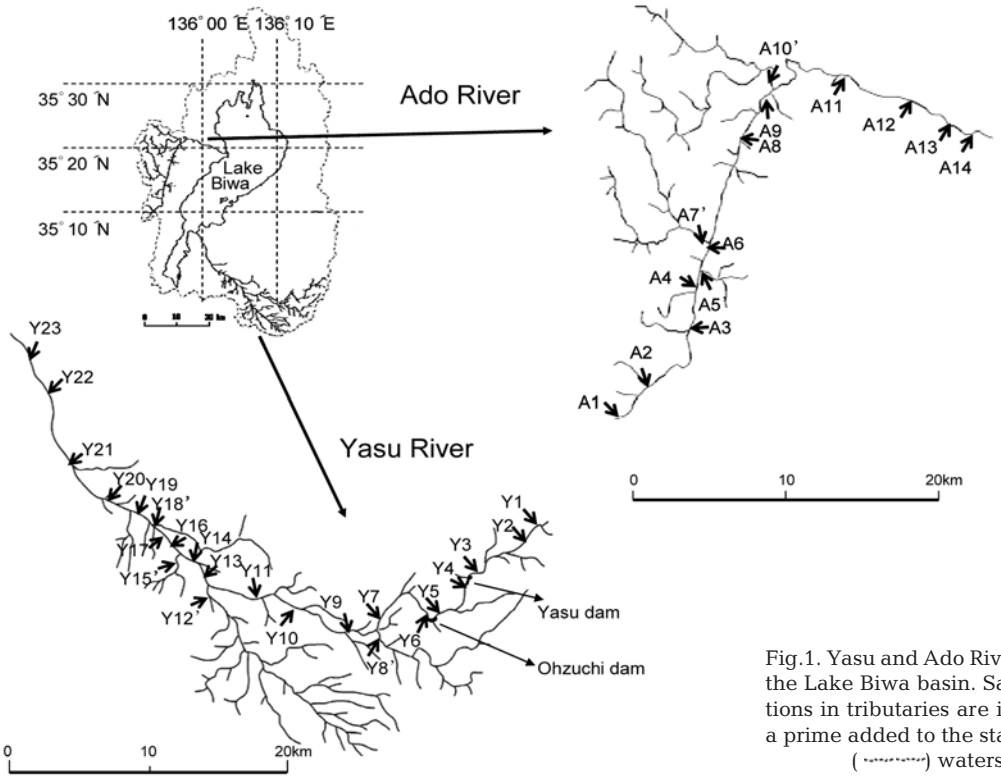

Fig.1. Yasu and Ado Rivers draining the Lake Biwa basin. Sampling stations in tributaries are indicated by a prime added to the station code; (·····) watershed

3631 (Yokogawa) in the Yasu, and a Stanley Model AEM1-D (Alec Electronics) in the Ado. pH and turbidity were determined with a pH meter (D-21ACT, Horiba) and a turbidometer (2100P, Hach), respectively.

Chemical analyses of river water. River water samples were filtered through 150 µm meshed screen and contained in polyethylene tanks for the measurement of particulate constituents (see below). For the measurements of nutrients and dissolved organic carbon (DOC), sample waters were filtered through precombusted (450°C, 5 h), acid-washed glass fiber filters (GF/F, Whatman). Filtrates were contained in acid-washed polystyrene (for nutrients) or polycarbonate (for DOC) bottles, kept cool, and brought back to the laboratory to be frozen for later analysis, except the analysis of ammonium concentration, which was conducted within 12 h (not frozen). Concentrations of nitrate, nitrite, and soluble reactive phosphorus (SRP) were determined with an auto-analyzer (AACS II, BRAN+LUEBBE) according to the manufacturer's manual. Ammonium concentration was determined fluorometrically with a TD700 fluorometer (Turner Designs) according to Holmes et al. (1999). Total dissolved nitrogen (TDN) and phosphorus (TDP) were measured by the wet oxidation method of Pujo-Pay & Raimbault (1994). Concentrations of particulate organic carbon (POC) and nitrogen (PON) were measured using a CHN analyzer (PE-240II, Perkin Elmer), whereas particulate phosphorus (PP) was determined as SRP after wet oxidization. The sum of the dissolved and particulate constituents was regarded as the concentration of total nitrogen (TN = TDN + PON) or total phosphorus (TP = TDP + PP). Concentration of DOC was analyzed with a Shimadzu TOC 5000A total organic carbon analyzer according to Kim et al. (2006). Biological oxygen demand (BOD) was determined as the decrease of dissolved oxygen concentration (determined by the dissolved oxygen meter; YSI 5100, YSI/Nanotech) in river water contained in BOD bottles during the incubation period of 5 d at 20°C.

Chemical analyses of epilithic materials. Epilithic materials were collected on precombusted GF/F filters. Concentrations of chlorophyll *a* were determined fluorometrically after extraction with acetone (Wetzel & Likens 2000). Concentrations of organic carbon (POC_{epi}) and nitrogen (PON_{epi}) and nitrogen isotopic compositions were determined using a continuous flow isotope ratio mass spectrometer (delta S, Finnigan) connected to the elemental analyzer (EA1108, Fisons) (Kohzu et al. 2008). The nitrogen stable isotope ratio was calculated in δ notation ($\delta^{15}N$) as the difference in parts per thousand (δ) from the standard:

$$\delta^{15}N = (R_{sample}/R_{standard} - 1) \times 1000$$

where R_{sample} and $R_{standard}$ are $^{15}N/^{14}N$ values of the sample and standard (atmospheric nitrogen), respectively.

DNA extraction. DNA contained in epilithic materials was extracted using the cetyl trimethyl ammonium bromide (CTAB) method (Murray & Thompson 1980) with modifications. Sodium dodecyl sulfate (SDS; 1%) and 0.2 mg ml^{-1} Proteinase K were added to the suspension of epilithic materials. After incubation at 65°C for 30 min, 1.0 M NaCl and 1% CTAB were added and incubated at 65°C for 30 min. DNA was extracted using an equal amount of chloroform:isoamyl-alcohol (CIAA; 24:1). The aqueous phase was concentrated using a microconcentrator (10 000 × *g*, 5 min, 4°C; Himac CF 15D2, Hitachi) to remove humic acids. After centrifugation (10 000 × *g*, 5 min, 4°C), the supernatant was transferred to new tubes. DNA was precipitated by adding 2.5 × the volume of 100% ethanol. To remove the ethanol, the DNA pellet was rinsed with 70% alcohol, dried, and suspended with Tris-EDTA (TE; pH 8.0). After RNase (0.1 mg ml^{-1}) treatment at 37°C for 1 h, the pellet was extracted with an equal amount of phenol:chloroform:isoamyl-alcohol (PCA; 25:24:1) and CIAA. After centrifugation (10 000 × *g*, 5 min, 4°C), the aqueous phase was transferred to new tubes, to which 0.3 M acetic acid and 2.5 × the volume of 100% ethanol were added. After centrifugation (10 000 × *g*, 5 min, 4°C), the ethanol was removed. The DNA pellet was resuspended in TE and then stored at –30°C until analysis. The variable regions were amplified using the following primers (Sigma-Aldrich): GC-341F (5′-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GCC TAC GGG AGG CAG CAG-3′), and 907R (5′-CCG TCA ATT CCT TT G AGT TT-3′) (Muyzer et al. 2004). The reaction mixture (25 µl) contained 5 µl of DNA extract in TE (10 to 20 ng DNA, determined for selected samples), 2.5 µl of PCR buffer, deoxynucleotide (Blend Taq kit, Toyobo), 3 µl of 5 pmol each of the universal primers (GC-341F and 907R, Sigma-Aldrich), and 0.2 µl of 2.5 U polymerase (Blend Taq kit, Toyobo). Thermocycling (iCycler, Bio-Rad) was preceded by a 3 min heating step at 95°C, followed by 32 cycles of denaturing at 95°C for 30 s, annealing at 52°C for 30 s, and extension at 72°C for 60 s, with a final extension step of 8 min at 72°C. The PCR product was quantified by agarose gel electrophoresis using a standard (molecular mass ruler, Takara Bio).

Denaturing gradient gel electrophoresis. DGGE was performed using the D-Code Universal Mutation Detection System (Bio-Rad) (Muyzer et al. 2004). The gel contained a gradient of denaturant ranging from 20 to 60% (100% denaturant was 7 M urea and 40% deionized formamide) in 1× TAE (40 mM Tris, 20 mM acetic acid, 1 mM EDTA). The PCR products (250 ng) containing loading buffer (Bio-Rad) were applied using markers (gel loading dye, DCode, Bio-Rad). DGGE was run at 50 V for 16 h at 60°C. The gel was stained with 1× SYBR Gold (Molecular Probes) for detection (image acquisition with a CCD camera under UV illumination) and positioning (EDAS120 version 3.0, Kodak Digital Science) of DNA bands. Samples were run twice using a different order of sample loading. The bands detected in one of these runs (10% of the total number of bands) were not included in the sequencing and the analysis of banding patterns to reduce errors associated with run-to-run variations in the detection of bands.

DNA was extracted from the gel by incubation in TE at 4°C for 24 h, reamplified using PCR (see above), run on DGGE, and then sequenced (see below). When sequencing was not successful, DGGE bands were cloned using the pGEM-T Vector System (Promega) before sequencing.

Sequencing and phylogenetic analysis. Sequencing of Yasu River samples was conducted using a DYEnamic direct cycle sequencing kit (Amersham Life Science) and a 373 DNA Sequencer (Applied Biosystems); sequencing of samples collected in the Ado River was outsourced (Macrogen). The sequencing primers were GC-341F and 907R (Muyzer et al. 2004). The sequence data were compared to those in GenBank using the BLAST function of the DNA Data Bank of Japan (DDBJ) server (www.ddbj.nig.ac.jp/Welcome-j.html), and the Classifier facility in the Ribosomal Database Project (rdp.cme.msu.edu/classifier/classifier.jsp). See Table 4 for GenBank accession numbers.

Determination of total prokaryotic abundance and fluorescence *in situ* hybridization analysis. Samples were fixed with 2% (final concentration) paraformaldehyde. The suspension of epilithic materials was mixed with 0.1% sodium pyrophosphate (1:9) and homogenized using Stomacher 80 (Biomaster Lab System, 10 s) and an ultrasonic bath (Branson 5510, 1 min); this procedure has been reported to yield the maximum cell abundance for counting biofilm samples (Buesing & Gessner 2002). The homogenate was filtered onto 0.2 µm polycarbonate membranes (Whatman).

Total prokaryotic abundance was counted by epifluorescence microscopy (BX61, Olympus) after staining with 10 µg ml^{-1} 4′6-diamino-2-phenylindole (DAPI) for 10 min. Bacterial community composition was determined using the FISH method with rRNA-targeted oligonucleotide probes (Nishimura & Nagata 2007). The hybridization solution contained 2.5 µg ml^{-1} probe, 0.9 mol l^{-1} NaCl, 20 mmol l^{-1} Tris-HCl (pH 7.2), 5 mmol l^{-1} EDTA, 0.01% SDS, and the concentration of formamide determined to achieve specificity for the bacterial groups targeted by the different probes (Table 1). Unlabeled competitors were added for probing *Beta-* and *Gammaproteobacteria*. All probes were commercially synthesized and labeled with Cy3 (Operon). After hybridization, the samples were transferred to a wash solution con-

taining 20 mmol l^{-1} Tris-HCl (pH 7.2), 5 mmol l^{-1} EDTA, 0.01% SDS, and the concentration of NaCl appropriate for each probe. The DAPI-positive cells and hybridized cells were counted manually under an epifluorescence microscope (Olympus BX50) equipped with optics described by Nishimura & Nagata (2007). For each subgroup, 20 microscopic fields (1164.9 μm^2 per field) were examined.

Statistical analyses of community patterns. The similarity of DGGE profiles derived from different bacterial communities was assessed using Bray-Curtis similarity (Quinn & Keough 2002). Patterns in the sample-by-sample matrix of Bray-Curtis similarity were examined using non-metric multidimensional scaling (NMDS) (Quinn & Keough 2002). The degree to which the plot matched the underlining similarity matrix was assessed using the Kruskal stress value formula. We performed 20 separate NMDS calculations for each plot using random starting configurations, and the best solution was that which produced the lowest stress. The BIOENV procedure (Clarke & Warwick 2001) was used to define the suite of environmental variables that best explained the bacterial assemblage structure. BIOENV analyzes the rank correlation between Euclidean distance similarity matrices for environmental variables and the Bray-Curtis similarity matrix computed for the DGGE fingerprint. These correlations were repeated for all possible combinations of the measured environmental variables (18 variables). The suite of environmental variables (or a single variable) that produced the highest correlation was considered the set that most accurately explained the community pattern. The above statistical analyses were conducted using PRIMER-E (version 6). NMDS and BIOENV have advantages owing to their lack of assumptions, but they also have their own weaknesses stemming from iteractive algorithms placing most weight on large distances (Clarke & Warwick 2001). Thus, we performed canonical correspondence analysis (CCA) to examine the relationship between the DGGE fingerprint and environmental variables using CANOCO 4.5 (ter Braak & Smilauer 2002).

RESULTS

Environmental variables

The physical, chemical, and microbiological parameters of the 2 rivers are summarized in Appendix 1, Tables A1 & A2. In the Yasu River, high concentrations of total nitrogen (TN; maximum value of 103 µM at Stn Y12′), nitrate (89 µM, Stn Y12′), ammonium (3.9 µM, Stn Y18′), total phosphorus (TP; 3.1 µM, Stn Y18′), and soluble reactive phosphorus (SRP; 1.4 µM, Stn Y18′) in the middle to downstream reaches indicated nutrient pollution (Appendix 1, Table A1). The average concentrations of TP, SRP, and electric conductivity (EC) at the downstream sites were significantly higher ($p < 0.05$) than those at the upstream sites (Table 2). Organic pollution in the middle reach was evident from significantly higher ($p < 0.05$) values of dissolved organic carbon (DOC) and biological oxygen demand (BOD) in the downstream sites (mean ± SE; 144.6 ± 9.6 µM and 0.8 ± 0.1 mg l^{-1} for DOC and BOD, respectively) than the upstream sites (64.3 ± 10.5 uM and 0.4 ± 0.1 mg l^{-1}) (Table 2). The amounts of organic carbon, nitrogen, and chl *a* per unit surface area of cobbles in the downstream sites were significantly higher ($p < 0.05$) than those in the upstream sites (Table 2). $\delta^{15}N$ of epilithic materials tended to increase downstream, with the lowest value at the upstream site (–2.5, Stn Y2) and the highest at the downstream sites (8.3, Stns Y17 and Y18′). These environmental variables along the entire Ado River were low and less variable compared to the data obtained in the Yasu River (Table 2 & Appendix 1, Table A2).

Spatial patterns in bacterial community composition revealed by DGGE and possible relationships with environmental factors

Spatial patterns in bacterial community composition within and between the 2 rivers were examined using NMDS analysis of the DGGE fingerprints (an example of the DGGE gel is presented in Appendix 1, Fig. A1.

Table 1. Probes used to examine the abundance of major prokaryotic groups in epilithic biofilms

Phylogenetic group	Probe	Probe sequence	Formamide conc. (%)	Source
Eubacteria	Eub338	GCT GCC TCC CGT AGG AGT	30	Amann et al. (1995)
Alphaproteobacteria	Alfa968	GGT AAG GTT CTG CGC GTT	30	Glöckner et al. (1999)
Betaproteobacteria	Beta42	GCC TTC CCA CTT CGT TT	30	Manz et al. (1992)
Gammaproteobacteria	Gam42a	GCC TTC CCA CAT CGT TT	30	Manz et al. (1992)
***Cytophaga– Flavobacteria* cluster**	CF319	TGG TCC GTG TCT CAG TAC	35	Manz et al. (1996)
Actinobacteria	HGC69a	TAT AGT TAC CAC CGC CGT	20	Glöckner et al. (2000)
Archaea	Arch915	GTG CTC CCC CGC CAA TTC CT	20	Amann et al. (1995)

We found 3 distinct clusters, each composed of specific geographical locations: the upstream reaches of the Yasu River (Cluster I), the downstream reaches of the Yasu River (Cluster II), and the Ado River (Cluster III, excluding the most upstream station, Stn A1) (Fig. 2). Because the goodness of fit of the stress value for the ordination with 2 dimensions was low (0.14), distances in the NMDS ordination reflect the magnitude of the dissimilarity in DGGE patterns (Clarke & Warwick 2001). The number of DGGE bands obtained for each station varied from 17 to 32 in the Yasu River and from 16 to 28 in the Ado River; in total, we detected 96 bands in the 2 rivers. Among these bands, 15 (16%), 7 (7%), and 21 (22%) were unique to Clusters I, II, and III, respectively, and the remaining 53 bands (56%) were common to 2 or 3 of the geographical clusters distinguished by NMDS.

Table 2. Summary of environmental variables in the Ado and Yasu Rivers. Variables are averaged for the upstream and downstream sites of each river in order to examine downward trends. Upstream sites of the Ado: Stns A1 to A7′, n = 7; and the Yasu: (Stns Y1 to Y12′, n = 12). Downstream sites of the Ado: Stns A8 to A14, n = 7; and the Yasu: Stns Y13 to Y23, n = 11. Student's *t*-test of the null hypothesis that the mean value of the upstream sites is equal to the mean value of the downstream sites; ns: not significant ($p > 0.05$)

Variables	River	Upstream Mean ± SE	Downstream Mean ± SE	p
Physical				
Water temperature (°C)	Ado	16.3 ± 0.6	20.1 ± 0.5	0.002
	Yasu	18.1 ± 1.0	22.2 ± 0.6	0.003
Flow rate (cm s^{-1})	Ado	48.7 ± 8.0	43.8 ± 7.9	ns
	Yasu	49.8 ± 9.8	35.9 ± 7.0	ns
Chemical (water)				
pH	Ado	7.3 ± 0.2	7.4 ± 0.0	ns
	Yasu	7.9 ± 0.1	8.1 ± 0.3	ns
EC (µS cm^{-1})	Ado	45.2 ± 6.3	58.1 ± 0.8	ns
	Yasu	98.4 ± 11.6	207.8 ± 28.7	0.001
Turbidity (NTU)	Ado	8.2 ± 3.4	4.0 ± 0.3	ns
	Yasu	2.1 ± 0.5	3.1 ± 0.5	ns
TN (µM)	Ado	47.5 ± 3.0	66.6 ± 4.7	0.005
	Yasu	67.9 ± 7.4	84.1 ± 2.9	ns
TP (µM)	Ado	0.8 ± 0.3	0.5 ± 0.0	ns
	Yasu	0.3 ± 0.1	1.2 ± 0.2	0.000
BOD (mg l^{-1})	Ado	0.3 ± 0.1	0.4 ± 0.0	ns
	Yasu	0.4 ± 0.1	0.8 ± 0.1	0.000
DOC (µM)	Ado	49.6 ± 2.5	103.3 ± 15.2	0.012
	Yasu	64.3 ± 10.5	144.6 ± 9.6	0.000
Nitrate (µM)	Ado	25.9 ± 2.5	39.5 ± 2.1	0.001
	Yasu	61.1 ± 6.9	60.0 ± 3.3	ns
Ammonium (µM)	Ado	0.3 ± 0.1	0.4 ± 0.1	ns
	Yasu	0.3 ± 0.1	1.0 ± 0.4	ns
SRP (µM)	Ado	0.4 ± 0.1	0.3 ± 0.0	ns
	Yasu	0.2 ± 0.1	0.7 ± 0.1	0.000
Chemical and biological (epilithic)				
Bacterial abundance	Ado	16.0 ± 4.1	30.2 ± 10.1	ns
($\times 10^6$ cells cm^{-2})	Yasu	25.0 ± 6.1	68.6 ± 9.1	0.001
Chl *a* (µg cm^{-2})	Ado	1.4 ± 0.4	3.2 ± 0.8	ns
	Yasu	1.6 ± 0.6	5.3 ± 1.5	0.033
POC_{epi} (mg cm^{-2})	Ado	0.135 ± 0.028	0.241 ± 0.053	ns
	Yasu	0.199 ± 0.055	0.453 ± 0.074	0.011
PON_{epi} (mg cm^{-2})	Ado	0.018 ± 0.004	0.034 ± 0.007	ns
	Yasu	0.027 ± 0.007	0.066 ± 0.012	0.011
C/N	Ado	9.3 ± 0.8	8.5 ± 0.6	ns
	Yasu	7.3 ± 0.1	7.2 ± 0.3	ns
$\delta^{15}N$ (‰)	Ado	0.4 ± 0.5	1.9 ± 0.8	ns
	Yasu	1.1 ± 0.7	6.5 ± 0.3	0.000

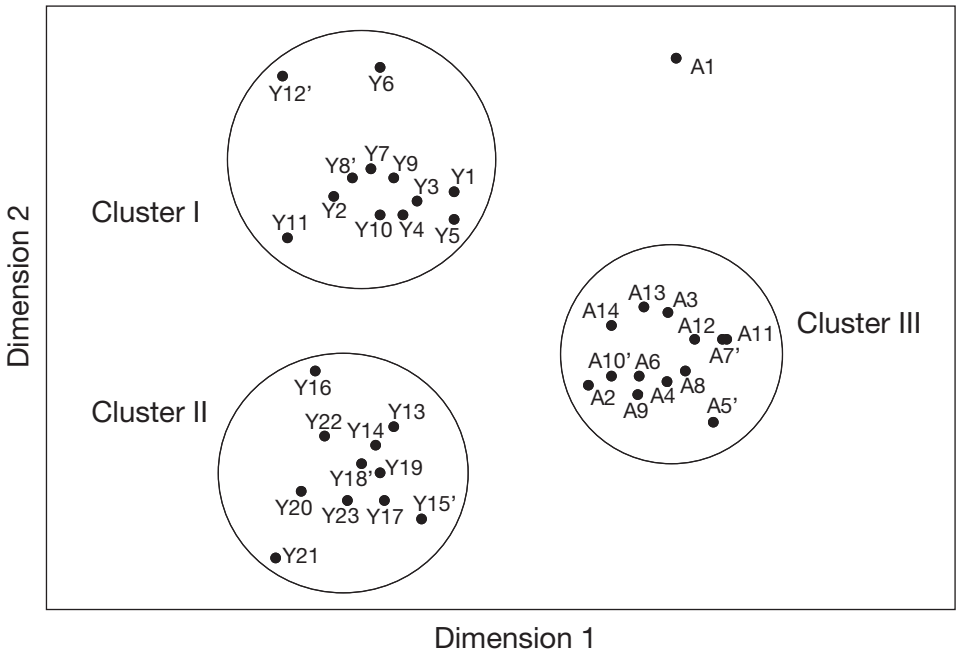

Fig.2. Non-metric multidimensional scaling analysis of the DGGE fingerprint (stress value = 0.14). Three clusters corresponded to geographical locations: Cluster I: upstream sites of the Yasu River (Stns Y1 to Y12'); Cluster II: downstream sites of the Yasu River (Stns Y13 to Y23); and Cluster III: Ado River (Stns A2 to A14, A1 excluded). See Fig. 1 for station locations

The BIOENV procedure (Clarke & Warwick 2001) was used to examine possible relationships between bacterial community composition and environmental variables (see Table 2). Within the entire dataset obtained from the Yasu and Ado Rivers, the highest correlation (ρ_w = 0.479) between bacterial composition and environmental variables was found for the combination of EC, TP, and nitrate (Table 3). The same analysis was conducted for the Yasu, and the highest correlation (ρ_w = 0.512) was found for the $\delta^{15}N$ of epilithic materials (Table 3). The second highest correlation (ρ_w = 0.510) was obtained for the combination of DOC and $\delta^{15}N$ (Table 3).

We also conducted CCA (ter Braak & Smilauer 2002) to explore spatial patterns in community structure and their possible relationships with environmental variables (Fig. 3). Among 18 variables determined (Table 2), 9 were selected by forward selection, i.e. variables were added until model improvement failed ($p < 0.05$). The selected variables, from strong to weak statistical significance (in parentheses), judged by Monte-Carlo permutation tests, were: nitrate ($p = 0.002$), $\delta^{15}N$ ($p = 0.002$), pH ($p = 0.002$), temperature ($p = 0.004$), TP ($p = 0.004$), SRP ($p = 0.010$), EC ($p = 0.014$), TN ($p = 0.04$), and DOC ($p = 0.048$). The sum of all eigenvalues ($\Sigma\lambda_i$) was 3.156, whereas the sum of all canonical eigenvalues ($\Sigma\lambda_{canonical}$) was 1.366. The variance in DGGE (ribotypes) explained by the environmental variables ($\Sigma\lambda_{canonical} / \Sigma\lambda_i \times 100$) was 43%. These results are generally consistent with the results obtained from the BIOENV analysis conducted for the entire dataset (Table 3), except that temperature was selected in the CCA but not in the BIOENV analysis. The relative positioning of the sampling sites in the ordination plot indicated the presence of 3 geographical clusters, which agrees well with that in the NMDS plot (Fig. 2).

Table 3. Results of the BIOENV analysis defining a subset of environmental variables that best explains the community structure. The analysis was conducted for the entire dataset obtained from the Yasu and Ado Rivers as well as those obtained only in the Yasu. A series of a single or a combination of environmental variables is presented according to the rank (low = 1, high = 5) of the magnitude of correlation between the community and environmental data matrices. Eighteen environmental variables (see Table 2) were used. Bac: bacterial abundance in the epilithon

Sites	Rank	Correlation (ρ_w)	Variables
Yasu + Ado	1	0.479	EC, TP, Nitrate
	2	0.468	pH, EC, TP, Nitrate
	3	0.467	EC, TP
	4	0.458	pH, EC, $\delta^{15}N$, TP, Nitrate
	5	0.457	pH, $\delta^{15}N$, Nitrate
Yasu only	1	0.512	$\delta^{15}N$
	2	0.510	$\delta^{15}N$, DOC
	3	0.508	Bac, $\delta^{15}N$, DOC
	4	0.500	Bac, $\delta^{15}N$, BOD, DOC
	5	0.498	Bac, BOD, DOC

Phylogenetic analyses of DGGE bands and biogeography of ribotypes

We successfully sequenced 25 DGGE bands. Among these, 20 ribotypes were matched to particular sequences of identified bacteria at a level of 97% similarity, all of which were assigned to major phylogenetic groups based on the most similar sequences in the database (Table 4). The most frequently sequenced phylogenetic groups were *Cyanobacteria* (32%), followed by *Betaproteobacteria* (28%), *Bacteroidetes* (20%), *Alphaproteobacteria* (12%), and *Gammaproteobacteria* (8%). Plastids were not detected.

Some ribotypes were detected at multiple sites covering the entire stretches of the Yasu and Ado Rivers. These ribotypes were affiliated with *Alphaproteobacteria* (ID 1, 2 and 3; see Table 4 for the ID description), *Betaproteobacteria* (ID 4, 7 & 9), *Gammaproteobacteria* (ID 12), *Bacteroidetes* (ID 15), and *Cyanobacteria* (ID 20 & 25). Other ribotypes displayed more restricted distributions: 8 ribotypes were found only in the Ado River (*Betaproteobacteria*, ID 5; *Bacteroidetes*, ID 13 & 16; *Cyanobacteria*, ID 19, 21, 22, 23 & 24), whereas 3 ribotypes were detected only in the Yasu River (*Betaproteobacteria*, ID 6 & 8; *Gammaproteobacteria*, ID 11). One ribotype affiliated with *Bacteroidetes* (ID 14, closest affiliation with 96% similarity is uncultured *Flavobacterium* sp.) was only detected at the less polluted sites, i.e. the sites upstream of the Yasu and Ado Rivers.

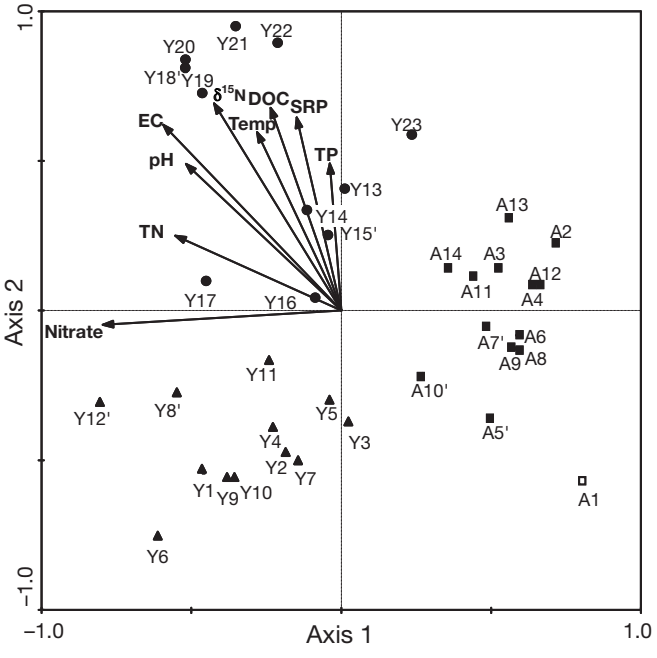

Fig. 3. CCA ordination of bacterial communities in relation to measured environmental variables (high-lighted in bold). The length and angle of the vectors relative to the axes indicate correlations of sampling sites with each environmental variable selected by forward selection. The relative positioning of the sampling sites is constrained by the set of environmental variables. ▲: Cluster I; ●: Cluster II; ■: Cluster III; □: Stn A1. Eigenvalues for Axes 1 and 2 are 0.410 and 0.329, respectively. The percentage of variance of ribotype data explained by Axes 1 and 2 are 13.0 and 9.6%, respectively, whereas the corresponding values of the ribotype–environment relationship are 30.0 and 22.2%

Spatial distribution of the major phylogenetic groups of prokaryotes and correlations with environmental variables

Prokaryotic community structure at the level of the domain or major phylogenetic group (division or subdivision) was analyzed using FISH (Fig. 4). For 11 out of 37 samples, we failed to obtain data because of high background fluorescence. Community structure did not exhibit systematic variation among the geographical clusters distinguished by NMDS and CCA of the DGGE bands (i.e. Clusters I, II, and III). The relative abundances of individual phylogenetic groups averaged for each cluster did not differ significantly among clusters (ANOVA, $p > 0.05$) (Table 5). The mean relative abundance of the domain *Bacteria* (Eub338 positive cells) was 78 ± 6%, but only 1 ± 1% for *Archaea* (Arch915 positive cells). Among *Bacteria*, *Alphaproteobacteria* were the most abundant group (34 ± 6%), followed by *Betaproteobacteria* (20 ± 6%), *Gammaproteobacteria* (8 ± 8%), and the *Cytophaga–Flavobacteria* cluster (CF cluster; 7 ± 5%). *Actinobacteria* were less abundant (<3%) at all examined sites. The relative abundance of each group averaged for each river is listed in Table 6.

Gammaproteobacteria were abundant at sites located just downstream of the dams, with relative abundances of 30 and 37% at Stn Y4 (downstream of the Yasu dam) and Stn Y6 (Ohzuchi dam), respectively (Fig. 4a). The relative abundance of the CF cluster varied greatly (2 to 26%); higher values (>10%) occurred in the upstream reach of the Ado River (Stns A2, A4 and A6) (Fig. 4b). Results of simple correlation analyses indicated that only the following variables were significantly correlated: *Alphaproteobacteria* and total bacterial abundance in the epilithon ($r = 0.49$, $p = 0.01$, $n = 26$), *Betaproteobacteria* and PON_{epi} ($r = 0.54$, $p = 0.004$, $n = 26$), and *Gammaproteobacteria* and POC_{epi} ($r = 0.42$, $p = 0.035$, $n = 26$).

DISCUSSION

We investigated broad-scale distribution patterns of epilithic bacterial communities and correlated these with extensive measurement of environmental vari-

Table 4. Sequences of partial 16S rRNA genes obtained from the DGGE bands. Station numbers at which a sequenced band was obtained are indicated by bold and underlined letters. In some cases, bands at the same position were sequenced at more than 2 stations, which resulted in the acquisition of the same sequences. In such cases, more than 2 station numbers are indicated by bold and underlined letters. Station numbers with regular letters indicate that a band was detected at the position of sequenced band(s). Identification numbers (ID) of OTUs are used to indicate the identity of individual ribotypes or bands on a gel (Fig. A1)

ID	Stns	Phylogenetic group	Closest affiliation	% Similarity	Source	Accession number
1	Y1, Y3, Y4, Y5, Y7, Y8′, Y9, Y10, Y13, Y14, Y23,A2, A3, A4, A5′, A6, A7′, A8,A9, A10′, A11, **A12**, **A13**, A14	*Alphaproteobacteria*	Alpha proteobacterium (AF531000)	97	Bruns et al. (2003)	AB275540
2	**Y5**, Y7, Y10, Y11, Y13, Y15′, Y17, Y19, Y21, Y23, A1, **A4**	*Alphaproteobacteria*	*Sphingomonas* sp. (AB299691)	95	L. M. Shi et al. (unpubl. data)	AB275568
3	Y8′, Y9, Y10, **Y15′**, Y18′, **A4**, A5′, A7′, A8, A9, A10′, A11, A12, A13, A14	*Alphaproteobacteria*	Alpha proteobacterium (EU770258)	98	K. Watanabe (unpubl. data)	AB275576
4	Y1, Y2, Y3, Y4, Y5, Y6, Y7, Y11, **Y13**, Y16, Y17, Y18′, **Y19**, Y20, Y22, A1, A2, A3, A4, A5′, A6, A7′, A8, A9, A10′	*Betaproteobacteria*	Uncultured beta proteobacterium (AY509483)	95	Eiler & Bertilsson (2004)	AB275579
5	A1, A6, A7′, A11, A12, **A13, A14**	*Betaproteobacteria*	Beta proteobacterium (AB426582)	99	P. A. Vaishampayan et al. (unpubl. data)	AB275543
6	Y1, **Y2**, Y3, Y4, Y5, Y6, Y7, Y8′, Y9, **Y12′**	*Betaproteobacteria*	Uncultured *Ralstonia* sp. (EU706276)	99	H. Morisaki (unpubl. data)	AB275554
7	Y1, **Y2**, Y3, Y4, Y7, Y8′,Y9, Y10, Y11, Y13, Y14, Y15′, Y16, Y17, Y18′, Y19, 20, Y21, Y22, Y23, **A2,** A3, A6, A10′, A14	*Betaproteobacteria*	Uncultured *Comamonadaceae* bacterium (EF562235)	99	Sahl et al. (2008)	AB275555
8	Y1, Y2, Y3, Y4, Y5, Y6, **Y7**, Y8′, Y9, Y10, Y11, Y12′, Y16	*Betaproteobacteria*	Uncultured *Burkholderiales* bacterium (EF667920)	98	Fraune & Bosch (2007)	AB275561
9	**Y13**, Y14, Y15′, **Y16**, **Y17**, Y18′, Y19, Y20, Y21, Y22, Y23, A4, A5′, A6, A9,A10′, A12, A13, A14	*Betaproteobacteria*	*Aquaspirillum* sp. (AY780907)	97	Grabovich et al. (2006)	AB275573
10	Y1, Y2, Y3, Y4, Y5, Y8′, Y9, Y10, Y13, **Y23,** A2, A3, **A8**, A9, A10′, A14	*Betaproteobacteria*	Uncultured *Janthinobacterium* sp. (EF111066)	99	Hoshino et al. (2005)	AB275588
11	Y5, **Y8′**, **Y12′**, Y20	*Gammaproteobacteria*	Uncultured *Aeromonadaceae* bacterium (AF513938)	99	Donachie et al. (2004)	AB275570
12	Y1, Y2, Y3, **Y4**, Y6, Y8′, Y9, Y10, Y23, A3, A6, A12, **A13**	*Gammaproteobacteria*	Uncultured *Aeromonas* sp. (EU718222)	99	L. Han & Y. Zhao (unpubl. data)	AB275557
13	A1, **A3, A4**	*Bacteroidetes*	*Flexibacteraceae* bacterium (DQ640009)	98	G. Wieshammer et al. (unpubl. data)	AB275542
14	Y4, **Y6**, Y7, Y8′, Y9, Y10, Y11, A7′, A8, A10′, A14	*Bacteroidetes*	*Flexibacteraceae* bacterium (DQ839329)	96	S. Lin et al. (unpubl. data)	AB275558
15	Y3, **Y5**, Y13, Y14, Y17, Y18′, Y19, Y21,Y23, **A2**, A3, A4, A5′, A6, A7′, A8, A9, A10′, A11, A12, A13, A14	*Bacteroidetes*	Uncultured *Flectobacillus* sp. (AM941709)	93	R. Sommaruga & E. O. Casamayor (unpubl. data)	AB275578
16	A3, **A12**, **A13**, A14	*Bacteroidetes*	Uncultured *Cytophagales* bacterium (AF361199)	99	Simek et al. (2001)	AB275538

Table 4. (continued)

ID	Stns	Phylogenetic group	Closest affiliation	% Similarity	Source	Accession number
17	Y1, Y2, Y3, Y4, Y5, Y6, Y7, **Y8'**, Y9, Y10, Y11, Y12', Y14, **Y20**, A14	*Bacteroidetes*	Uncultured *Flectobacillus* sp. (AM946201)	98	A. Hervas (unpubl. data)	AB275562
18	Y1, Y3, Y4, Y6, **Y7, Y9**, Y10, A1, A3, A4, A13, A14	*Cyanobacteria*	Uncultured cyanobacterium (EF662346)	98	K. Jangid et al. (unpubl. data)	AB275559
19	A1, **A6**, **A7'**, A9, A13, A14	*Cyanobacteria*	Uncultured Antarctic cyanobacterium (EU032358)	99	Wood et al. (2008)	AB275527
20	**Y1**, Y2, Y4, **Y5,** Y7, Y8', Y9, Y10,Y11, Y12', Y13, Y14, Y22, A1, A12, A13	*Cyanobacteria*	Uncultured cyanobacterium (EF438248)	97	L. C. Kelly et al. (unpubl. data)	AB275566
21	**A5'**, **A6**	*Cyanobacteria*	Uncultured cyanobacterium (EU297382)	98	K. Jangid et al. (unpubl. data)	AB275523
22	**A3**, **A4**, A6, A7', A8, A10', A11, A12, A14	*Cyanobacteria*	Uncultured cyanobacterium (AY323170)	96	O. Pantos et al. (unpubl. data)	AB275535
23	**A3**, **A5'**, A7', A11	*Cyanobacteria*	Uncultured cyanobacterium (EU780335)	97	S. E. Godwin et al. (unpubl. data)	AB275515
24	A1, **A9**, **A11**, A12, A13	*Cyanobacteria*	Uncultured cyanobacterium (EF662391)	98	K. Jangid et al. (unpubl. data)	AB275534
25	Y1, Y2, Y3, Y4, Y6, Y7, Y8', Y9,Y10, **Y13**, **Y14**, Y16, Y17, Y18', Y19,Y20, Y22, Y23, A1, A6, A9, A10', A11, A13, A14	*Cyanobacteria*	Uncultured cyanobacterium (EF662346)	93	K. Jangid et al. (unpubl. data)	AB275582

ables. The examination of geographical patterns in bacterial community composition can lead to hypotheses regarding the dominant mechanisms by which bacterial communities are structured (Fierer et al. 2007, Beier et al. 2008). However, we were limited in our ability to fully capture the complexity of the controls of epilithic communities due to local conditions, including hydrodynamics of the surface boundary layer (Besemer et al. 2007) and biotic interactions (Sutherland et al. 2004, Huws et al. 2005). Mineral composition and texture of the cobble surfaces might also affect epilithic communities (Gleeson et al. 2006), although changing patterns in the community structure could not be accounted for by geological settings of the investigated region, i.e. bed rocks are mainly sedimentary throughout the regions (see 'Materials and methods'). Furthermore, our small sample sizes may not fully represent whole epilithic communities in individual river reaches. Despite these limitations, the PCR-DGGE fingerprints of epilithic bacterial communities revealed that the Yasu River can be divided into 2 stretches, composed of upstream (Stns Y1 to Y12′; Cluster I) and downstream (Stns Y13 to Y23; Cluster II) sites (Figs. 2 & 3). This clear shift in epilithic bacterial community composition at the middle reach of the river appears to be related to anthropogenic inputs of nutrients and organic matter from the watershed. In support of this hypothesis, the BIOENV results suggest a link between bacterial community composition and the $\delta^{15}N$ of epilithic materials ($\rho_w = 0.512$) (Table 3). The $\delta^{15}N$ of epilithic materials can serve as an indicator of the extent of sewage-derived nitrogen loading, because nitrate derived from sewage effluent is enriched with ^{15}N (Kendall et al. 2007), and this isotopic signal is transferred to primary producers such as epilithic algae through the utilization of nitrate (Cole et al. 2004, Kohzu et al. 2008a,b), although isotopic fractionation associated with denitrification in rivers may also result in ^{15}N-enrichment of nitrate (Kendall et al. 2007). The correspondence in the locations of discontinuity (between Stns Y12′ and Y13) in both bacterial community composition and increases in DOC concentrations also suggests that the organic matter loading from the watershed plays a role in shaping bacterial community structure. The combination of $\delta^{15}N$ and DOC ($\rho_w = 0.510$) clearly contributed to spatial variation in bacterial community composition in the Yasu River.

In contrast, we failed to detect any systematic, spatial patterns in community shifts along river stretches in the Ado River, with the exception that community composition at the most upstream site (Stn A1) differed from that at other sites in the NMDS (Fig. 2). Sampling from the Yasu and Ado Rivers was conducted during different seasons (Yasu in May and Ado

in September), which may in part explain the observed differences in community patterns between the 2 rivers. Previous studies have reported that bacterial community composition in epilithic biofilms exhibits seasonal variations, presumably in response to changes in physical conditions such as temperature (Hullar et al. 2006), and flow rate and substrate conditions (Olapade & Leff 2006). Differences in nutrient and organic matter loading from the watershed may also explain the variation in bacterial community structure between the 2 rivers. This hypothesis is consistent with the BIOENV results indicating that, for the entire dataset, indicators of anthropogenic effects on water chemistry (EC, TP, nitrate), rather than physical variables (temperature and flow rate), were selected as the set of environmental parameters most closely related to bacterial community composition (Table 3).

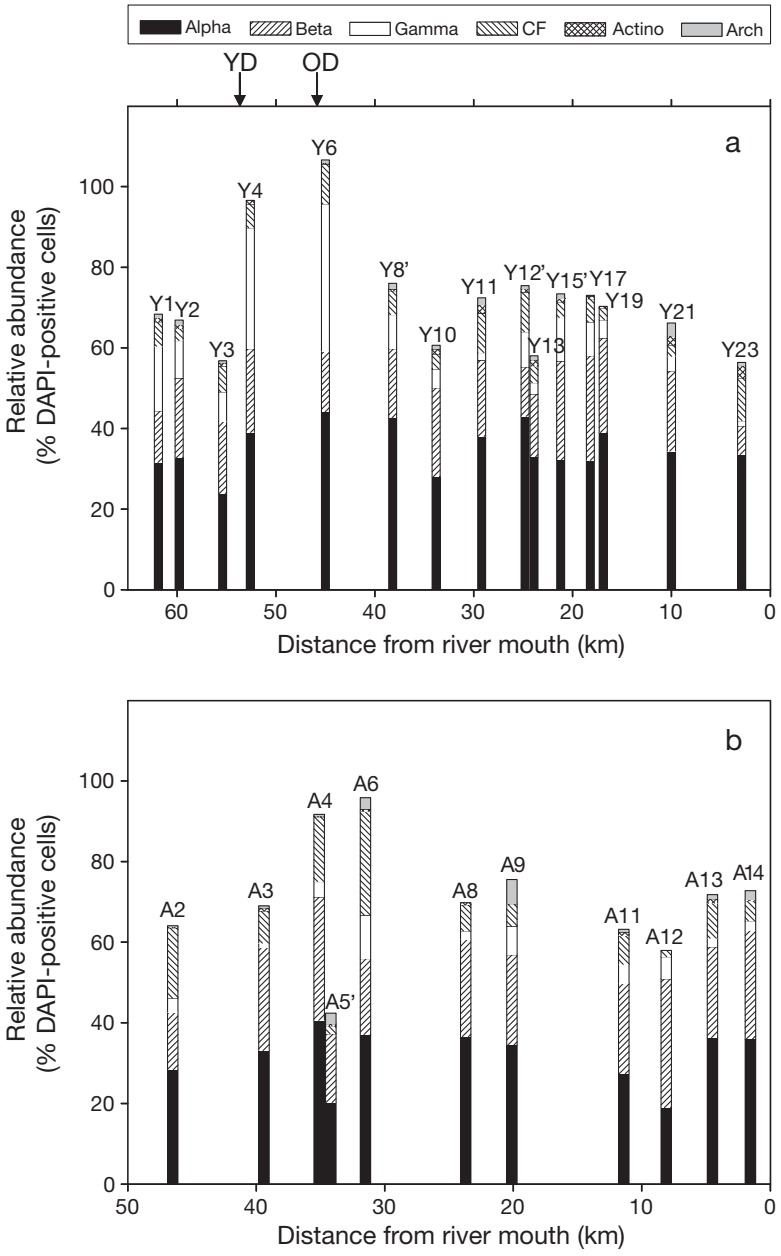

Fig. 4. Longitudinal variations of the relative abundance of different phylogenetic groups of prokaryotes in epilithic biofilms of the (a) Yasu and (b) Ado Rivers. Arrows indicate positions of the dams. YD: Yasu dam; OD: Ohzuchi dam; Alpha: *Alphaproteobacteria*; Beta: *Betaproteobacteria*; Gamma: *Gammaproteobacteria*; CF: *Cytophaga–Flavobacteria* cluster; Actino: *Actinobacteria*; Arch: *Archaea*. See Fig. 1 for location of sample stations given above bars

Epilithic bacterial community composition, as revealed by FISH, varied little among the different geographical clusters distinguished by the NMDS and CCA of DGGE fingerprints. Regardless of site, epilithic bacteria were dominated by *Alphaproteobacteria* and *Betaproteobacteria*: these 2 groups accounted for nearly half of the total prokaryotic cells. The differences observed between DGGE and FISH results may reflect differences in methodology. Community composition revealed by DGGE may include errors due to potential biases associated with PCR (Polz & Cavanaugh 1998), which can result in under- or over-representation of particular phylogenetic groups of bacteria (Castle & Kirchman 2004, Webster & Negri 2006). However, our data on nucleotide sequences of the DGGE bands indicated that *Alphaproteobacteria* and *Betaproteobacteria* accounted for a large fraction (40%) of the total number of sequenced bands, which was consistent with the results obtained by FISH. DGGE fingerprints may also be biased by the inclusion of plastid DNA extracted from epilithic algae. However, this effect was probably minor in our samples, because plastid DNA was not detected among the sequenced ribotypes. Thus, the differences in community patterns obtained by DGGE and FISH likely reflect differences in the taxonomic resolution of community analyses: spatial variability in community composition was much more pronounced at the finer phylogenetic level (as revealed by DGGE) than at the level of the major phylogenetic group (as revealed by FISH).

Previous studies have found that *Alphaproteobacteria* and *Betaproteobacteria* often dominate epilithic communities in river systems, including the Mahoning (Olapade & Leff 2004, 2006, Rubin & Leff 2007), Elbe (Brümmer et al. 2000), Spittelwasser (Brümmer et al. 2000), Albegna (Fazi et al. 2005), Fiora (Fazi et al. 2005), and Kanzaki (Araya et al. 2003)

Table 5. Summary of the relative abundances of different phylogenetic groups of prokaryotes including those affiliated with *Bacteria* (Eub), *Alphaproteobacteria* (Alpha), *Betaproteobacteria* (Beta), *Gammaproteobacteria* (Gamma), the *Cytophaga–Flavobacteria* cluster (CF), *Actinobacteria* (Actino), and *Archaea* (Arch). The mean ± SE (range) are given for the data obtained in each geographical cluster (Cluster I, II, or III) distinguished by the non-metric multidimensional scaling of the denaturing gradient gel electrophoresis patterns (Fig. 2). Corresponding values for the entire sampling site are also presented (Yasu + Ado). ANOVA indicated that, for all the phylogenetic groups examined, there was no significant ($p > 0.05$) difference in mean values of the relative abundance among different geographical clusters

Locations	n	% Total prokaryotic abundance						
		Eub	Alpha	Beta	Gamma	CF	Actino	Arch
Yasu (upstream) Cluster I	9	77 ± 5 (70–85)	35 ± 7 (23–40)	18 ± 3 (12–22)	13 ± 12 (2–37)	6 ± 2 (3–10)	1 ± 1 (0–2)	1 ± 0 (0–2)
Yasu (downstream) Cluster II	6	79 ± 4 (71–82)	36 ± 4 (32–47)	19 ± 8 (7–26)	6 ± 4 (1–11)	6 ± 3 (3–11)	1 ± 1 (0–3)	1 ± 1 (0–3)
Ado (all) Cluster III	11	77 ± 7 (67–87)	31 ± 7 (19–41)	23 ± 5 (14–32)	4 ± 3 (0–10)	9 ± 7 (2–26)	1 ± 0 (0–1)	2 ± 2 (0–6)
Yasu + Ado	26	78 ± 6 (66–87)	34 ± 6 (19–44)	20 ± 6 (7–32)	8 ± 8 (0–37)	7 ± 5 (2–26)	1 ± 1 (0–3)	1 ± 1 (0–6)

Rivers, although high abundances of other groups, including the CF cluster (O'Sullivan et al. 2002, Rubin & Leff 2007) and *Actinobacteria* (O'Sullivan et al. 2002) have also been reported (Table 6). In Italian rivers, Fazi et al. (2005) found that *Alphaproteobacteria* tend to become more abundant than *Betaproteobacteria* in epilithic biofilms in riffles, whereas the reverse was true for samples collected in pools of the same rivers. Our data confirm and supplement these previous results, which suggests that species and clones affiliated with a small number of the major phylogenetic groups, i.e. *Alphaproteobacteria* and *Betaproteobacteria*, can occupy a wide range of epilithic habitats. This assertion is consistent with the results of Brümmer et al. (2003), who found that *Betaproteobacteria* in biofilms of polluted rivers in Germany consisted of various clones exhibiting characteristic distributional patterns, most likely as a reflection of species- or clone-specific adaptations to environmental conditions.

Although community composition determined by FISH at the level of the broad phylogenetic group was less variable than that revealed by DGGE, the distribution of *Gammaproteobacteria*, which were highly abundant (>30% of total prokaryotes) at 2 sites downstream of the dams of the Yasu River, was noteworthy. *Gammaproteobacteria* consist of a dynamic group of bacteria (e.g. *Vibrionales*) characterized by multiple genotypes (Polz et al. 2006), fast growth (Yokokawa et al. 2004), adaptation to organically polluted waters (Watkins & Cabelli 1985), and active production of extracellular polymers that provide the matrices of biofilms (Hammer & Bassler 2003). Although these features might explain the high abundance of *Gammaproteobacteria*, future studies must identify which environmental conditions favor the occurrence of this bacterial group in epilithic communities downstream of dams. The CF cluster also displayed large variability in relative abundance (2 to 26%). Although the relative abundance of this phylogenetic bacterial group was not significantly correlated with any of the environmental variables studied, the CF cluster tended to be abundant (>20%) in the upstream reaches of the Ado River. Given that this group competitively uses high molecular weight organic carbon, including cellulose (Kirchman 2002), the observed pattern may reflect allochthonous organic carbon loading from the forested watershed of the Ado River.

In conclusion, bacterial community composition revealed by PCR-DGGE in epilithic biofilms of the Yasu River displayed a longitudinal pattern, possibly in response to anthropogenic nutrient- and organic matter-loading from the watershed. These results suggest that epilithic bacterial community composition can serve as a biotic indicator of pollution and eutrophication in rivers. FISH results revealed that *Alphaproteobacteria* and *Betaproteobacteria* dominated epilithic bacterial communities across both study reaches and rivers, indicating that species and clones affiliated with a limited number of major phylogenetic groups occupy diverse epilithic habitats, including both polluted and less polluted environments.

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Table 6. Relative abundance of the major phylogenetic groups of bacteria in epilithic biofilms in rivers. Data obtained by using artificial substratum (glass plates, ceramic tiles, and polycarbonate slides) are included. Abbreviations as in Table 5. Data were obtained by FISH using oligonucleotide probes targeted to small subunit rRNA except for the data obtained by the construction of clone library in the Taff River (the percentage of the occurrence of each phylogenetic group in the library is indicated in parentheses). Alpha: *Alphaproteobacteria*; Beta: *Betaproteobacteria*; CF: *Cytophaga–Flavobacteria*; Eub: *Eubacteria* nd; Gamma: *Gammaproteobacteria*; Actino: *Actinobacteria*; Arch: *Archaea*; nd: not determined

River	Country	Subsratum type	Dominant phylogenetic group	Relative abundance (%)							Source
				Eub	Alpha	Beta	Gamma	CF	Actino	Arch	
Yasu	Japan	Cobbles	Alpha	78.0 ± 1.2	35.2 ± 1.5	18.2 ± 1.3	10.3 ± 2.6	6.0 ± 0.7	1.2 ± 0.2	0.9 ± 0.2	Present study
Ado	Japan	Cobbles	Alpha/Beta	77.1 ± 2.1	31.7 ± 2.1	23.3 ± 1.6	3.9 ± 0.9	9.4 ± 2.2	0.5 ± 0.1	1.5 ± 0.5	Present study
Mahoning	USA[a]	Cobbles	Alpha/CF	52.9 ± 9.2	6.5 ± 0.9	4.0 ± 0.5	0.9 ± 0.1	4.6 ± 0.6	1.6 ± 0.3	1.6 ± 0.2	Olapade & Leff (2004)
Mahoning	USA[b]	Ceramic tiles	Alpha/CF	42.1 ± 12.3	4.1 ± 2.0	2.2 ± 0.4	1.1 ± 0.3	3.5 ± 0.9	1.3 ± 0.3	1.1 ± 0.3	Olapade & Leff (2006)
Mahoning	USA[c]	Cobbles									Rubin & Leff (2007)
West Branch				61.3 ± 12.3	21.2 ± 4.5	24.8 ± 4.7	19.7 ± 4.6	22.8 ± 7.7	nd	nd	
Upstream				24.7 ± 4.6	12.1 ± 3.1	13.4 ± 3.0	13.7 ± 3.7	10.6 ± 4.7	nd	nd	
Downstream				19.0 ± 3.2	11.0 ± 4.0	11.7 ± 3.2	12.4 ± 5.1	11.9 ± 5.6	nd	nd	
Elbe	Germany[d]	Glass plates	Beta	35.9 ± 1.5	9.2 ± 0.6	11.7 ± 1.8	3.1 ± 0.2	5.6 ± 0.4	2.2 ± 0.2	nd	Brümmer et al. (2000)
Spittelwasser	Germany[e]	Glass plates	Beta	43.2 ± 1.6	11.2 ± 0.6	20.6 ± 1.9	2.2 ± 0.2	4.8 ± 0.4	2.3 ± 0.1	nd	Brümmer et al. (2000)
Kanzaki	Japan[f]	Polycarbonate slides	Beta	77.6 ± 0.8	25.2 ± 1.4	43.4 ± 1.0	22.0 ± 0.7	31.1 ± 1.8	nd	nd	Araya et al. (2003)
Albegna	Italy	Stones (pool)	Alpha/Beta	30.4	5.6	7.9	2.1	1.2	nd	nd	Fazi et al. (2005)
		Stones (riffle)	Alpha	38.4	15.4	5.6	2.6	7.0	nd	nd	
Ente	Italy	Stones (pool)	Alpha/Beta	30.4	7.0	8.4	2.6	0.9	nd	nd	Fazi et al. (2005)
		Stones (riffle)	Alpha	38.4	21.4	3.5	1.4	1.4	nd	nd	
Fiora	Italy	Stones (pool)	Beta	30.4	7.7	14.0	0.9	2.3	nd	nd	Fazi et al. (2005)
Taff	UK[g]	Stones	(CF)	(98.3)	(19.1)	(7.9)	(13.1)	(24.9)	(13.0)	nd	O'Sullivan et al. (2002)

[a]Mean ± SE of the data obtained for every month during one year (n = 12)
[b]Mean ± SE of the seasonal measurement (n = 5)
[c]Mean ± SE of the seasonal measurement (n = 4)
[d]Mean ± SE of the monthly measurement (n = 11)
[e]Mean ± SE of the data obtained for every month during one year (n = 12)
[f]Mean ± SE of the seasonal measurement (n = 3)

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Appendix 1. Summaries of geographic, demographic and environmental variables of the Yasu and Ado rivers, and banding of the 16S rRNA gene amplicons on a DGGE gel for samples collected in the Ado River

Table A1. Geographic and demographic features and environmental variables at the sampling stations of the Yasu River. WT: water temperature; Flow: water flow rate; EC: electric conductivity; Chl *a*: chlorophyll *a* in epilithon; POC_{epi}: particulate organic carbon in epilithon; PON_{epi}: particulate organic nitrogen in epilithon; TN: total nitrogen in river water; TP: total phosphorus in river water; BOD: biological oxygen demand in river water; DOC: dissolved organic carbon in river water; SRP: soluble reactive phosphorus in river water

Stn	Sampling time (MM/DD/HH:MM)	Position	Distance from mouth (km)	Altitude (m)	Population density (no. km^{-2})	WT (°C)	Flow (cm s^{-1})	pH	EC[a] (µS cm^{-1})	Turbidity (NTU)
Y1	05/28/07:30	35°00.525′ N, 136°24.462′E	61.9	710	0.0	12.4	17	8.0	43.2	0.4
Y2	05/28/08:30	35°00.083′N, 136°23.522′E	59.8	480	0.0	13.4	26	7.8	58.0	0.3
Y3	05/28/09:30	34°59.332′N, 136°22.145′E	55.4	400	0.0	14.5	42	7.7	62.3	0.2
Y4	05/28/10:00	34°58.617′N, 136°20.752′E	52.6	360	3.6	15.4	22	7.9	66.0	6.2
Y5	05/28/11:00	34°57.293′N, 136°19.592′E	48.5	300	26.6	16.8	8	7.8	76.1	1.9
Y6	05/28/11:30	34°57.500′N, 136°18.143′E	45.0	260	23.7	19.5	77	8.3	82.1	2.4
Y7	05/28/13:30	34°56.487′N, 136°16.278′E	39.0	220	34.4	20.5	119	7.8	93.6	4.5
Y8′	05/28/12:00	34°55.872′N, 136°16.780′E	38.2	220	66.5	20	26	7.9	148.8	1.6
Y9	05/28/14:00	34°56.112′N, 136°15.635′E	37.6	215	52.4	21	89	8.0	117.2	1.3
Y10	05/28/15:00	34°57.153′N, 136°13.020′E	33.8	200	74.5	21.1	66	7.9	125.3	1.2
Y11	05/28/15:30	34°57.922′N, 136°11.132′E	29.2	170	96.4	23.9	37	8.2	144.6	3.9
Y12′	05/29/07:30	34°58.345′N, 136°07.777′E	24.8	140	204.9	18.6	69	7.5	163.1	1.4
Y13	05/28/16:30	34°57.587′N, 136°08.353′E	23.9	140	289.3	23.2	51	8.5	140.1	4.3
Y14	05/29/09:00	34°59.012′N, 136°07.253′E	22.8	140	232.7	19.3	23	7.5	150.5	3.5
Y15′	05/29/09:30	34°59.190′N, 136°06.770′E	21.2	140	197.8	17.8	15	7.5	108.4	3.3
Y16	05/29/11:30	34°53.492′N, 136°06.473′E	20.6	140	241.5	21.9	20	7.5	152.2	2.8
Y17	05/29/10:30	35°00.113′N, 136°05.753′E	18.2	130	243.2	20.8	80	7.5	152.4	2.7
Y18′	05/29/12:30	35°00.353′N, 136°05.603′E	18.1	130	540.2	23.2	70	7.3	462.6	7.1
Y19	05/29/13:30	35°00.870′N, 136°04.217′E	16.9	130	277.7	23.5	42	8.9	213.9	2.4
Y20	05/29/14:00	35°01.667′N, 136°01.933′E	12.4	110	308.2	24.2	20	9.4	235.2	1.7
Y21	05/29/15:00	35°02.662′N, 136°01.093′E	10.0	105	308.0	23.1	12	9.3	229.9	1.4
Y22	05/29/16:00	35°04.483′N, 136°00.382′E	6.7	95	321.1	23.2	21	8.9	220.1	1.6
Y23	05/29/17:00	35°06.083′N, 135°53.387′E	2.9	87	319.9	23.6	43	7.3	220.3	3.4

Stn	Bacterial abundance (×10^6 cells cm^{-2})	Chl *a* (µg cm^{-2})	POC_{epi} (mg cm^{-2})	PON_{epi} (mg cm^{-2})	C:N	$\delta^{15}N$ (‰)	TN (µM)	TP (µM)	BOD (mg l^{-1})	DOC (µM)	Nitrate (µM)	Ammonium (µM)	SRP (µM)
Y1	11.0 ± 3.1	0.831	0.110	0.014	8.0	–1.6	44	0.1	0.3	36.4	37	0.1	0.1
Y2	11.6 ± 3.3	0.186	0.040	0.006	6.9	–2.5	36	0.1	0.3	35.2	29	0.1	0.0
Y3	4.9 ± 1.3	0.263	0.038	0.005	7.1	–2.1	37	0.1	0.3	30.3	30	0.1	0.1
Y4	9.4 ± 4.3	0.792	0.260	0.036	7.3	–0.5	45	0.1	0.3	60.6	34	0.2	0.0
Y5	23.8 ± 8.7	2.573	0.323	0.040	8.0	–0.4	57	0.2	0.4	58.6	43	0.3	0.1
Y6	63.2 ± 17.4	5.366	0.719	0.095	7.5	1.6	50	0.2	0.9	75.0	74	0.3	0.5
Y7	11.2 ± 3.7	0.187	0.078	0.010	7.5	2.4	75	0.3	0.5	46.4	68	0.5	0.1
Y8′	23.0 ± 7.8	0.647	0.126	0.019	6.7	4.0	92	0.3	0.3	59.5	83	0.3	0.2
Y9	23.9 ± 8.8	0.419	0.147	0.022	6.7	1.6	88	0.2	0.4	64.0	82	0.2	0.2
Y10	12.0 ± 5.2	0.270	0.072	0.011	6.6	1.7	92	0.2	0.2	46.7	86	0.2	0.2
Y11	37.4 ± 11.1	1.824	0.178	0.023	7.8	4.2	96	0.9	0.6	165.5	78	1.5	0.6
Y12′	68.3 ± 14.5	6.379	0.301	0.042	7.1	4.8	103	0.5	0.6	93.2	89	0.4	0.3
Y13	35.1 ± 10.2	0.660	0.327	0.050	6.5	5.8	75	1.4	1.1	188.9	48	1.0	0.8
Y14	74.5 ± 20.9	4.181	0.465	0.066	7.0	5.8	93	1.0	0.7	138.9	39	0.6	0.0
Y15′	96.5 ± 18.8	0.386	0.356	0.037	9.5	6.3	91	1.5	1.2	130.5	67	2.8	1.1
Y16	32.0 ± 15.3	0.898	0.148	0.023	6.4	5.0	88	0.8	0.8	135.9	68	0.4	0.6
Y17	33.2 ± 10.4	8.626	0.903	0.145	6.2	8.3	91	1.0	0.6	134.6	72	0.3	0.6
Y18′	57.7 ± 18.1	18.054	0.847	0.134	6.3	8.3	101	3.1	0.9	223.2	75	3.9	1.4
Y19	92.2 ± 19.4	5.586	0.378	0.052	7.3	7.5	85	1.0	0.7	138.4	66	0.4	0.7
Y20	120.8 ± 25.9	6.687	0.591	0.083	7.2	6.9	70	0.9	0.8	130.8	59	0.4	0.6
Y21	69.5 ± 17.6	4.661	0.405	0.060	6.7	5.5	79	0.8	0.7	126.1	56	0.3	0.6
Y22	46.9 ± 14.3	1.758	0.128	0.016	8.0	5.9	74	0.9	0.7	124.3	54	0.4	0.6
Y23	96.4 ± 14.8	6.687	0.433	0.057	7.6	6.4	77	0.8	0.6	119.5	55	0.6	0.5

[a]High EC values were mainly due to high concentrations of sodium and chloride, possibly derived from industrial and other anthropogenic discharges (T. Nagata, unpubl. data)

Table A2. Geographic and demographic features and environmental variables at the sampling stations of the Ado River. Abbreviations as in Table A1

Stn	Sampling time (MM/DD /HH:MM)	Position	Distance from mouth (km)	Altitude (m)	Population density (no. km^{-2})	WT (ºC)	Flow (cm s^{-1})	pH	EC (µS cm^{-1})	Turbidity (NTU)
A1	09/27/09:00	35°09.600′N, 135°47.950′E	50.1	645	0.1	13.9	24	6.2	20.8	28.1
A2	09/27/10:00	35°10.788′N, 135°49.669′E	46.5	555	4.4	15.1	41	7.2	30.8	6.8
A3	09/27/11:30	35°12.582′N, 135°51.330′E	39.4	405	6.7	18.1	24	7.6	63.0	2.0
A4	09/27/12:30	35°14.427′N, 135°51.885′E	35.1	315	7.8	17.9	65	7.7	61.3	6.5
A5′	09/27/13:30	35°14.875′N, 135°52.015′E	34.2	310	0.6	17.1	47	7.3	35.2	4.3
A6	09/27/14:30	35°15.858′N, 135°52.448′E	31.5	280	8.2	18.3	60	7.6	59.4	5.5
A7′	09/28/08:30	35°15.892′N, 135°52.287′E	31.3	280	1.0	17.4	81	7.3	45.9	4.0
A8	09/28/09:30	35°18.070′N, 135°53.213′E	23.7	195	10.8	18.1	61	7.4	55.2	3.2
A9	09/28/10:00	35°21.065′N, 135°55.147′E	20.1	170	14.8	19.5	63	7.3	55.1	2.8
A10′	09/28/11:00	35°21.312′N, 135°55.212′E	19.4	165	3.5	19.1	38	7.4	58.6	3.7
A11	09/28/12:00	35°21.773′N, 135°58.257′E	11.4	120	21.0	20.0	16	7.4	58.7	4.3
A12	09/28/13:30	35°20.988′N, 136°00.040′E	8.1	105	23.9	20.9	15	7.4	59.3	4.7
A13	09/28/14:30	35°20.353′N, 136°02.132′E	4.5	90	25.1	21.3	61	7.5	59.6	4.8
A14	09/28/15:30	35°19.553′N, 135°03.798′E	1.6	85	25.2	21.7	52	7.6	60.1	4.7

Stn	Bacterial abundance (×10^6 cells cm^{-2})	Chl *a* (µg cm^{-2})	POC_{epi} (mg cm^{-2})	PON_{epi} (mg cm^{-2})	C:N	$\delta^{15}N$ (‰)	TN (µM)	TP (µM)	BOD (mg l^{-1})	DOC (µM)	Nitrate (µM)	Ammonium (µM)	SRP (µM)
A1	7.9 ± 0.8	0.135	0.071	0.006	14.1	2.2	62	2.8	0.5	50.4	20	0.5	0.6
A2	18.7 ± 3.3	1.023	0.186	0.025	8.7	1.1	40	0.9	0.3	60.1	16	0.2	0.6
A3	32.0 ± 2.9	3.108	0.277	0.040	8.1	1.3	44	0.4	0.1	47.4	26	0.7	0.3
A4	3.3 ± 1.3	1.082	0.111	0.016	8.3	−0.5	42	0.6	0.2	55.7	23	0.1	0.4
A5′	7.9 ± 4.3	1.003	0.106	0.013	9.9	−1.7	52	0.3	0.1	40.1	35	0.1	0.1
A6	28.0 ± 9.5	2.326	0.118	0.018	7.5	−0.8	42	0.5	0.1	43.0	29	0.1	0.3
A7′	14.1 ± 5.0	1.122	0.074	0.010	8.8	0.9	51	0.3	0.5	49.9	32	0.1	0.3
A8	18.8 ± 7.3	2.782	0.173	0.028	7.2	−0.2	48	0.5	0.4	46.5	31	0.1	0.3
A9	48.3 ± 18.1	3.207	0.259	0.042	7.2	−0.1	54	0.3	0.3	93.3	34	0.2	0.3
A10′	11.2 ± 3.9	0.834	0.087	0.012	8.8	−0.2	77	0.3	0.4	127.9	48	0.1	0.3
A11	5.6 ± 4.8	0.366	0.058	0.006	10.8	3.8	65	0.4	0.4	69.3	41	0.8	0.3
A12	5.7 ± 3.1	3.596	0.417	0.046	10.6	1.9	69	0.4	0.5	87.4	42	0.5	0.4
A13	74.4 ± 32.6	4.702	0.329	0.048	7.9	3.4	83	0.5	0.3	140.3	42	0.8	0.4
A14	47.3 ± 12.2	6.616	0.366	0.006	7.2	4.6	71	0.7	0.3	158.6	38	0.5	0.4

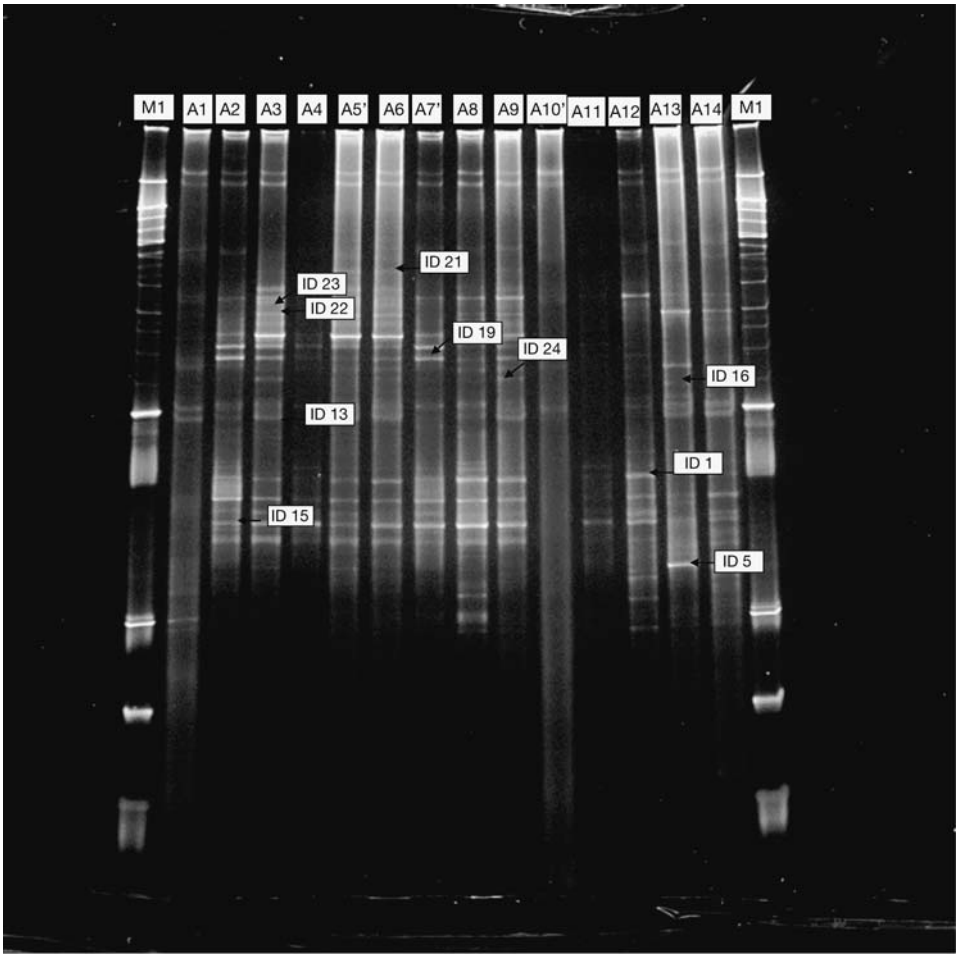

Fig. A1. Example of the DGGE gel (samples are from the Ado River). M1: marker; A1–A14: sampling stations; (⟶) bands applied for sequencing. ID numbers as in Table 4

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