Longitudinal Changes in the Bacterial Community of the Danube by 16S rDNA Profiling: A Whole River Approach

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Introduction

Running waters are important links in the global biogeochemical cycles. They act as natural integrators of surficial processes, including human activities, within their drainage basins and transport large amounts of nutrients and organic matter. Rivers change the quantity and quality of material transported to the ocean through transformation, consumption and production of nutrients and organic material (Hopkinson et al. 1998). The net effect of processes such as storage, breakdown and transformation of matter is a basic ecological property of lotic ecosystems. The bacterial community is an important component mediating these ecosystem processes. Along the riverine continuum, they catalyse the flux of energy and matter and are the link to the riverine metazoan food web (Pusch et al. 1998). The activity of the microbial community is influenced by the community composition in relation with environmental factors such as availability of organic matter and nutrients (Sinsabaugh and Foreman 2001).

Basic concepts of river ecology such as the river continuum concept (RCC, Vannote et al. 1980) or the serial discontinuity concept (SDC, Ward and Stanford 1995) focus on resource availability, input of organic matter, and autochthonous productivity in the longitudinal dimension of lotic ecosystems. One of the predictions of these concepts is a high diversity of soluble organic compounds in low order rivers due to the various terrestrial inputs and this diversity of organic compounds decreases with increasing autochthonous production in higher order rivers (Vannote et al. 1980). Numerous reports on the link between DOC quantity and quality with the microbial community (e.g. Sun et al. 1997) suggest that prokaryotic species richness might be linked to these changing inputs of terrestrial organic matter along a river (Leff 2000). Also, lateral exchange of matter as discussed by the FPC (flood pulse concept, Junk et al. 1989) and other papers (Ward and Stanford 1995, Tockner et al. 2000) as well as autochthonous production (Thorp and Delong 2002) are expected to have an impact on bacterial species richness and community dynamics. Additionally, in human impacted rivers such as the Danube River, the relationship of the microbial community with organic matter may be influenced by damming (Friedl and Wüest 2002).

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The population dynamics and longitudinal changes of bacterial communities in large rivers have not been studied with appropriate resolution. There exists only one study from the Changjiang River (China) investigating the bacterial population changes over its entire length (Sekiguchi et al, 2002). However, in this study bacterial community composition was determined at only eight sampling stations separated by a distance of approximately 100-500km. Thus bacterial species richness and community dynamics along rivers and their determining factors still remain unclear. This is also the case for large rivers like the Danube River.

The aim of this study was to investigate the longitudinal dynamics and changes of the bacterial community in the River Danube during JDS 2001 with high spatial resolution. In total 98 samples along the course of the River Danube and its major tributaries were taken, covering a distance of 2581 km. By means of polymerase chain reaction (PCR), denaturing gradient gel electrophoresis (DGGE), and DNA sequencing of 16S rRNA gene fragments the bacterial community composition, species richness, and population dynamics was studied in an unprecedented spatial resolution of 20-40 km.

Methods

Water samples. Sampling was performed during the Joint Danube Survey (JDS) project (13 August-19 September 2001). Details about the project and the sampling locations can be found in the report of the International Commission for the Protection of the Danube River (ICPDR) . All large capitals (Vienna, Budapest, Belgrade and Bucharest) and river inflows were sampled upstream and downstream in a frequency of every 20-40 km. On a total of 98 stations, 50-150ml of water, collected 30cm below the water surface, were filtered through 0.22µm pore-size filters to collect the prokaryotic cells on the filters, representing the total in situ prokaryotic community. The filters were kept frozen until DNA extraction.

DNA Extraction. Precursor experiments have shown that it is essential for subsequent PCR amplification to avoid contamination of the DNA extracts by inhibiting agents such as humic acids, commonly found in water samples from the river Danube. Thus, extraction of the DNA from the filters was performed using the UltraClean Soil DNA Kit (MO BIO Laboratories) according to the manufacturer's instructions. The kit is efficiently separating DNA from contaminating substances and, thus, is the most appropriate extraction method for the samples. After extraction all samples were analysed for the presence of amplifiable bacterial DNA as checked by applying a universal bacterial PCR assay.

PCR – DGGE analysis. An approximately 600 bp long fragment of the bacterial 16S rRNA gene was amplified from the DNA extracts by PCR using the primer pair 341F (5'-CCT ACG GGA GGC AGC AG-3') and 907R (5'-CCG TCA ATT CMT TTG AGT TT-3'). A 40bp long GC-clamp (5'-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G-3' was attached to the 5' end of the primer 341F to obtain amplicons which can be used for DGGE. Five µl of nucleic acid extract was used as template in PCR reactions. The PCR reaction (50µl) contained the following: 5 µl of 10x Taq buffer (MBI Fermentas; 100 mM Tris-HCl [ph 8.8], 500 mM KCl, 0.8% Nonidet P40), 4 µl of 25 mMol $MgCl_2$ (MBI Fermentas; final concentration: 2 µMol), 6.25 µl of 2 mMol dNTP-Mix (MBI Fermentas #R0241; final concentration: 250 µMol each), 0.5 µl of 100 µMol primer 341F and 907R (MWG-Biotech AG; final concentration: 1 µMol), and 0.25 µl of 5 U μl^{-1} Taq-polymerase (MBI Fermentas; #EP0401). Initial denaturation was at 95°C for 1 min followed by 30 cycles with denaturation at 95°C for 1min, annealing at 56°C for 1 min, and elongation at 72°C for 1 min and a final elongation step was performed at 72°C for 30 min. Standard agarose gel electrophoresis was used to size and quantify the PCR fragments. DGGE analysis was

performed on a DCode Universal Mutation Detection System (Bio-Rad). The PCR products obtained from a single PCR reaction of each sample were loaded on 6% polyacrylamide gels containing linear gradients of formamide and urea of 20-70%. Electrophoresis was performed at 100V and 60°C for 16h in 1x TAE buffer (40 mM Tris, 20 mM Acetic acid, 1 mM EDTA, pH 8.3). The gels were stained with SYBR Green I (Molecular Probes; 1:10,000 dilution of stock solution) for 30 min before digitized gel images were obtained using a GelDOC 2000 (Bio-Rad) gel documentation system equipped with a charge-coupled device camera. Further methodical details are given in Winter et al. (2006)

Results

During the JDS 2001, all 98 filtrations of 50ml to 150ml of River Danube water followed by DNA extraction and PCR –DGGE analysis resulted in complex and well focused 16S rRNA gene DGGE band patterns (c.f. Fig.1). Apparent bacterial richness (i.e. numbers of separated DGGE 16S rRNA gene bands) ranged from 18 to 32 OTUs (operational taxonomic units). According to apparent bacterial richness, the River Danube could be divided into distinguishable sections. The upstream (reaches 2-3, JDS nomenclature, km2581-km1816) and downstream sections (reaches 6-8, km1202-km12) showed significantly higher apparent bacterial richness of on average 27 and 30 bands, respectively, as compared to the middle sections (reaches 4 –5, km1816-1206) with an average of 21 bands. In the tributaries apparent bacterial richness ranged from 15 to 33 bands. Bacterial richness was apparently influenced by primary production as there was a negative correlation between the concentration of Chl-a ($r = -0.54$; $p<0.005$) to the number of observable DGGE bands.

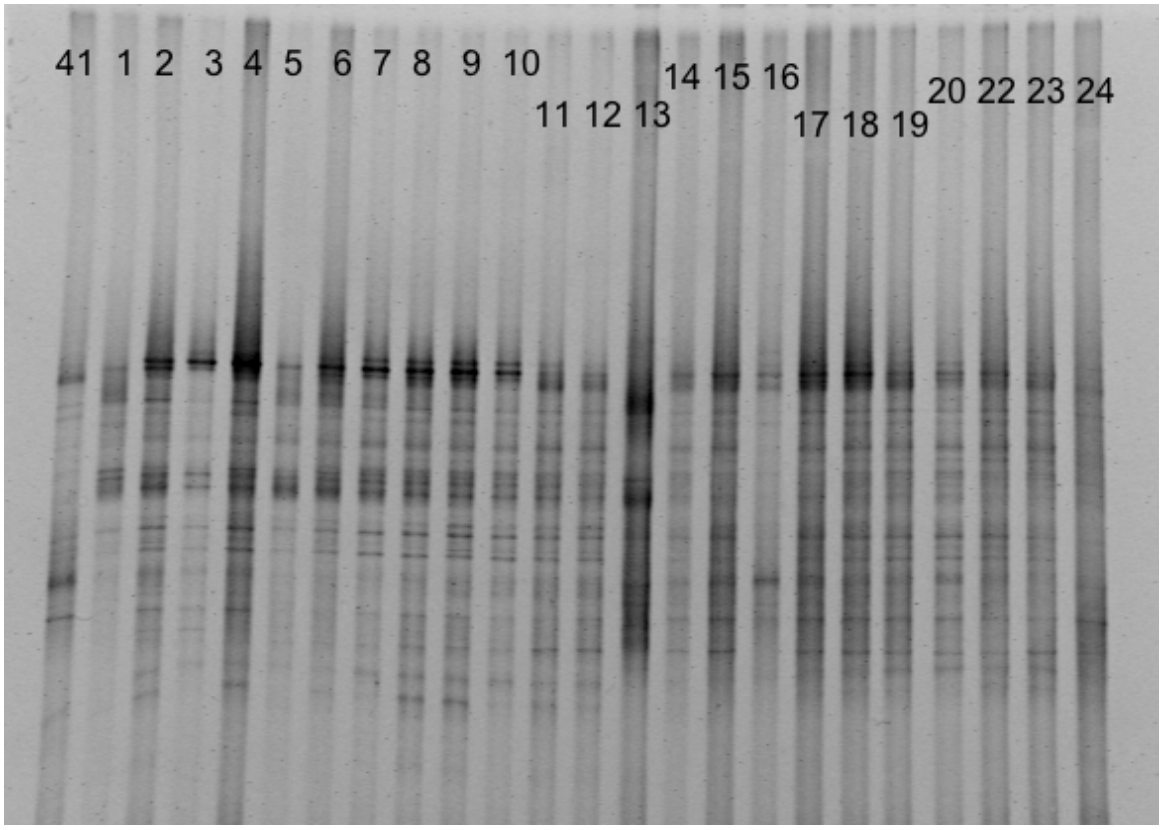

Fig. 1. Exemplarily selected results of the PCR – DGGE analysis covering 768km of the upstream section of the German (lane numbers 1, 2, 3, 4, 6) Austrian (lane numbers 7 –15) and Slovakian (16 – 21) River Danube JDS 2001 sampling stations (from km2580 to km1812). Lanes 5,13,16 are tributaries (see below for details). Lane 41 is given as an "outgroup" band pattern of the given upstream sequences, representing a downstream location at km1533. Given DGGE band patterns of the analysed sampling locations are a result of the encountered bacterial

community composition. The sequence of lanes reflect the longitudinal bacterial community dynamics in the river. One band represent an operational taxonomic unit (OTU) and the sum of detected OTUs are taken as an indication of the bacterial species richness. Lane numbers refer to the following sampling stations (abbreviations: DE =Germany, AT = Austria; SK = Slovakia, HU = Hungary): River Danube: 1 = km2580 Neu-Ulm (DE); 2 = km2412 Kelheim (DE); 3 = km2358 upstream dam Geisling Regensburg (DE); 4 = km2327 upstream dam Kachlet Passau (DE); 6 = km Jochenstein (DE/AT); 7 = km2165 upstream dam Aschach (AT); 8 = km2120 upstream dam Abwinden-Asten (AT); 9 = km2096 Wallsee (AT); 10 = km2061 upstream dam Ybbs-Persenbeug (AT); 11= km1950 upstream dam Greifenstein (AT); 12 = km1942 Klosterneuburg (AT); 14 = km1895 Wildungsmauer (AT); 15 = km1881 upstream Morava Hainburg (AT); 17 = km1869 Bratislava (AT); 18 = km1856 Gabcikovo reservoir entrance (SK); 19 = km1852 Gabcikovo reservoir (SK); 20 = km1846 Gabcikovo reservoir 2 (SK); 21 = km1812 Sap outlet channel (SK); 41 = km1533 Paks (HU); Tributaries: 5 = Inn km0,5 (DE/AT;) 13 = Schwechat km0,1 (AT); 16 = Morava km0,08, (AT/SK).

A total of 43 distinct bands could be detected within the whole set of DGGE banding patterns. Sequencing of selected bands revealed phylotypes closely related to typical freshwater bacterioplankton including taxa of *Cyanobacteria*, *α-*, *β-*, *γ- Proteobacteria*, *Cytophaga – Flavobacterium - Bacteroides* group and *Actinobacteria*. Analysis of the longitudinal development of the DGGE profiles in the River Danube during the JDS 2001 indicated that the bacterial community developed gradually over the studied distance of over 2500km (see Fig. 1 bands 1-24 as selected example of the gradual development of the upstream sections km2580 to km1812). The similarity of the bacterial communities related to the first sampling station decreased with increasing distance and the Jaccard dissimilarity index ranged from approximately 0,25 (2412 km) to 0,65 (12 km). However, the gradual community development was significantly disrupted in reach 5 (1659-1202 km) by a significant decrease in similarity. This abrupt community shift was also reflected by a sharp decrease of apparent bacterial richness as described above. Cluster analysis of the bacterial community composition also followed these changes, dividing the communities in a distinguishable “upstream” and “downstream“ cluster. Interestingly, large impoundments did not reveal a detectable influence on the bacterial community. A detailed analysis, discussion, and mathematical modelling of the data is given in Winter et al. (2006).

Discussion and Summary

For the first time a comprehensive snapshot of the dynamics of the planktonic bacterial community composition in the Danube River was obtained by PCR – DGGE based analysis and sequencing. Bacterial communities, despite the influence of major tributaries, showed a continuous change in their populations along their longitudinal journey in the Danube River. Internal river metabolism had a strong influence on bacterial richness and community dynamics, as demonstrated by the observed phytoplankton bloom in the middle section between the cities of Budapest and Belgrade. This decreasing effect of the phytoplankton bloom on bacterial richness may be explained by increased algae exudates availability likely leading to the selection of abundant but less divers populations adapted to the respective substrate conditions.

The study clearly demonstrates that investigating the microbial ecology of large rivers on appropriate resolution scales has become possible and further applications of such approaches will undoubtedly contribute to a better understanding of larger river systems. In this respect, further studies will also have to link bacterial activity with the given community structure to learn more about functional diversity and furthermore should include also river bottom and sediment compartments for a more comprehensive system interpretation basis.

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