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Development of the Bacterial Compartment Along the Danube River: a Continuum Despite Local Influences

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Abstract Microbial food webs dominate heterotrophic food webs in large rivers with bacterial metabolism being a key component of carbon processing. Thus, analysis of bacterial population dynamics is critical to understanding patterns and mechanisms of material cycling and energy fluxes in large rivers. Within the frame of the Joint Danube Survey (JDS) 2007, the longitudinal development of the natural bacterial community in the Danube in terms of bacterial numbers, morphotype composition, and heterotrophic production of the suspended and particle-attached fractions was followed at a fine spatial resolution of approximately 30 km for the first time in such a large river along a 2,600-km stretch. Twenty-one major tributaries and branches were also included. This allowed us to investigate whether bacterial standing stock and production undergo continuous, linear changes or whether discontinuities and local processes like the merging of tributaries or the potential impact of sewage input drive the bacterial population in the Danube. The presented investigation revealed surprising continuous patterns of changes of bacterial parameters along the Danube River. Despite the presence of impoundments or hydropower plants, large municipalities, and the discharge of large tributaries, most bacterial parameters (standing stock, morphotype succession, and attached bacterial production) developed gradually, indicating that mainly broad-scale drivers and not local conditions shape and control the bacterial community in the midstream of this large river. As most important broad-scale drivers, nutrients (inorganic and organic) and changes in particle concentrations were identified. These data are also in remarkable accordance with the patterns of changes of the genetic bacterial community composition, observed during the first JDS (2001) 6 years before. In contrast, bacterial activity did not follow a continuous trend and was mainly controlled by the input of sewage from large cities in the middle section, leading to a bloom of phytoplankton. The observed patterns and the comparison between the Danube, its tributaries and other large rivers worldwide indicate that the bacterial community in rivers has a powerful indicator function for estimating the ecological status of large river ecosystems once enough information has been collected at various temporal and spatial scales.

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Introduction

River networks fundamentally differ from most other ecosystems because (1) they are open systems with tight functional linkages to their adjacent ecosystems, and (2) they are nested systems with their physical and ecological structure and function changing over several spatial and temporal scales. Their hierarchical organization and their tight link to adjacent terrestrial and subterranean ecosystems have stimulated the development of concepts such as the River Continuum Concept [42] and more recent concepts on river floodplain functioning [38, 40]. Traditional perceptions focus on the question whether the longitudinal continuum or the lateral connectivity driven by flood pulses [2, 21] controls organic matter supply. More recent concepts discuss the importance of physical discontinuities [1, 32] and local processes in rivers [39] as substantial contribution to understand habitat structure and carbon cycling in lotic ecosystems.

Since the integration of the microbial loop concept for aquatic ecosystems [31], it has been recognized that a major part of the organic carbon from primary production is channeled through the bacterial compartment. However, this concept was developed for lentic ecosystems, and there is only limited information whether it is also applicable for lotic systems, especially for large rivers [30]. Rivers differ from lakes in the way that allochthonous inputs of organic matter are of increased significance in comparison to primary production to fuel the bacterial compartment with carbon and energy [1]. From this, it can be deduced that the microbial food web has an even higher importance in rivers and that bacterial metabolism is a key component of carbon processing [5]. Thus, analysis of bacterial population dynamics is critical to understanding patterns and mechanisms of material cycling and energy fluxes in large rivers [32]. Despite its primordial importance for river system functioning, the bacterial compartment has not been considered in international regulations like the European Water Framework Directive (WFD; 11), where biological quality elements were defined for assessing the ecological status of aquatic ecosystems.

Large rivers, as the river Danube, are known to experience the impact of sewage emission from point and non-point sources and an important number of tributaries. While there is little doubt that these may influence the physico-chemical and biological development of the midstream, the magnitude of this influence on the water body in the midstream of the river is still a matter of debate. The Danube River is characterized by the input of sewage from nine riparian countries with five capitals and the tribute of some 40 rivers merging into the main stream on its way to the Black Sea. The whole catchment area covers approximately 801.500 km^2 and with its total length of 2,780 km, the Danube is the second longest river in Europe [35].

Within the frame of the Joint Danube Survey (JDS) 2007, organized by the International Commission for the Protection of the Danube River (ICPDR) to assess the ecological status of the Danube according to the WFD, a representative number of stations between Kelheim (Germany, river km 2,600) and the Danube Delta (Romania, rkm 6) were sampled. For the first time, the longitudinal development of the natural bacterial community in terms of bacterial numbers, morphotype composition, and secondary production was followed on a wide spatial scale at a fine spatial resolution of approximately 30-km intervals in such a large river. Sampling was focused on the midstream, but also major tributaries or branches were included. During the first JDS (2001) 6 years before, Winter et al. [48] observed a continuous development of the bacterial community composition based on the variation of 16S ribosomal RNA (rRNA) gene denaturing gradient gel electrophoresis (DGGE) band patterns, which was only interrupted by one major shift after Budapest, Hungary. Thus, we tested in this study whether the patterns observed for bacterial community composition are also reflected in terms of cell abundance, cell volumes, morphotype composition, and secondary production. We asked whether continuous linear changes are found for the recorded bacterial parameters or whether discontinuities and local processes like the potential impact of tributaries and sewage control the bacterial compartment in the midstream. By relating the changes of the bacterial parameters to other concomitantly investigated ecological variables, a clearer picture of the driving forces of the bacterial community in this large river could be drawn.

Materials and Methods

Study Area and Sampling

The Danube is the world's most international river collecting water from the territories of 19 countries in Central and Eastern Europe [37]. A characteristic feature is the alternation between wide alluvial plains and constrained sections along the main stem. Approximately 30% of the length of the main stem is impounded through major hydraulic structures which significantly alter sediments and groundwater regimes [37]. The Danube is currently navigable for 87% of its total length (rkm 2,410). *S*amples for bacteriological analyses and for the determination of environmental variables were collected during the JDS 2 (Aug 15, 2007 till Sept 26, 2007) along the longitudinal

stretch of the River Danube from the upper section (rkm 2,600) to the delta (rkm 6). In sum, 96 samples were taken, of which 75 were collected from the midstream and 21 from the major tributaries and branches. A detailed map of the Danube river basin with the specific sampling points can be found in Kirschner et al. [23, 24]. Average water depth of the Danube sampling sites was with the exception of the first sampling site (0.5 m) always more than 2 m and reached a maximum of 20 m in the Iron Gate Reservoir. Average water depths of the sampling sites in the tributaries/branches varied between 1 m and 4 m. Water flow in the Danube without impoundments ranged from 0.5 to 2.7 m s^{-1}, with an average value of 1.2 m s^{-1}. Until rkm 2,233, a high-water situation was faced, which was progressively dampened by the series of the hydropower plants. For all figures, calculations and interpretations, station 1 was excluded as it was not considered as representative for a large river. At all Danube and large tributary sampling stations, water samples were collected directly from the bow of the cruise ship in the midstream of the river, after the ship has moved approximately 30 s against the flow, immediately before the ship was anchored to prevent influence of particles resuspended by the ship. Samples were taken with sterile 1-L glass flasks fixed to a sampling rod at a water depth of approximately 30 cm. For smaller tributaries and branches, samples were taken in the same manner from small boats. Samples were immediately divided into two fractions by filtration through 3-μm pore size polycarbonate filters (Cyclopore, Whatman, Germany) applying a maximal vacuum of 200 mbar to differentiate between free-living (<3 μm) and attached (>3 μm) bacterial communities. A pore size of 3 μm was considered appropriate as particle sizes in the Danube were reported to range from 4 to 25 μm [22].

Bacterial Numbers and Biometry

Bacterial numbers were estimated with the acridine orange direct count method according to a protocol of Kirschner and Velimirov [25]. Ten milliliters of water sample (total and 3 μm filtrate) were fixed with 0.5 ml of 37% formaldehyde for 1 h. Subsamples of 0.5 to 5 ml were immediately filtered (on board) through black polycarbonate membranes (0.2 μm pore size, 25 mm diameter, Whatman) at a maximum pressure difference of 200 mbar, stained with acridine orange, and mounted on glass slides for long-term storage at room temperature. Room temperature was chosen because for acridine orange-stained cells, the signal remained more stable than when frozen. For security reasons, the rest of each fixed sample was stored in the dark at 4°C. Within 1 to 3 months, at least 200 bacterial cells were counted per filter in an epifluorescence microscope (Diaplan, Leica, Vienna, Austria; excitation wavelength, 450–490 nm; cutoff filter, 515 nm) and grouped into four morphological classes (rods, cocci, vibrio-shaped and filamentous cells). In most of the total samples, bacteria attached to particles were easily detectable, as particles were rather small and transparent. In case particles were larger and non-transparent, stored samples were sonicated (15 W, 3×20 s) before the staining procedure. For a series of representative stations (n>10), bacterial numbers were quantified from both immediately fixed and from stored samples. Results of these duplicates were always within a range of maximally 8% (data not shown). Bacteria were sized by an eyepiece micrometer. Cell volume estimations were derived from the assumption that bacteria have spherical or cylindrical shape with two hemispherical caps. At least 100 bacteria per sample were measured. Cellular carbon content, expressed in femtograms of carbon per cell, necessary for biomass estimations was calculated from cell volumes (V; μm^3) assuming the allometric relation $C = 120 \times V^{0.72}$ after Norland [29].

Leucine Incorporation—Heterotrophic Bacterial Production

Heterotrophic bacterial production was determined for both untreated and 3-μm filtered samples following the protocol of Eiler et al. [10]. Four 1-ml subsamples and two blanks were amended with 100 nM (final concentration) of ^{3}H-leucine as a tracer for the incorporation of amino acids into the bacterial protein pool. Blanks were stopped immediately with 60-μl trichloroacetic acid (TCA, final concentration 5%). After 30-min incubation at in situ temperature in the dark, samples were stopped with TCA, and proteins were precipitated with 100 μl of 35% NaCl and purified in several extraction/centrifugation steps according to Kirschner and Velimirov [26]. All vials with the purified proteins were stored at −20°C on board until transfer to the University laboratory (maximum of 1 month). There, the vials were thawed, scintillation cocktail was added, and radioactivity in the proteins was measured in a scintillation counter (Perkin Elmer, TriCarb 2300 TR) and converted to units of carbon using the conversion factor of Simon and Azam [36]. Additionally, eight saturation experiments along the Danube with increasing amounts of ^{3}H-leucine (10–150 nM) were conducted which verified that the administered radioisotope concentration was adequate to saturate the uptake by the respective bacterial population (data not shown).

Environmental Variables

Temperature, pH, and conductivity were measured according to international standards [7, 16, 19] immediately after sampling in water samples collected with a built-in pump,

with the exception of some tributaries that were not accessible by ship. Pumped water samples were stored in appropriate clean glass vessels and used for further analysis. Samples for NH_4, NO_3, and PO_4 were filtered through 0.45-μm pore-size membranes and were analyzed directly on board according to international standards [15, 17, 20]. Total phosphorus and organic nitrogen were determined in the laboratory after storage at −20°C [8, 20]. For dissolved organic carbon (DOC) determination, the 3-μm filtrates were filtered through 0.2-μm polycarbonate membranes (Whatman), prewashed with 0.5% HNO_3, and acidified with 100-μl 25% HNO_3 in muffled 40-ml glass vials. The 0.2-μm filtrates were stored frozen on board of the ship at −20°C until analysis. Analysis was performed according to the manufacturer's instructions with a Phoenix 8000 TOC Analyzer (Tekmar-Dohrmann, JCT, Wiener Neustadt, Austria). Total suspended solids (TSS) were separated by filtering an aliquot of 0.2–1 L onto pre-combusted, pre-weighed glass fiber filters (GF/F). After drying (105°C, 4 h), TSS were determined by gravimetric analysis. The filter was further combusted in a muffle furnace (500°C, 4 h). The difference in the weight before and after ignition is assumed to be roughly equivalent to organic substances (particulate organic matter). Chlorophyll a concentration was analyzed using hot extraction technique [18]. An aliquot of 0.2 to 2 L was filtered onto GF/C filters, stored at −35°C, and extracted and analyzed in the laboratory. Extinction was measured at 665 nm corrected for background absorption using the extinction at 750 nm before and after acidification. For zooplankton analysis, 50 L of water were filtered through a 50-μm plankton net, preserved with 4% formaldehyde (final conc.), and stored at 4°C until analysis. The quantitative and qualitative composition of zooplankton was determined in the laboratory using a stereomicroscope [48]. As a detailed presentation of all mentioned environmental variables would be far beyond the scope of this paper, the data were only used for correlation analysis with the observed variation of microbiological parameters. Details on the longitudinal development of these variables can be retrieved from ICPDR [14] and from the public website http://www.icpdr.org/danubis.

Statistical Analysis

All statistical analyses were performed with SPSS 17.0. Correlations between all variables were made by Pearson's *r* test; variables not fitting normal distribution were log10-transformed before correlation. *P* values <0.005 were regarded as significant, according to the Bonferroni correction method. Only statistically significant correlations are reported in the text. For principal component analysis, Varimax rotation with Kaiser normalization was applied.

Results

Danube River Midstream

Bacterial Numbers Along the complete length of the Danube, total bacterial numbers varied between 0.77×10^9 L^{-1} and 5.1×10^9 L^{-1} and exhibited a significant increasing trend ($r=0.41$, $p<0.001$) as the river approached the Black Sea (Fig. 1a). Elevated values were also recorded for the stations from rkm 2,354 to rkm 2,204 and in the stretch after Belgrade between rkm 1,132 and 1,040, as well as between rkm 629 and rkm 500, a stretch with intensive agriculture. Free bacteria (<3 μm fraction) constituted on average 73% of total bacterial numbers (Table 1) and, with the exception of the last three stations in the Danube Delta, they followed the same pattern as total bacterial numbers ($r=0.81$, $p<0.001$). Bacteria attached to particles (>3 μm fraction) amounted on average to 27% of total bacterial numbers (Table 1), but increased to 83–92% in the Danube Delta (Fig. 1a).

Cell Volumes In contrast to bacterial numbers, average cell volume variations displayed a steady significant decrease ($r=-0.45$; $p<0.001$) from the upper course of the Danube towards the Delta (Fig. 1b). The largest average cell volumes were recorded between river kilometers 2,400 and 1,800, with values up to 0.360 μm^3. The mean cell volume over the whole river was 0.197 μm^3. Bacterial cell volumes of free bacteria followed the variation pattern of the cell volumes of total bacteria with a mean cell volume of 0.163 μm^3. For the attached bacterial fraction, no direct determination of cell volumes was possible, but volumes (V_{att}) could be calculated according to the formula $V_{att} = (V_{total} \times BN_{total} - V_{free} \times BN_{free}) \times (BN_{att})^{-1}$, where BN are bacterial numbers. Cell volumes of attached bacteria were markedly higher (average, 0.319 μm^3; range, 0.071–0.893 μm^3, data not shown) than the ones of the free bacterial population.

Morphotype Composition Changes in bacterial morphotype composition in both untreated and filtered samples (Fig. 2) showed that the percentage of cocci ($r=0.65$, $p<0.001$) and vibrio-shaped cells ($r=0.38$, $p=0.001$) became steadily more abundant as the river approached the Danube Delta, while the percentage of rod-shaped cells continuously decreased ($r=-0.71$, $p<0.001$). On average, rods, cocci, and vibrio-shaped cells contributed 58.3%, 25.5%, and 15.7% to total bacterial numbers. The contribution of filamentous bacteria remained below 2.3% and 1.6% in untreated and filtered samples, respectively; nevertheless, a significant continuous decrease towards the Delta was observed ($r=-0.46$, $p<0.001$). Another significant trend

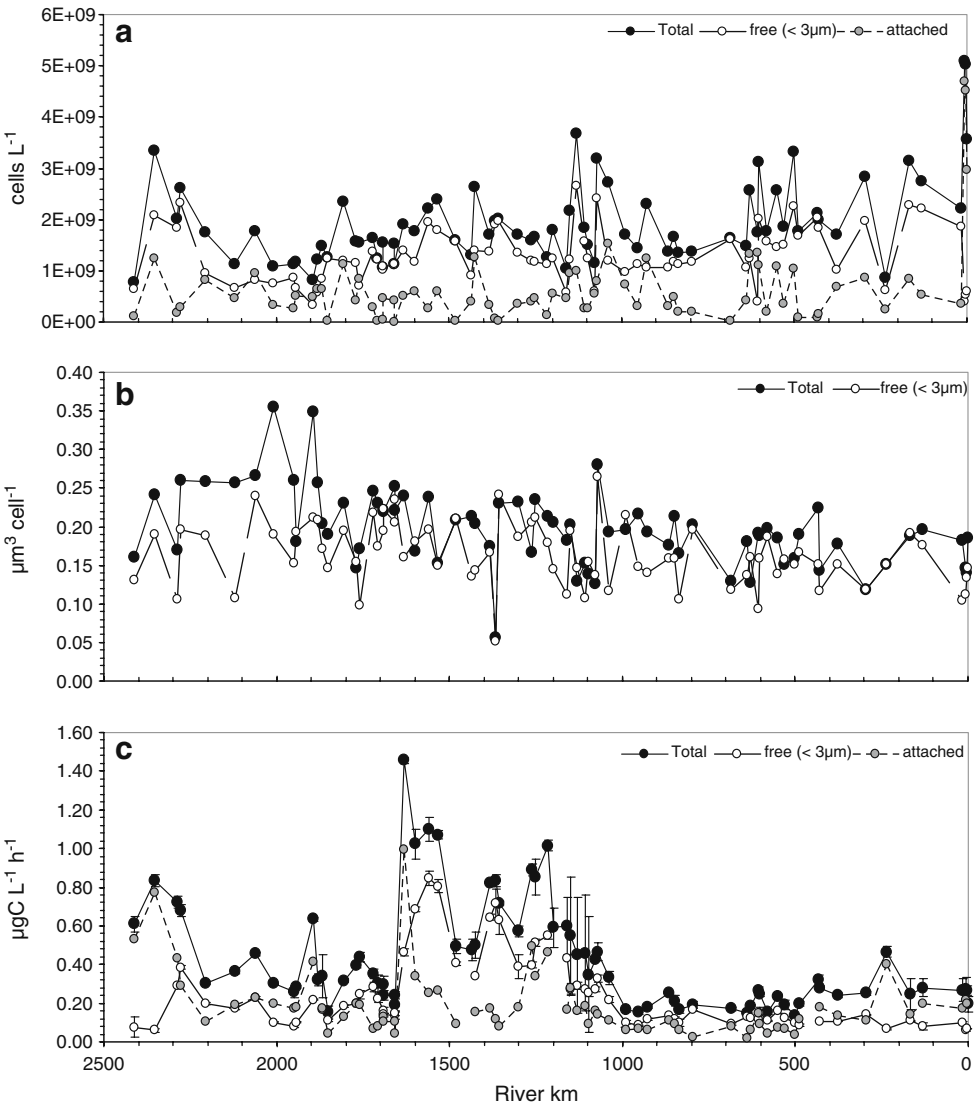

Figure 1 Variation of bacterial numbers (**a**), cell volumes (**b**), and heterotrophic production (**c**) along the Danube River. Total, free (<3 μm), and attached (>3 μm) bacteria are displayed. *Bars* for bacterial production indicate ± standard deviation of four measurements

that could be observed was the decrease in cell volume of vibrio-shaped cells along the Danube (r=−0.54, p<0.001), while cell volumes of the other three morphotypes fluctuated without any discernible pattern.

Bacterial Production Heterotrophic bacterial production in the midstream of the Danube River (Fig. 1c) ranged from 0.20 to 1.46 μg C L^{-1} h^{-1}, with a mean of 0.44 μg C L^{-1} h^{-1}. For the majority of the stations, the values remained well below 0.8 μg C L^{-1} h^{-1}. In contrast to cell numbers, cell volumes, and morphotype composition, no continuous trend of bacterial production over the whole river length was observed. Highest production rates were observed in the middle section of the Danube between

Table 1 Median percentage (and range) of free and attached bacterial numbers, biomass, and secondary production in Danube River samples, samples from tributaries and branches as well as over all samples

	Bacterial numbers		Bacterial biomass		Bacterial production	
	Free	Attached	Free	Attached	Free	Attached
Danube	72.7	27.3	66.2	33.8	58.1	41.9
	7.9–99.1	0.9–92.1	6.6–93.7	6.3–93.3	7.1–88.5	11.5–92.9
Tributaries and branches	73.8	26.2	64.7	35.3	53.6	46.4
	15.8–99.2	0.8–84.2	14.7–93.1	6.9–85.3	4.1–91.4	9.6–95.9
All	72.8	27.2	66.1	33.9	57.3	42.7
	7.9–99.2	0.8–92.1	6.6–93.7	6.3–93.3	4.1–91.4	9.6–95.9

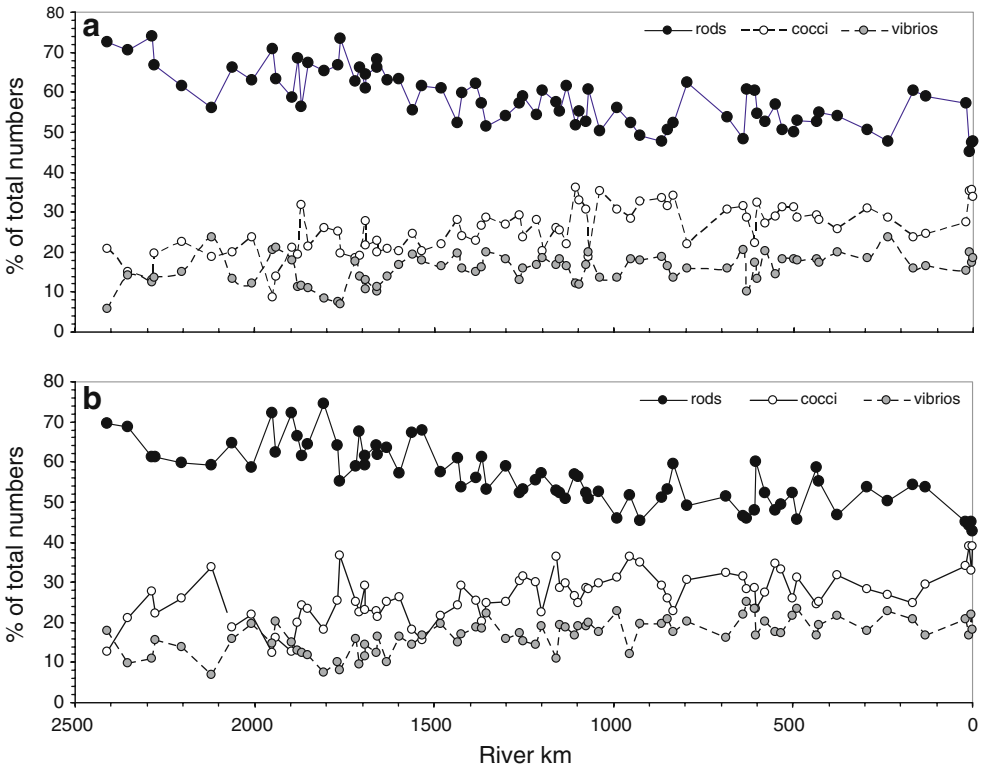

Figure 2 Percentage of rods, cocci, and vibrio-shaped cells to total bacterial numbers along the Danube River. **a** Total community, **b** free-living (<3 μm) fraction

rkm 1,632 (after Budapest) to rkm 1,071 (before the Iron Gates). Also, from rkm 2,412 to 2,278 and at rkm 1,895 (after Vienna), production values >0.6 μg C L^{-1} h^{-1} were observed. On average, 58.1% of the bacterial production was due to free bacteria (Table 1). The variation patterns for total and free bacteria were similar for the majority of the samples (r=0.85; p<0.001), with the exception of samples in the uppermost stretch of the Danube (rkm 2,412–2,354) and from rkm 430 towards the Delta. In these two sections, the highest percentages of attached bacterial production were recorded with values of more than 70–90%. Although no continuous trend could be observed for total bacterial production, there was a significant decreasing contribution (r=−0.74, p<0.001) of attached bacteria to total production from the upper towards the middle section of the Danube (rkm 1,355) and a significant increasing contribution (r=0.61, p<0.001) from the middle section towards the Delta (Supplementary Information, Fig. S1).

Correlation with Environmental Variables There were several environmental variables that showed a significant linear trend along the Danube. Nitrate (r=−0.72, p<0.001), pH (r=−0.51, p<0.001), and the biomass of rotifers (r=−0.38, p=0.001) decreased significantly, while organic nitrogen (r=0.41, p<0.001), total phosphorus (r=0.35, p<0.002), and total suspended solids (r=0.56, p<0.001) increased. Bacterial numbers showed significant correlation only with phosphate (r=0.34, p=0.002), while bacterial production was positively correlated with pH (r=0.36, p<0.002), chlorophyll a (r=0.43, p<0.001), nitrate (r=0.44, p<0.001), and rotifer biomass (r=0.52, p<0.001). Cell volumes were also positively correlated with pH (r=0.48, p<0.001) and nitrate (r=0.40, p<0.001) and negatively with phosphate (r=−0.35, p=0.002). Interestingly, also at the level of morphotype composition, significant relationships were found. The percentage of rods was positively correlated with pH (r=0.59, p<0.001) and nitrate (r=0.64, p<0.001) and negatively with ammonium (r=−0.37, p=0.001); for cocci, the situation was exactly inverse. The percentages of vibrio-shaped cells and filaments showed no significant correlation to any environmental variable.

Principal component analysis revealed that river kilometer—as a surrogate for the distance and the time the flowing wave has moved downstream—and the related variables pH and NO_3 are significantly linked with the bacterial compartment (factor 1, Table 2). Especially changes in morphotypes (cocci, rods, and filaments) are correlated to these abiotic variables, as well as bacterial cell volume. In factor 2, particulate matter concentrations are positively related with the percentage of attached bacteria to total bacterial numbers and production. Bacterial production was mainly linked to indicators of the trophic situation (factor 3) like chlorophyll a (positive correlation) and PO_4 (negative correlation) as well as with pH (positive correlation). Bacterial numbers are clustered in factor 4 with conductivity and nitrate, while the percentage of total bacterial numbers contributed by vibrio-shaped cells is clustered with N_{org} in factor 5.

Table 2 Principal component analysis of the Danube data set

Variable	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5
$BN_{\%coc}$	−0.770				
CV	0.758				
RKM	0.736				
$BN_{\%fil}$	0.723				
NO_3	0.637			0.564	
$BN_{\%rod}$	0.597				
TSS		0.814			
$BP_{\%att}$		0.807			
POM		0.645			
$BN_{\%att}$		0.599			
CHL_a			0.927		
PO_4			−0.624		
BP			0.632		
pH	0.526		0.600		
COND				0.797	
BN				0.629	
$BN_{\%vib}$					0.841
N_{org}					0.519
expl. variance	18.3%	12.1%	11.0%	8.9%	7.7%

Only variables with correlation values >0.5 are indicated

TSS total suspended solids, *POM* particulate organic matter, $BP_{\%att}$ percentage of bacterial production (BP) contributed by attached bacteria, $BN_{\%att}$ percentage of bacterial numbers (BN) contributed by attached bacteria, *ROT* rotifer biomass, CHL_a chlorophyll a, *COND* electrical conductivity, N_{org} organic nitrogen, *CV* bacterial cell volume, $BN_{\%coc}$ percentage of BN contributed by cocci, $BN_{\%fil}$ percentage of BN contributed by filaments, $BN_{\%rod}$ percentage of BN contributed by rods, $BN_{\%vib}$ percentage of BN contributed by vibrio-shaped cells, *RKM* river kilometer

Tributaries and Branches

Bacterial Numbers Bacterial numbers in tributaries and branches were on average four times higher than those from Danube River samples (Fig. 3a), ranging from 1.26×10^9 to 73.2×10^9 cells L^{-1}. Maximum values of total and filtered samples were recorded for the Arges River which revealed maximum values for all measured bacterial parameters. Lowest concentrations were surprisingly registered in the four largest tributaries Inn, Drava, Tisza, and Sava. On average, 73.8% of all bacteria were free-living (Table 1). In the tributaries Drava, Sava, and Olt as well as in the Soroksar branch, the overwhelming majority of all bacteria (>90%) were free-living, while in the tributaries Morava and Prut as well as Russenski Lom branch, more than 70% of all bacteria were attached to particles >3 μm.

Cell Volumes Bacterial cell volumes in the tributaries ranged from 0.121 to 0.627 μm^3, with an average of 0.254 μm^3 (Fig. 3b) which was some 25% higher than the average cell volume in the midstream of the Danube. Volumes of free bacteria ranged from 0.107 to 0.494 μm^3, with an average of 0.209 μm^3. The calculated average cell volume of the attached cells was 0.388 μm^3 (range, 0.111–1.090 μm^3) and markedly higher than of the free-living cells.

Morphotype Composition The percentages of rods, cocci, and vibrio-shaped cells were on average 59.1%, 23.3%, and 13.6% of total bacterial numbers and thus of similar composition as in the Danube. The percentage of filaments was on average only 1.9%, but a maximum value of 15.8% was recorded for the river Arges.

Bacterial Production Bacterial production rates in the tributaries ranged from 0.30 to 14.8 μg C L^{-1} h^{-1} (average, 1.77 μg C L^{-1} h^{-1}) and were much higher than in the Danube River (Fig. 3c). Highest production values were observed in the river Arges and in the Soroksar I branch, lowest values were again observed in the four largest tributaries Inn, Drava, Tisza, and Sava, and in addition, in the river Olt, with values below 0.5 μg C L^{-1} h^{-1}. On average, about 50% of total production was due to free-living and 50% due to bacteria attached to particles <3 μm (Table 2). The highest contribution of free-living bacteria to total bacterial production was recorded in the river Vah (91%), the lowest contribution of 6% and 4%, or—vice versa—the highest contribution of attached bacteria were recorded in the rivers Morava and Prut, respectively.

Discussion

Continuous Trends Versus Local Processes Influencing the Bacterial Community Along the Danube

Despite the fact that the anthropogenic impact on the Danube in the last century (destruction of flood plains, use as water way, construction of impoundments, and hydro-power plants) dramatically changed river morphology,

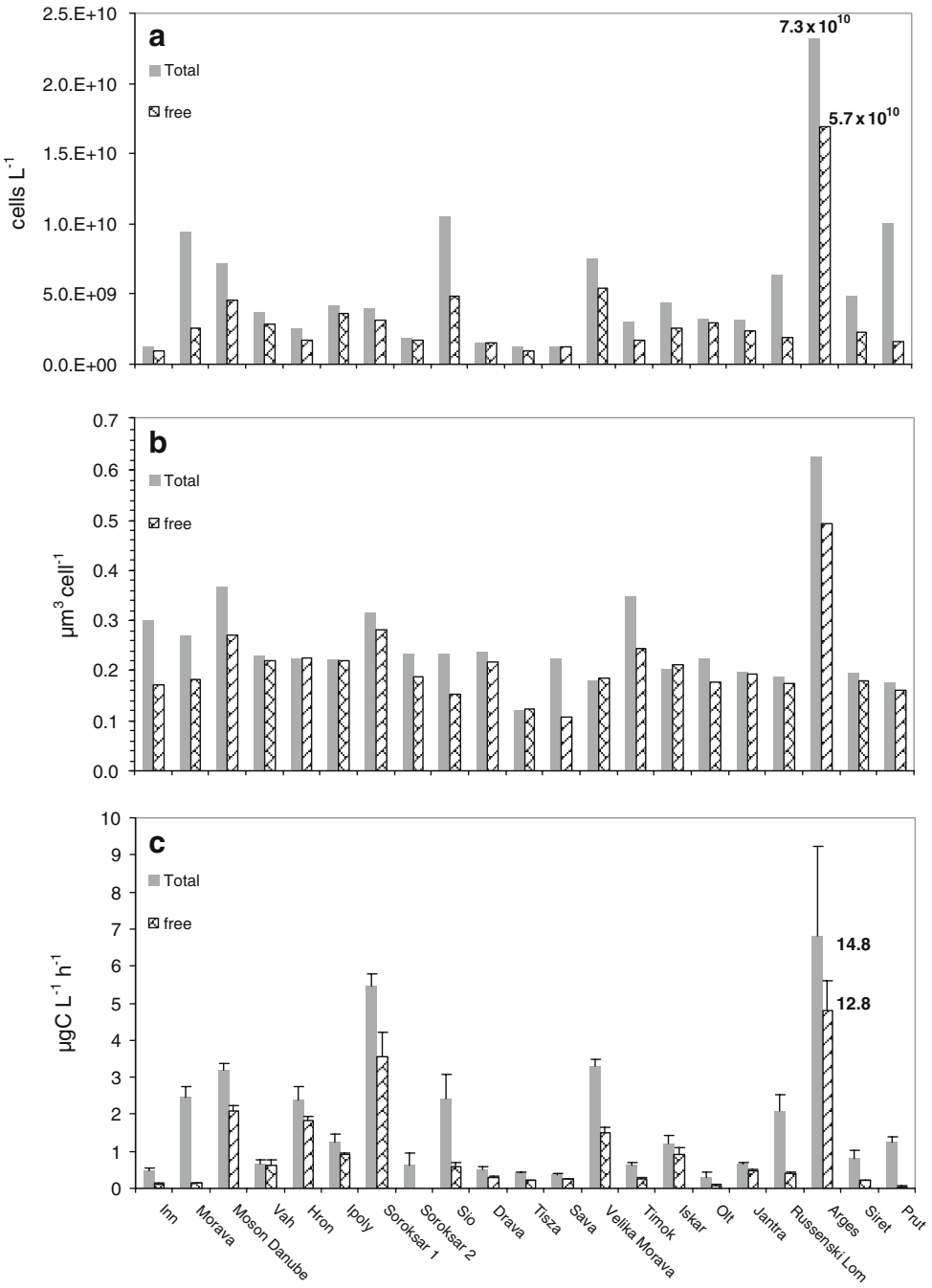

Figure 3 Bacterial numbers (**a**), cell volumes (**b**), and heterotrophic production (**c**) in the tributaries and branches of the Danube. Total and free (<3 μm) bacteria are displayed. *Bars* for bacterial production indicate ± standard deviation of four measurements

statistically significant longitudinal trends along the investigated 2,400-km long stretch of the Danube River could be identified for several bacterial variables, along with gradual changes in inorganic nutrient and particle concentration. We are aware that the period in which the Danube flows to the Delta lasts approximately 32 days while sampling was performed within 43 days. However, because no high water or rainy weather situation was experienced during this period (with the exception of the first few stations), we are convinced that possible temporal changes in bacterial variables would have no relevant influence on the results and their interpretation.

Bacterial numbers showed a significant increasing trend, and these changes were correlated with phosphate concentrations. The general trend observed in our study fits well to earlier investigations in the Danube [22], where a steady increase in bacterial biomass from rkm 1,868 to rkm 93 was observed. Similar trends were also reported for the Australian Murray River [33] and the river Rhine [12]. Nevertheless, some local discontinuities were also identified. The higher values between rkm 2,354 and rkm 2,204 were due to a high-water situation experienced during the beginning of the survey and not considered representative for normal flow conditions, like they were observed during

the rest of the survey. The elevated bacterial numbers after Belgrade and in a stretch of Romania/Bulgaria between rkm 629 and rkm 500 with intensive agriculture indicate some influence of wastewater input from municipalities and/or agronomy on the bacterial load in the Danube.

A continuous pattern reverse to bacterial numbers was observed for cell volumes, decreasing significantly from a level around 0.25 μm^3 to values around 0.16 μm^3 per cell along the Danube's way towards the Delta and showing negative correlation with phosphate and positive correlation with nitrate. Smaller cell volumes are usually an indication for limited nutrient situation [44]. A gradual nutrient depletion occurs in the water column when nutrient uptake by bacteria is not balanced by a corresponding input from phytoplankton or allochthonous organic matter. It has been found that the refractory portion of dissolved organic matter increases with downstream transport, leading to lower quality for bacterial degradation in the potamal region of large rivers [41, 42]. A limiting-nutrient situation for bacteria is mostly accompanied with changes in cell morphology from rod-shaped cells to more coccoid forms [28]. In this study, we could indeed find significant trends for the longitudinal development of the different morphotypes fitting to this postulation. For vibrio-shaped cells, a significant reduction in cell volume was observed. The percentage of rods (total and free-living cells) significantly decreased towards the Delta, while the percentage of cocci significantly increased following concomitant changes of inorganic nutrients (nitrate and ammonium).

Total bacterial production showed no continuous pattern along the Danube, but was obviously influenced by section-specific conditions. Highest production rates occurred from rkm 1,632 downwards to rkm 1,071 and in the uppermost section from rkm 2,412 to rkm 2,278. The peak in the upper section was most probably caused by the high-water situation at the beginning of the cruise, as it is known that during periods of heavy rainfall increased surface—runoff and sewage overflow from wastewater treatment plants increase bacterial load in rivers. The maximum in the middle section was triggered by the high chlorophyll a and inorganic nutrient values caused by the intense sewage input to the river after Budapest until some 100 km after Belgrade [23]. River phytoplankton exudes low molecular weight substances which are readily taken up by bacteria stimulating bacterial production [6]. Winter et al. [48] had observed a marked change in community composition in this stretch of the Danube suggesting that certain high-nutrient-adapted bacterial species are promoted in comparison to the bacteria dominating before. Surprisingly, bacterial numbers and cell volumes were not elevated in this stretch. Thus, the observed rise in bacterial production is obviously counterbalanced by increased loss factors like grazing by protozoa or metazoa. There are strong indications from the literature that bacteria in the water column of large rivers are mainly controlled by protozoan zooplankton [5, 45]. Albeit protozoa were not quantified during this investigation, a significant impact on the bacterial community can also be assumed for the river Danube.

In contrast to total bacterial production, we observed significant continuous patterns of the contribution of attached bacterial production to total production along the Danube. From rkm 2,412 to rkm 1,355, the percentage significantly decreased (r=0.74, p<0.001); thereafter, a significant increase (r=0.61, p<0.001) towards the Delta was observed. The section around 1,200 to 1,300 rkm marks the turning point between the two trends. It is conspicuous that this section comprises the merging sites of the three largest tributaries, Drava (rkm 1,379), Tisza (rkm 1,215), and Sava (rkm 1,170), which may influence the ecosystem of the Danube. While the high values for attached bacteria in the uppermost part of the Danube can be explained by the specific high-water situation with increased input of particles from surface runoff, the high percentages in the lower Danube seem to be a common phenomenon in large rivers. Ochs et al. [30] reported that in the Lower Mississippi, the large majority of water column bacterial production was due to attached bacteria, which was correlated to the increased sediment load when the large river approaches the mouth. Changes in particle concentrations obviously have a significant influence on the bacterial community and, thus, on biogeochemical processes in the Danube. The percentages of attached bacterial numbers and production were significantly linked to total suspended solids and particulate organic matter concentrations. Furthermore, particles seem to be hot spots of bacterial activity. Particle-attached bacterial numbers amounted on average to 27% of total numbers while attached production amounted on average to 42% of total production. Calculated cell volumes of attached bacteria were more than 70% higher than of free bacteria. For tributaries, these values were even higher. Similar observations on the role of particles in large rivers were made by Luef et al. [27] who reported that attached bacteria in the Danube had an 86-fold higher per cell activity than free-living bacteria and by Wilczek et al. [47], who found that attached bacteria contributed only 5–17% to total bacterial numbers, but contributed up to 30–58% of extracellular enzyme activity in the Elbe River.

Our findings on continuous trends versus local processes regulating bacterial standing stock and production in the Danube are in remarkable accordance with the patterns of changes of the 16 S rRNA gene-based bacterial community composition, observed during the first JDS (2001) 6 years before [48]. The authors could show that—despite of the merging of large tributaries and the presence of large impoundments—there was a continuous change in bacterial

community composition, as determined as Jacard's dissimilarity index of operational taxonomic units on DGGE gels. The only exception was the stretch between Budapest (rkm 1,632) and Belgrade (rkm 1,170), where bacterial richness decreased dramatically and an up-shift in dissimilarity occurred, coupled with the extremely high chlorophyll a concentrations found in this stretch (see above).

Influence of Tributaries on the Midstream Samples

A surprising implication of our data is that the impact of tributaries on the continuous development of the bacterial community in the midstream of the river Danube may be mostly negligible. Except for the four largest tributaries Inn, Drava, Tisza, and Sava, confluent rivers had usually higher bacterial numbers, cell volumes, and higher bacterial production rates than the specific Danube region where they merge. Yet, this import of bacteria is not reflected in the midstream samples, fitting well to similar observations on the genetic bacterial community composition in the Danube [48]. Mass calculations to compare the expected bacterial biomass load in the Danube River after the tributaries input with observed biomass load at the following sampling station showed that—with the exception of Iskar, Olt, and Tisza—the expected bacterial biomass values were always between 7% and 63% higher (median, 21%) than the monitored biomass values (Table 3). In the case of Tisza, a decrease of bacterial biomass was expected for the downstream Danube station, but a marked increase was observed. It may therefore be speculated that mixing of the tributaries water masses and their organic load with the water of the midstream could be a slow process. Even though mixing models for tributaries merging into large rivers are problematic in their application for predicting the horizontal transport across the river, rough estimations of the order of magnitude of the distance between the site of discharge and the site of achieved mixing are possible [9, 34] (Table 4). These estimations support the view of a slow mixing of the tributary water masses with those of the river Danube. The water of the river Arges, for instance, merging at rkm 432, would experience complete horizontal mixing only after 529 km which would imply that the water mass of the Danube has reached the Black Sea before complete mixing is achieved. Similarly, the river Timok, which merges at rkm 845, would need 982 km for complete horizontal mixing. Even though

Table 3 Discharge and bacterial biomass of the 17 tributaries at the confluence with the Danube

Tributary	Discharge		Bacterial Biomass				
	Tributary (confl.) $m^3\ s^{-1}$	Danube (upstr.) $m^3\ s^{-1}$	Tributary (confl.) $\mu g\ C\ L^{-1}$	Danube (upstr.) $\mu g\ C\ L^{-1}$	Expected (downstr.) $\mu g\ C\ L^{-1}$	Observed (downstr.) $\mu g\ C\ L^{-1}$	Observed (downstr.) %
Inn	730	530	62.5	115.6	84.9	77.0	90.8
Morava	120	1,780	437.0	54.0	78.2	54.2	69.3
Vah	200	1,950	151.2	44.6	54.5	50.9	93.3
Hron	55	2,155	102.2	68.5	69.4	49.9	72.0
Ipoly	25	2,215	163.8	68.5	69.6	49.9	71.7
Sio	10	2,340	430.4	72.7	74.2	60.0	80.9
Drava	620	2,350	61.9	56.8	57.9	21.6	37.4
Tisza	800	2,970	32.9	47.6	44.5	66.9	150.0
Sava	1,600	3,770	49.3	66.9	61.7	34.6	56.1
V.Morava	250	5,370	255.3	55.3	64.2	50.8	79.2
Timok	25	5,620	161.7	63.8	64.3	42.9	66.7
Iskar	15	5,645	163.4	50.8	51.1	69.3	135.6
Olt	175	5,660	132.7	62.9	65.0	109.5	168.3
Jantra	35	5,838	114.9	88.9	89.0	60.7	68.2
Arges	70	5,870	6140.5	83.1	154.5	57.5	37.2
Siret	230	5,940	176.2	111.2	113.6	99.6	87.7
Prut	110	6,190	338.4	111.2	115.2	99.6	86.5

Discharge and bacterial biomass of the Danube upstream the confluence and the expected and observed bacterial biomass in the Danube downstream the confluence with the tributaries. The four largest tributaries Inn, Drava, Tisza, and Sava exhibit bacterial biomass values lower than or comparable to the Danube. With the exeption of Iskar, Olt, and Tisza, the observed bacterial biomass in the midstream of the Danube was always lower than the expected value after merging with the tributary

Table 4 Horizontal and vertical hydraulic mixing models according to Rutherford [34] and Doneker and Jirka [9]

Model	Formula
Horizontal mixing model	$LMH = 0.5 \times B^2 \times U \times (Ey)^{-1} = 5 \times B^2 \times (h \times \alpha y)^{-1}$
LMH	Length of horizontal mixing [m]
B	Width of the river [m]
U	Average flow velocity [$m \times s^{-1}$]
h	Depth of the river [m]
Ey	Horizontal diffusivity $= \alpha y \times u^* \times h = \alpha y \times 0.1 \times U \times h$
αy	0.5±50%
u*	Rotation speed of the turbulent eddies, approx., U×0.10
Vertical mixing model	$LMV = 0.5 \times U \times h^2 \times (Ez)^{-1}$
LMV	Length of vertical mixing [m]
U	Average flow velocity [$m \times s^{-1}$]
h	Depth of the river [m]
Ez	Vertical diffusivity $= \alpha z \times u^* \times h = \alpha z \times 0.1 \times U \times h$
αz	0.07±50%
u^*	Rotation speed of the turbulent eddies, approx., U×0.10

the prerequisite of the model is an idealized river bed which does not account for enhanced mixing due to meandering, heterogeneity in the bottom profile of the river bed and shipping traffic, the order of magnitude of these values supports nonetheless our mass calculations. We may therefore assume that the impact of a tributary on the midstream of the River Danube is probably negligible when in the following stretch of the River Danube the number of meanders is low and devoid of hydropower plants. These conditions are mainly found in the lower Danube section (rkm 850 to the Delta) and in the middle section (rkm 1,650 to rkm 1,250). Tributary water may therefore be submitted to a filter effect of the river bed sediments along to the river bank where it merges, while experiencing slow mixing with water of the midstream. This hypothesis is further supported by a vertical mixing model [9, 34] (Table 4) which predicts for the river Timok a distance of only 393 m for the complete vertical mixing of the tributary water with the first turbulent eddy of the midstream and some 429 m for the river Arges. Such short-term vertical mixing cycles suggest that the water of the river bank may be effectively cleaned by benthic filter feeders like bivalves or amphipods [12, 43, 46] which represent the most important functional feeding groups at the Danube's river bottom [13]. We are aware that the conclusions presented here are based on midstream samples only, and are in this way rather hypothetical. For validation of the formulated hypotheses, sampling at both river sides is necessary in addition to sampling of the midstream. However, our data clearly show that tributaries, even those with high bacterial load, have negligible influence on the bacterial community in the midstream, and that broad-scale factors prevail shaping the bacterial community in the midstream of the Danube.

The Bacterial Compartment as an Indicator of the Ecological Status of Rivers

The Danube showed, with the exception of the four largest tributaries Inn, Drava, Tisza, and Sava, four times lower values of bacterial numbers and production than its tributaries. Based on a comparison among European rivers, it was observed that larger rivers (River Rhine) had up to threefold lower bacterial abundances than smaller rivers (Saar and Moselle) [5]. The same pattern was also observed for the Mississippi river which exhibited a four to 20-fold lower bacterial production than its tributaries Tennessee, Ohio, and Cumberland [30], indicating a general principle in the spatial distribution of standing stock and bacterial productivity in river networks. Such general principle can also be seen in the continuous trends observed for several bacterial parameters along the Danube. Continuous increases in bacterial numbers and production have also been observed for an important Austrian tributary of the Danube (Schwechat), on the way from its pristine source to the merging site (Supplementary Information Fig. S2), indicating accumulating pollution from anthropogenic sources. Also, in the Danube, elevated values of total bacterial production and bacterial numbers in some stretches indicated pollution from point or non-point sources.

To use these bacterial parameters for assessing the ecological status of rivers is—to date—far from realization. First, a standardized methodology for assessing these parameters is necessary. In our study, the bacterial abundances along the longitudinal profile of the Danube were of similar magnitude as long-term data measured at rkm 1,938 upstream Vienna (range, 1.4×10^9 to 4.1×10^9 L^{-1}; unpublished data, n=67). In contrast, bacterial

production estimates from that study were much higher (range, 4.8 to 120 μg C L^{-1} day^{-1}; n=76, Supplementary Information, Fig. S3) than observed during the JDS (3.4–35 μg C L^{-1} day^{-1}). Values of similar magnitude were also observed by Kasimir [22] (range, 23 to 116 μg C L^{-1} day^{-1}) and Berger et al. [4] (range, 3.1 to 350 μg C L^{-1} day^{-1}). All those investigations were based on the incorporation of ^{3}H-thymidine into bacterial DNA, indicating possible incoherencies due to methodology. Parallel measurements with both methods would be necessary. Second, the spatial and temporal scale of such investigations has to be carefully considered. For example, all values from the river Danube obtained during the studies mentioned above refer to sampling stations close to the river bank, where the influence of large cities or tributaries may by higher than in the midstream. Also, sampling at one date may not be representative, ignoring diurnal, seasonal and inter-annual variation. Finally, and most sophisticated, there is the precondition—because historical data are missing—that an extensive data set has to be created, gathering information from all representative eco-morphological elements of a river network at varying time scales.

Conclusions

This investigation revealed surprising continuous patterns of changes of the bacterial community along the Danube River. Despite the presence of impoundments or hydropower plants, large municipalities and the discharge of large tributaries, many investigated bacterial parameters (standing stock, morphotype succession, and attached bacterial production) developed gradually, indicating that primarily broad-scale drivers and not local conditions shape and control the bacterial community in the midstream of this large river. As most important broad-scale drivers, nutrients (inorganic and organic) and changes in particle concentrations were identified. In contrast, total bacterial activity did not follow a continuous trend but was mainly controlled by the phytoplankton bloom in the river as triggered by the impact of the large cities in the middle section (Budapest, Belgrade). These findings are in remarkable accordance with the molecular biological observations on the 16 S rRNA gene-based bacterial community dynamics and development in the Danube [48]. This accordance is all the more striking because the same patterns arose from two "snapshots" of bacterial population dynamics along the Danube despite different methods used and a period of 6 years between the investigations. We therefore conclude that the midstream of large rivers like the Danube exhibits a continuum of living conditions for bacterial communities, and influences of tributaries/wastewater may be visible mainly in the boundary water masses of the river. This hypothesis has to be validated by additional sampling on both river sides during the next JDS. The comparison between the Danube and its tributaries and other large rivers worldwide revealed that within a river network, the main river transports lower numbers of bacteria and exhibits lower bacterial production rates than its tributaries. All these arguments suggest that the bacterial community may have a powerful indicator function for estimating the ecological status of large river ecosystems, a point which should be followed in future investigations.

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