Multiparametric monitoring of microbial faecal pollution reveals the dominance of human contamination along the whole Danube River

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Abstract

The microbial faecal pollution of rivers has wide-ranging impacts on a variety of human activities that rely on appropriate river water quality. Thus, detailed knowledge of the extent and origin of microbial faecal pollution is crucial for watershed management activities to maintain safe water use. In this study, the microbial faecal pollution levels were monitored by standard faecal indicator bacteria (SFIB) along a 2580 km stretch of the Danube, the world’s most international river, as well as the Danube’s most important tributaries. To track the origin of faecal pollution, host-associated \textit{Bacteroidetes} genetic faecal marker qPCR assays for different host groups were applied in concert with SFIB. The spatial resolution analysis was followed by a time resolution analysis of faecal pollution patterns over 1 year at three selected sites. In this way, a comprehensive faecal pollution map of the total length of the Danube was created, combining substantiated information.

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on both the extent and origin of microbial faecal pollution. Within the environmental data matrix for the river, microbial faecal pollution constituted an independent component and did not cluster with any other measured environmental parameters. Generally, midstream samples representatively depicted the microbial pollution levels at the respective river sites. However, at a few, somewhat unexpected sites, high pollution levels occurred in the lateral zones of the river while the midstream zone had good water quality. Human faecal pollution was demonstrated as the primary pollution source along the whole river, while animal faecal pollution was of minor importance. This study demonstrates that the application of host-associated genetic microbial source tracking markers in concert with the traditional concept of microbial faecal pollution monitoring based on SFIB significantly enhances the knowledge of the extent and origin of microbial faecal pollution patterns in large rivers. It constitutes a powerful tool to guide target-oriented water quality management in large river basins.

Keywords
Standard faecal indicator bacteria; Microbial source tracking; Host associated genetic faecal markers; Bacteroidetes; qPCR; Large river; Joint Danube survey

1 Introduction
The microbial faecal pollution of rivers negatively impacts a variety of human activities that rely on good river water quality. Among other reasons, the production of drinking water from river bank filtration, water used for crop irrigation and the watering of animals, and river stretches used for recreation are the most relevant river ecosystem services that directly affect the health of humans and animals. Because rivers are widely used as receiving waters for wastewater treatment plant (WWTP) effluents, the direct inflow of untreated wastewater and diffuse pollution, the microbial river water quality is often severely impaired (Kirschner et al., 2009; Servais et al., 2007). Even in regions with state-of-the-art wastewater treatment, high levels of microbial faecal pollution may occur (Mayer et al., 2016), unless WWTPs are equipped with a final disinfection step such as UV irradiation (Das, 2001). In Europe, the environmental water quality is generally regulated by the EU Water Framework Directive (European Parliament and Council, 2000) that defines environmental targets for all surface and ground waters in the European Union. However, the microbial water quality targets are only indirectly addressed and mentioned in the Urban Wastewater Treatment Directive (European Council, 1991), the Bathing Water Directive (European Parliament and Council, 2006) and the Drinking Water Directive (European Council, 1998). There are no target values or regulatory values regarding microbial faecal pollution for river water at the European level, which may potentially lead to conflicts between upstream and downstream countries if a transboundary river is highly polluted.

The river Danube is the second-longest river in Europe with a total length of 2780 km, and it is the most international river in the world. Its catchment area covers 801,500 km², with approximately 81 million inhabitants in 19 countries (Schmedtje et al., 2005). Drinking water production from river bank filtrates and the supply of water for domestic, agricultural and industrial use are of major importance in all of these countries. Moreover, the Danube is
an important international transportation route and recreation area (Kirschner et al., 2009). Microbiological pollution along the Danube may originate from point sources such as discharges of treated or untreated sewage from human sources or livestock enterprises and from non-point sources such as urban and agricultural runoff or wildlife. Microbiological contamination from faecal pollution by anthropogenic sources is considered to be a crucial problem throughout the Danube River basin, posing a threat to all types of water uses (Kirschner et al., 2009). Thus, detailed knowledge on the extent and origin of microbial faecal pollution is crucial for watershed management activities to maintain safe water use. For example, in the case of drinking water production from river bank filtrates, this knowledge can help water companies to derive performance targets for water safety plans, based on quantitative microbial risk assessment (Derx et al., 2016).

The extent of recent microbial faecal pollution in surface waters is usually determined by the use of standard faecal indicator bacteria (SFIB) such as *E. coli* and intestinal enterococci. According to the EU Bathing Water Directive (European Parliament and Council, 2006), the assessment of the microbiological bathing water quality is exclusively based on these two parameters. However, the determination of the SFIB does not provide information about the pollution source. In the past decade, microbial source tracking (MST) has become an indispensable tool for uncovering the origin of microbial faecal pollution in different environmental water systems (Hagedorn et al., 2011). Among the several available approaches (Caldwell et al., 2011; Hagedorn and Weisberg, 2011; McQuaig and Noble, 2011; Wuertz et al., 2011), host-associated genetic Bacteroidetes markers have been shown to be one of the most reliable targets for MST (Kildare et al., 2007; Reischer et al., 2013). Based on qPCR detection, this approach is highly sensitive, specific, accurate and precise for MST in combination with SFIB determination (Mayer et al., 2016), and it has been successfully applied in a wide variety of aquatic ecosystems (Farnleitner et al., 2011; Liu et al., 2015; Reischer et al., 2007; Stapleton et al., 2009).

In 2001 and 2007, the microbial faecal pollution of the river Danube was mapped along a stretch of approximately 2600 km during two Joint Danube Surveys organized by the International Commission for the Protection of the Danube River (ICPDR) (Kavka and Poetsch, 2002, Kirschner et al. 2008, 2009). However, in those investigations, only midstream samples were analysed without considering the site-specific cross-sectional pollution pattern. It has been hypothesized that in large rivers such as the Danube, the mixing of polluted water masses from wastewater influents or tributaries only occurs slowly and may take several dozen kilometres before being detectable midstream (Velimirov et al., 2011). Thus, potential pollution hot-spots may have been overlooked. In the current study, microbial faecal pollution levels were therefore monitored in the midstream and at both riversides as well as in the Danube’s most important tributaries. To track the origin of faecal pollution, host-associated genetic faecal markers for different host groups were determined simultaneously at all the sampling sites along the river and the tributaries. The spatial resolution analysis during the Joint Danube Survey 2013 was followed by a time resolution analysis of faecal pollution patterns over one year in 2014 at three selected sites along the river. In this way, a comprehensive, updated and extended faecal pollution map of the Danube was created, combining substantiated information on both the extent and origin of
microbial faecal pollution over the total length of a river that extends over a continental scale.

2 Materials and methods

2.1 Joint Danube Survey

The Joint Danube Survey (JDS) was organized by the ICPDR in 2001, 2007 and 2013. It is the world's biggest and most international river expedition with dozens of scientists and laboratories involved. In 2013, samples were taken by boat in the summer between Aug 12 and Sep 26 at 70 sampling stations along the longitudinal profile of the Danube in consecutive order from the upper section (river km 2581) to the Delta (river km 18), including the major tributaries and branches at their confluence with the Danube. The sampling sites were positioned within regions that cover the whole range of different land-use classes such as large municipalities, areas with intensive agriculture, forests, grasslands or wetlands. A detailed map of the river and the sampling stations can be found in the Suppl. Information (Fig. S1). In addition to the 68 official JDS stations (Fig. S1), samples were taken at two additional stations for microbiological analysis only; at the Inn tributary (rkm 2225) and downstream from the city of Vienna (rkm 1930). During the JDS, dry weather conditions and baseflow conditions prevailed, with a single precipitation event (25 mm on August 27), leading to an increase in the discharge from approx. 1200 m$^3$/s to 2000 m$^3$/s between sampling sites 10 (rkm 1895) and 13 (rkm 1869). Details on the discharge measurement and data can be found in the Supplemental information, Table S1. During the JDS, the Danube water temperatures ranged from 18 to 23 °C.

2.2 Sampling

Water samples were collected by hand from small boats (anchored at the primary ship) at a water depth of approx. 30 cm, according to ISO 19458:2006 (International Organization for Standardization, 2006), in two sterile 1-L glass flasks (flask A and B) from 54 sampling stations along the Danube, and from 16 tributaries or branches. Because the vertical mixing of water masses in the Danube is most likely a rapid process (Velimirov et al., 2011), samples from a 30 cm depth are regarded as representative for the whole water column. At all the Danube stations (except station JDS1) and at the Inn, Drava, Tisza, and Sava tributaries, samples were taken from the middle and the left and right sides of the river. The other tributaries and branches were sampled only from the middle of the river. Tributaries were sampled at a maximum of 500 m upstream of the confluence with the Danube. Samples from the left and right sides were taken approx. 15 m away from the shoreline, and samples from the middle were taken from the middle of the cross-sectional profile. All the samples were immediately processed in the on-board laboratory of the primary ship.

2.3 Microbiological analysis

Escherichia coli and intestinal enterococci were used as general indicators of faecal pollution (World Health Organization, 2003), and they were determined using standard cultivation-based methods. Genetic faecal Bacteroidetes markers associated with human faecal pollution (BacHum, HF183II), ruminant faecal pollution (BacR) and porcine faecal
pollution (Pig2Bac) were determined via quantitative PCR (qPCR). For quality control purposes, the AllBac marker was included in the analysis.

**Escherichia coli**—*E. coli* concentrations were determined according to ISO 9308–2:2012 (International Organization for Standardization, 2012) with Colilert 18 (IDEXX, Ludwigsburg, Germany), using two sample volumes (100 ml, 1 ml). The samples were incubated at 36 ± 2 °C for 18–22 h and analysed in a UV cabinet. Quantitative values were obtained by comparing the results with the MPN table provided by the manufacturer.

**Intestinal enterococci**—Enterococci concentrations were determined by the standard method according to ISO 7899–1:1998 (International Organization for Standardization, 1998) with commercially available MUD/SF microtitre plates (BIORAD, Vienna, Austria) using two dilutions (1:2 and 1:20). The microtitre plates were incubated at 43 ± 2 °C for 32–40 h and analysed in a UV cabinet. Quantitative values were obtained by comparing the results with the MPN table given in ISO 7899–1:1998.

**Classification system for faecal pollution**—To enable the assessment of the faecal pollution levels, the faecal indicators were classified by a system of 5 microbiological water quality categories according to Kavka et al., (2006) and Kirschner et al., (2009) (Supplemental Information Table S2). To set up this scheme, two concentrations derived from the EU Bathing Water Quality Directive 2006 (European Parliament and Council, 2006) were used as anchor points (1000 CFU/MPN for *E. coli* and 400 CFU/MPN for enterococci). Faecal pollution levels in quality classes I and II are below these values, and quality classes III, IV, and V exceed these values. The EU Bathing Water Directive and the assessment of the bathing water quality could not be applied to the JDS data set since the bacterial indicator data for faecal pollution generated during the JDS are single measurements. According to the EU Bathing Water Directive, the assessment of the bathing water quality should always comprise at least 16 samples that are compiled in relation to the current bathing season and the three preceding bathing seasons, based upon 95-percentile and 90-percentile evaluations, respectively.

**DNA filtration and extraction**—From each sample, duplicate subsamples (one from flask A and one from flask B) were filtered through 0.2 μm polycarbonate filters with a diameter of 47 mm. Depending on the turbidity of the samples, the filtration volume was between 100 and 300 ml. Clean control filters were frozen and stored alongside the sample filters at each sampling station. The filters were immediately frozen at −20 °C, and after no more than 3 weeks, all the filters were transferred to a −80 °C freezer in the home laboratory. DNA was extracted from the filters by phenolchloroform extraction combined with bead-beating (Reischer et al., 2008). The extracted DNA was dissolved in 100 μl of Tris buffer (10 mM, pH 8.0) and stored at −80 °C until qPCR analysis. Extraction controls were routinely run alongside each extraction batch.

**Microbial faecal source tracking assays**—All the flask A samples were analysed using microbial faecal source tracking assays. In parallel, an additional 40 flask B samples were analysed to check for sample variability. For qPCR quality assurance and inhibition control, all the DNA extracts were measured by AllBac qPCR assay (Layton et al., 2006),
which targeted the abundant Bacteroidetes phylum to ensure the presence of amplifiable bacterial DNA in duplex with an internal amplification control (IAC) (Anderson et al., 2011), targeting the ntb2 gene from tobacco to rule out PCR inhibition. Furthermore, all the samples were quantified in 1:4 and 1:16 dilutions for the additional assessment of PCR inhibition. For microbial source tracking, the human-associated faecal markers BacHum (Kildare et al., 2007) and a recently modified version of HF183II (Green et al., 2014) were determined by qPCR, indicating human-associated faecal pollution. The ruminant-associated BacR qPCR assay (Reischer et al., 2007) and the pig-associated Pig2Bac qPCR assay (Mieszkin et al., 2010) were included as methods for detecting these specific animal faecal pollution sources. All the qPCR assays were adapted to run on the Rotor-Gene Q thermocycler by using the Rotor-Gene Multiplex PCR mastermix (Qiagen Inc.).

Quantification was performed by running plasmid standard dilution series of known concentration. No-template controls were applied to all the instrument runs. For the source-associated assays, all the samples (1:4 dilutions) were run in duplicate. None of the samples showed evidence of PCR inhibition, and all the filtration/extraction controls and PCR no-template controls were negative. PCR efficiencies (eff.) and coefficients of determination ($r^2$) as calculated from linear regression of the standard dilution series for the applied assays were: AllBac (eff. 100–105%, $r^2 > 0.96$), BacHum (eff. 98–105%, $r^2 > 0.97$), HF183II (eff. 100–105%, $r^2 > 0.99$), BacR (eff. 96–103%, $r^2 > 0.99$), Pig2Bac (eff. 98–101%, $r^2 > 0.99$).

### 2.4 Annual cycle

To assess the temporal variability in the faecal pollution, monthly samples were taken at three critical sampling stations along the Danube in 2014, downstream from Vienna, downstream from Budapest and downstream from Belgrade. Vienna (1.7 million inhabitants) has been equipped with a secondary WWTP since 1980; it was upgraded with a second biological treatment step in 2005, and it cleans all the city’s municipal wastewater (2 MPE). Budapest (1.7 million inhabitants) has had a central WWTP since 2010 that cleans the majority of the municipal wastewater (1.6 MPE). Belgrade (1.35 million inhabitants) does not have a WWTP.

In Vienna and Budapest, monthly samples were taken from bridges (left, middle, and right) downstream from the treated wastewater inflow. In Belgrade, samples were taken from small boats downstream from the city. The samples were immediately brought to the home laboratories in insulated cooling boxes and processed as described above. In Belgrade and Budapest, DNA samples were stored at −20 °C and brought to Vienna in July 2014 and February 2015. MST marker determinations were performed by qPCR as described above.

### 2.5 Statistical analysis

All the statistical analyses were performed with SPSS 22 (IBM, New York, USA). All the microbiological data were log10-transformed after adding 1 to each value. A Spearman rank correlation and a simple linear regression were used to study the relationships among the different faecal indicators and source tracking markers. Significance was accepted at $p \leq 0.05$. To study the relationship between the microbial faecal parameters and the background environmental data, a principal component analysis (Varimax rotation with Kaiser Normalization) was performed. All the JDS 2013 background data were retrieved from the...
ICDPR Danubis database (https://www.icpdr.org/wq-db/) with the kind permission of the ICPDR.

3 Results

3.1 Longitudinal faecal pollution patterns reveal critical pollution in the middle section of the Danube, several hot-spots along the whole river and in specific tributaries

Along the upper section of the Danube (Germany, Austria and Slovakia, rkm 2600 to 1800), all the midstream samples exhibited low to moderate *E. coli* pollution levels (Fig. 1A; the same graph including all WWTPs > 40,000 population equivalents can be found in the Supplemental Information, Fig. S2). However, at three sampling stations, the left and right near-shore sites showed critical and strong pollution. Surprisingly, at Kelheim (Germany), the left riverbank sample exhibited the highest *E. coli* value of all the Danube samples (34,500 MPN/100 ml). Elevated pollution levels were also observed in Austria at two near-shore sites (Oberloiben left, 2420 MPN/100 ml and downstream from Vienna right, 2430 MPN/100 ml). The higher *E. coli* numbers from the right riverside downstream from Vienna can be explained by the inflow of the Danube channel a few hundred metres upstream of the sampling station. This channel holds the recipient water from the Vienna WWTP effluent. No satisfactory explanation could be found for Kelheim and Oberloiben, except that both sites are situated next to a pier. All the other near-shore sites showed similar *E. coli* levels as the respective midstream sites. The three major investigated tributaries joining the Danube in this river section had 0.5 log (Inn, Morava) to 1.2 log (Moson Danube) higher *E. coli* concentrations than the Danube, with the Moson Danube receiving wastewater from the Hungarian city of Győr.

In the middle section (Hungary, Croatia, and Serbia, rkm 1800 to 900), several midstream stations showed critical *E. coli* pollution levels (Fig. 1A). At Dunaföldvár, which is situated approximately 60 km downstream from Budapest, the midstream sample showed the highest *E. coli* values of all the midstream Danube samples (12,500 MPN/100 ml). From downstream from Budapest (rkm 1632) until Banatska Palanka (rkm 1071), not a single Danube sample was classified as having low *E. coli* pollution. Specifically, in the stretch from Novi Sad to Velika Morava that includes the cities of Belgrade and Pancevo, all the midstream samples were critically polluted. Downstream from Novi Sad, the left riverside sample was markedly more polluted (6100 MPN/100 ml) than the midstream sample (980 MPN/100 ml), and downstream from Belgrade, even samples from both the left and right riversides showed strong *E. coli* pollution levels (11,600 and 12,500 MPN/100 ml, respectively). At the end of the middle Danube section, the large reservoirs for the two Iron Gate hydropower plants significantly reduced the *E. coli* pollution levels to low, most likely due to sedimentation and longer water residence times. At Vrbica (Serbia)/Simijean (Romania) (rkm 926), the lowest *E. coli* value of all the Danube samples was recorded for the midstream sample (2 MPN/100 ml), while both the left (2400 MPN/100 ml) and right-side samples (980 MPN/100 ml) showed critical and moderate pollution levels. The tributaries and branches joining in this Danube section had contrasting levels of pollution such as the critically polluted Drava and Velika Morava and the little polluted Rackeve-
Soroksar arm and Sava (midstream sample). The left-side Sava sample was moderately polluted with \textit{E. coli}, such as the Tisza and Vah tributaries.

In the lowest Danube section (Romania, Bulgaria, Ukraine; rkm 900 to 18), all but one midstream sample showed low to moderate \textit{E. coli} pollution levels. Three right and two left riverside samples exhibited critical pollution levels. Amongst those, the sites downstream from Russenski Lom (rkm 488, right) and downstream from Arges (rkm 429, left), the two most polluted tributaries/branches of the whole river basin, were found. The other three sites were downstream from Zimmicea/Svistov (rkm 550, right) and at Chiciu/Silistra (rkm 378, both sides). The Timok tributary (860 MPN/100 ml) was much more polluted than the river Danube in terms of \textit{E. coli}, leading to a higher downstream value at the respective riverside (rkm 834, left). The two tributaries Iskar and Jantra showed little \textit{E. coli} pollution, while Siret and Prut were critically and moderately polluted, respectively.

In general, the faecal pollution pattern derived from intestinal enterococci resembled the \textit{E. coli} pattern (Fig. 1B). Both parameters were highly significantly inter-correlated in the Danube (rho = 0.685; \(p < 0.001\)) and its tributaries (rho = 0.596; \(p \leq 0.001\)) (Table 1). In the upper section, Kelheim (left; > 34,600 MPN/100 ml), Oberloiben (left; 480 MPN/100 ml) and downstream from Vienna (right; 390 MPN/100 ml) showed the highest enterococci concentrations. In the middle section, downstream from Budapest (right; 940 MPN/100 ml), Dunaföldvár (middle; 2500 MPN/100 ml), the Drava tributary (460 MPN/100 ml) and the section between Novi Sad and Velika Morava (mostly left and right riverside samples; 110–2300 MPN/100 ml) showed critical pollution values. Additionally, at Iza/Szony (rkm 1761), the left riverside was critically polluted. After a significant drop along the Iron Gates, the enterococci concentrations were elevated for the left and right riversides at Vrbica (Serbia)/Simijan (Romania) (rkm 926). In the lowest section, Rusenski Lom (46,900 MPN/100 ml) and Arges (3100 MPN/100 ml) showed the highest concentrations of all the investigated tributaries and branches, with both leading to significantly elevated downstream concentration levels at their respective Danube riversides. In addition, the sampling station downstream from Zimmicea/Svistov (rkm 550; left) exhibited strong faecal pollution (27,700 MPN/100 ml).

### 3.2 Cross-sectional patterns reveal the strong representative status of midstream samples but also specific local lateral discontinuities

Over the total length of the Danube, midstream samples representatively depicted the microbial faecal pollution levels at the respective river sites (Fig. 2). The midstream \textit{E. coli} concentrations were significantly correlated with the left-side (rho = 0.55; \(p < 0.01\)) and right-side concentrations (rho = 0.56; \(p < 0.01\)). A similar pattern was observed for enterococci, but the correlation coefficients were lower (rho = 0.35 for the left and 0.28 for right sides, \(p < 0.05\)) due to the higher detection limit of the microtitre plate method. However, specific left and right riverside samples showed significantly increased pollution levels in comparison to the midstream sample. This trend was mostly observed downstream of large municipalities with and without wastewater treatment or polluted tributaries (Fig. 2). With the exception of downstream Jantra (enterococci), only the results from Dunaföldvár
(60 km downstream Budapest) showed higher SFIB values for the midstream sample than for both riverside samples.

3.3 Microbial faecal pollution constitutes an independent principal component

To analyse the potential relationships to the ancillary environmental data, chemophysical data (oxygen, pH, conductivity, temperature, and discharge), nutrient data (dissolved organic carbon, total phosphorus, and total nitrogen), biological data (chlorophyll $a$, total and large bacterial cell numbers) and chemical tracers related to anthropogenic pollution (acesulfame, carbamazepine, and caffeine) were included in the principal component analysis together with the microbiological data. Within the multidimensional component matrix, the microbial faecal pollution parameters constituted an independent principal component apart from the other variables (Table 2, Fig. 3). Even the chemical tracers of anthropogenic origin (caffeine, carbamazepine, and acesulfame) did not cluster with the microbiological parameters. When examining the specific left and right riverside samples with surprising critical, strong or excessive pollution (e.g. Kelheim, Oberloiben), the ancillary data could not satisfactorily explain the high observed pollution levels.

3.4 Several midstream stations showed lower pollution levels in JDS 3 than in JDS 2

A comparison between the midstream data from 2013 (JDS 3) and 2007 (JDS 2) revealed a similar pollution pattern (see (Kirschner et al., 2009)) and a highly significant correlation between the years (rho = 0.738; $P < 0.001$, $n = 48$). However, the median concentrations were slightly lower in 2013 in comparison to those of 2007 (with a median difference of 0.4 log for both $E. coli$ and enterococci) and at several stations, markedly (>1 log) lower $E. coli$ concentrations occurred in 2013 (Fig. 4, left panel). This trend towards lower values in 2013 was also valid when the data were normalized to the discharge, i.e., when the total load was calculated (Fig. 4, right panel). Among the stations with markedly lower $E. coli$ levels are the stations downstream from large municipalities with wastewater discharge such as Budapest, Wildungsmauer (that is 35 km downstream Vienna), downstream from Linz, Bratislava, and downstream from Svistov. In addition, the two branches (Moson Danube and Rackeve-Soroksar arm) also showed markedly (approx. 2 log) lower concentrations in 2013 (data not shown). Enterococci were not included in the comparison because there were too many missing data and data below or at the detection limit (15 MPN/100 ml).

3.5 Microbial faecal pollution in the Danube and its major tributaries is dominated by human faecal pollution

The human-associated faecal marker BacHum was detected in 92.4% of all the investigated Danube samples ($n = 159$) and in 100% of all the tributary samples ($n = 22$) (Table 3). The concentrations ranged from 250 to $1.3 \times 10^6$ marker equivalents (ME)/100 ml in the Danube and from 260 to $4.6 \times 10^6$ ME/100 ml in the tributaries. The highest concentration in the Danube was observed downstream from Arges (left). The tributary with the highest concentration was Rusenski Lom, followed by Arges ($4.5 \times 10^6$ ME/100 ml). The BacHum concentrations in the midstream samples were 0.1–0.2 log (median values) and 0.3 to 0.6 log (maximum values) lower compared to the left and right riverside samples, but at all three positions, > 90% positive results were obtained. The second human-associated faecal marker
HF183II was present in >80% of all the investigated Danube samples, while in the tributaries, it was detected in 59% of the samples (Table 3). The concentrations were approximately 0.5 log lower than the BacHum concentrations, with a maximum value of $4.0 \times 10^5$ ME/100 ml in the Danube (downstream Arges) and $1.5 \times 10^6$ ME/100 ml in the tributaries (Rusenski Lom). In both the Danube and its tributaries, BacHum and HF183II were highly significantly inter-correlated (Table 1) with rho = 0.894 (p < 0.001) and rho = 0.903 (p < 0.001), respectively. In general, the results from flask A and flask B matched very well for both BacHum (rho = 0.910, p < 0.001, n = 40) and HF183II (rho = 0.938, p < 0.001, n = 40), showing low sample variability and corroborating the high reliability of the chosen assays (Supplemental information, Fig. S3).

In contrast to human-associated markers, the ruminant (BacR) and pig (Pig2Bac)-associated markers were of minor importance along the whole Danube River and its major tributaries. Only between 4 and 9% of the samples were positive for both markers, with equal percentages for the Danube and its tributaries (Table 3). In addition, the maximum concentrations were 2–3 logs lower than the human-associated markers. The highest Pig2Bac concentration ($6.9 \times 10^3$ ME/100 ml) was found in the Sulina arm of the Danube Delta (left, rkm 26), and the highest BacR concentration ($2.9 \times 10^3$ ME/100 ml) was found in the Jantra tributary (merging at rkm 537).

### 3.6 Human-associated faecal markers showed a highly significant correlation with the standard faecal indicators in the Danube

Both *E. coli* and enterococci were significantly correlated with the human-associated markers of faecal pollution (Table 1). The highest and most significant (p < 0.001) correlation was found for *E. coli* in the Danube with rho = 0.776 and 0.848 for BacHum and HF183II, respectively. In the tributaries, the correlation was significant at a level of p < 0.05. The enterococci showed correlation coefficients with human faecal markers between 0.494 and 0.685 (p < 0.001) in the Danube. In the tributaries, no significant correlation to human faecal markers (0.338, p > 0.05 and rho = 0.326, p > 0.1 for BacHum and HF183II, respectively) was observed. Fig. 5 shows that for the whole data set, 67% and 59% of the *E. coli* variability was explained by HF183II and BacHum, respectively. For the Danube, the coefficients of variation were 65% and 60%, respectively, and for the tributaries, they were 78% and 64%. Similar values were obtained for left-side, midstream and right-side data (Fig. S4, Supplemental Information). Due to the low quantity of positive signals, no correlations were calculated for ruminant and pig-associated markers.

### 3.7 Annual patterns in faecal pollution

At the three stations downstream of the large capitals (Vienna, Budapest, and Belgrade), the samples were analysed monthly over one year in 2014. Despite some temporal variability, the data confirmed the basic findings from the JDS 3 "snapshot" analysis. In Vienna, only low and moderate pollution levels were recorded (Fig. 6A and B). The right-side samples always showed significantly higher faecal pollution than the midstream samples (T-test, t = 2.91; p < 0.01 for *E. coli* and t = 3.38; p < 0.01 for enterococci) due to the inflow of the WWTP effluent on this side. The left-side samples showed no significant differences relative to the midstream samples (p > 0.1). In Budapest, the pollution was mostly found to be at
moderate levels, with a few critical samples (midstream and right-side only) and two samples with strong pollution levels (enterococci only) (Fig. 6C and D). The right-side concentrations of both *E. coli* and the enterococci were significantly higher than the midstream concentrations (*t* = 1.90; *p* < 0.05 and *t* = 2.71; *p* < 0.05, respectively), and the left-side concentrations were significantly lower (*t* = 2.90; *p* < 0.01 and *t* = 3.63; *p* < 0.01, respectively). In Belgrade, the majority of all the samples showed critical pollution levels. At several time-points, the midstream samples showed moderate pollution levels, while strong pollution was observed in the right riverside. Significantly higher pollution levels occurred in both river sides than in the midstream segments (*p* < 0.05 for the left riverside and *p* < 0.001 for the right riverside).

The human-associated faecal markers BacHum and HF183II showed similar spatial distribution patterns (Fig. 7), with the highest marker concentrations on the right riversides for all three stations. In general, the marker concentrations downstream from Vienna and Budapest were of comparable magnitude, while downstream from Belgrade, they were approximately one log higher (Table S3, Supplemental information).

All the faecal-associated parameters were highly significantly inter-correlated (*rho* = 0.510 to 0.963, *p* < 0.001, and *n* = 111; see Table S5, Supplemental Information). BacHum and HF183II explained 39% and 42% of the *E. coli* variability, respectively (Fig. S5, Supplemental Information).

### 4 Discussion

The chosen approach of using standard faecal pollution indicators together with advanced microbial source tracking markers over a large spatial and temporal scale allowed us to comprehensively characterize the microbial faecal pollution patterns along the whole Danube River. This study was built on two previous whole-river surveys, in which the longitudinal development of faecal pollution was only based on the SFIB concentrations that were determined for the midstream area of this large river (Kirschner et al., 2009, 2015). Here, we extended this sampling concept by including left and right riverside samples, different host-associated source tracking markers and a one-year analysis at three specific sites to assess the temporal variability of faecal pollution. To the best of our knowledge, no study of comparable size and sampling depth characterizes the microbial faecal pollution patterns of such a large river.

In general, the different methods used to determine the microbial faecal pollution (standard faecal indicators and human genetic faecal markers) were highly and significantly inter-correlated, and they yielded congruent pictures of the pollution patterns along the Danube. Nevertheless, the *E. coli* measurements performed according to ISO 9308–2:2012 (International Organization for Standardization, 2012; Colilert) had a more satisfactory resolution than the enterococci measurements performed in accordance with ISO 7899–1:1998 (International Organization for Standardization, 1998; microtitre plate method) because for the latter, many values at and below the detection limit were recorded. However, in the principal component analysis, all the microbial faecal pollution parameters constituted...
a separate component and did not cluster with any of the environmental background data, including chemical tracers of anthropogenic origin.

The general microbial faecal pollution pattern during JDS 2013 was similar to the one observed in 2007, with the highest pollution levels in the middle section of the Danube and faecal pollution hot-spots in the Rusenski Lom (BUL) and Arges (ROM) tributaries (compare to (Kirschner et al., 2009)). However, in the stretch downstream from Budapest (rkm 1632–1434), lower pollution levels were observed in 2013, including the Rackeve-Soroksar branch, which was a hot-spot of faecal pollution in 2007. Clearly, the implementation of the Central Budapest WWTP with a capacity of 1.6 Mio population equivalents did have a positive effect on the faecal pollution levels of the Danube in this stretch, with the E. coli concentrations being 0.4 to 1 log lower in 2013 than in 2007. Only in the midstream at Dunaföldvár (rkm 1560) were similarly high values observed during both years. Because the wastewater of the Budapest WWTP is dumped in the middle of the Danube, we hypothesize that the plume is still visible even 60 km downstream due to the inefficient mixing of the water masses (Velimirov et al., 2011). Apart from Dunaföldvár, the highest contamination levels in the midstream of the Danube occurred in Serbia, in the stretch between Novi Sad and the Velika Morava tributary. Here, two large municipalities, Novi Sad and the Serbian capital Belgrade, are without state-of-the-art wastewater treatment.

When interpreting the differences between the years, the specific river regime must be considered. In 2013, baseflow conditions prevailed along the whole Danube (Supplemental Information, Table S1). For 2007, an incomplete set of discharge data is available. During that year, the discharge levels during the JDS were 33%–48% higher in the upper stretch (rkm 2204–1942) and 88%–128% higher in the lower stretch (rkm 1071–130) of the Danube. In the middle stretch (rkm 1560–1200), the discharge during JDS 2007 was slightly (15%–28%) lower than it was during JDS 2013 (Supplemental Information, Table S1). From Fig. 4 it became obvious that the lower concentrations observed in 2013 in comparison to 2007 are not attributed to a dilution effect but to a general reduction of faecal pollution in the Danube. This reduction could be caused by more effective wastewater treatment (e.g., the implementation of the central WWTP at Budapest). Additionally, reduced diffuse pollution may have reached the river, because baseflow conditions were experienced during JDS 2013 throughout the whole expedition, and only a single precipitation event occurred downstream from Vienna.

In comparison to 2007, the additional sampling from both riversides in 2013 allowed for the identification of further and partly surprising hot-spots of faecal pollution. In general, the midstream samples appropriately reflected the faecal pollution status of the Danube at the specific river sites. It has been hypothesized that the horizontal mixing of the water masses along the Danube is a slow process, which may occur over several hundreds of kilometres (Velimirov et al., 2011). Therefore, local pollution at the riverside may not be reflected in the midstream samples. This trend was found not only for several stations in the middle and the lower sections of the Danube (downstream from Ruse or from Arges) but also for two stations in the upper section of the Danube that exhibited critical or strong pollution levels at the left riverside, Kelheim (GER) and Oberloiben (AUT). Because both countries perform state-of-the-art wastewater treatments and both stations are far downstream of large
municipalities, local peculiarities (vicinity to a pier) are assumed as the reasons for the high observed levels of microbial faecal pollution. A third station with critical pollution levels was downstream from Vienna, which is obviously caused by the inflow of the Vienna WWTP. Other stations with a striking difference in faecal pollution levels between the midstream and the riversides were observed primarily in the lower section at rkm 926 (Vrbica-SRB/Simian-ROM), where the E. coli and enterococci levels were 2–3 orders of magnitude higher at the riversides than in the middle of the Danube, and at rkm 550 (downstream Svistov-BUL/Zimnicea-ROM), where excessive enterococci pollution levels were observed.

Large municipalities obviously have a tremendous effect on the microbiological water quality of the Danube. After the large cities Vienna, Budapest, Novi Sad, and Belgrade, the microbial faecal pollution levels were generally increased. In addition, the tributaries that are used as the receiving waters of municipal waste-water such as the rivers Arges (Bucharest-ROM) and Rusenski Lom (Ruse-BUL) showed strong or even excessive pollution levels, indicating that human faecal pollution is a major source of microbial faecal pollution. With the microbial source tracking markers used in this study, we could now convincingly demonstrate that human faecal pollution is dominant throughout the whole length of the river Danube. Human-associated genetic faecal Bacteroidetes markers were present in 78% (HF183II) to 96% (BacHum) of all the samples, and BacHum (Kildare et al., 2007) exhibited higher prevalence and concentrations in comparison to HF183II (Green et al., 2014). A recent study investigating the human-associated marker concentrations in wastewater and faecal samples has shown that HF183II is slightly less abundant and more source-specific than BacHum in the faecal sources (unpublished data). The human-associated markers were highly significantly inter-correlated and correlated with the E. coli and enterococci concentrations as well. For the Danube and its tributaries, the E. coli concentrations explained 59%–78%, respectively, of their variability, and HF183II showed slightly higher coefficients of determination. By contrast, ruminant (BacR; (Reischer et al., 2007)) and pig-associated (Pig2Bac; (Mieszkin et al., 2010)) markers were only rarely detected (<10% positive samples), and the median concentrations were one to three orders of magnitude lower than the human-associated marker concentrations, despite the fact that animal farming and pastureland occur along the whole river. It must be considered that baseflow conditions prevailed during the whole river expedition, with only a single precipitation event downstream from Vienna. Low surface runoff may have led to reduced, diffuse pollution entering the river, leading to an underestimation of the non-point animal faecal pollution sources. Another potential source of animal faecal pollution that was not considered in this study may be faeces from wild and livestock birds. No general marker for all bird species is available so far, but markers for gulls (Lee et al., 2013), ducks (Kobayashi et al., 2013) and poultry (Ryu et al., 2014) exist. Moreover, horses and fish may also partly contribute to the faecal pollution in the Danube River, but no reliable genetic faecal markers associated with those two groups are known. However, because the ruminant and pig markers that also target wildlife such as deer and boar were relatively unimportant, we consider the role of the other animal groups to be of similarly little importance to the general faecal pollution of the Danube River.
During the annual cycle that covered a wide range of hydrological conditions at all three selected sites, the investigations revealed considerable seasonal variability in all the measured microbial faecal pollution parameters, but they corroborated the conclusions drawn from the Joint Danube Survey. In comparison between the three capitals, Vienna showed the lowest (little to moderate) pollution levels, followed by Budapest, while Belgrade (without wastewater treatment) exhibited critical to strong faecal pollution. In nearly all cases, the midstream values were lower than the values observed at the left and right riversides for all the measured parameters, including the genetic faecal source tracking markers. It is obvious that human faecal pollution is dominating at all three stations downstream from the capitals with approx. 1.5 to 2 Mio inhabitants. However, the prevalence and abundance of ruminant and pig markers in the midstream, where faecal pollution should reflect the mixed picture of all upstream contamination sources, were not different from the ones observed at both river-sides and not higher than those observed during the JDS. This finding clearly supports the results from the spatial analysis in which human faecal pollution is the dominant source along the whole Danube.

5 Conclusions

In addition to traditional cultivation, the future monitoring of microbial faecal pollution will increasingly rely on the implementation of advanced culture-independent techniques that simultaneously allow for the identification of the sources of contaminations. For the first time, standard indicators of faecal pollution were applied in combination with several host-associated genetic faecal markers for the whole length of a large international river at a high spatial resolution. In addition to identifying the hot-spots and general patterns of faecal pollution, we could show that the microbial faecal pollution constituted an independent component within the environmental matrix. We could also clearly demonstrate that human faecal pollution is the primary source of microbial faecal contamination along the whole Danube River. It turned out that for whole river monitoring purposes, midstream samples are largely representative of the faecal pollution levels at a specific river site and its longitudinal development along a large river. However, at a few somewhat unexpected sites, high pollution levels occurred along the lateral zones of the river, although the midstream zone had good microbial water quality. Therefore, if site-specific considerations have to be performed (e.g. a certain location shall be used as a source to provide water for irrigation) a specific assessment of the microbiological water quality at the site of interest is recommended, including a detailed temporal and spatial (cross-sectional) investigation, even at stretches where good microbiological water quality is obvious from the midstream monitoring data. We are convinced that this study provides a solid scientific basis for guiding the authorities and decision makers towards optimally designed monitoring schemes for state-of-the-art microbial faecal pollution management in large rivers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.
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Fig. 1.
Longitudinal development of *E. coli* (A) and Enterococci concentrations (B) along the increasing discharge of the Danube in the midstream, at the left (upper line) and the right river-side (lower line) and in the tributaries sampled during JDS 2013. Not-sampled tributaries are also indicated on the x-axes. Symbol size varies according to log-transformed *E. coli* and Enterococci concentrations [(MPN+1/100 ml)]. Colours depict the faecal pollution levels according to Table S2 (Suppl. Information): blue - little, green - moderately, yellow - critically, orange - strongly and red - excessively polluted. Danube stations with
remarkable pollution levels are named. Total discharge of the Danube (grey-shaded area) can be calculated by adding left and right-side contributions. Grey-shaded triangles indicate discharge contributions of the main tributaries. RMD-canal: Rhine-Main-Danube canal; RSD-arm: Rackeve-Soroksar Danube arm. The position of Iron Gate I and II are shown in red.
Fig. 2.
Scatterplots of *E. coli* and Enterococci concentrations for comparing the results from the midstream with results from both river-sides. The stations where the concentrations were above 100 MPN/100 ml ($\log_{10} = 2$) and differed by more than 1 log are named. The middle dashed lines indicate the 1:1 curve, the outer dashed lines indicate 1 log difference.
Fig. 3.
Two-dimensional component plot of the principal component analysis. The microbiological faecal parameters constitute an independent component (surrounded by square). ENT: Enterococci, ECOLI: E. coli; BacHum & HF183: human genetic faecal markers; BP: bacterial production; OXY: oxygen; CHLA: chlorophyll a; BNLRG: large bacterial cell numbers, BNTOT: total bacterial cell numbers; PTOT: total phosphorus; NTOT: total nitrogen; TEMP: water temperature; DOC: dissolved organic carbon; COND: electrical conductivity; DISCH: discharge; CARB: carbamazepine; CAF: caffeine; ACE: acesulfame; all data was retrieved from the ICDPR Danubis database (https://www.icpdr.org/wq-db/) with kind permission of the ICPDR.
Fig. 4.
Scatterplots of the *E. coli* concentrations (left) and the load (right) for comparing results from 2013 to 2007 (midstream only). Those stations where the concentrations differed by more than 1 log are named (except Dunaföldvar). The middle dashed lines indicate the 1:1 curve, the outer dashed lines indicate 1 log difference.
Fig. 5.
Regression analysis between the *E. coli* concentrations and the concentrations of the human-associated genetic faecal markers BacHum and HF183II for all data, for the Danube and for the tributary data set.
Fig. 6. 
*E. coli* and Enterococci concentrations during the annual cycle (January 2014–January 2015) at the three selected Danube stations downstream from Vienna, downstream from Budapest and downstream from Belgrade. Colours depict the faecal pollution levels according to Table S2 (Suppl. Information). Large circles: midstream, small circles: left-side and small diamonds: right-side samples.
Fig. 7.
Box-whisker plots of the human-associated genetic faecal marker concentrations (BacHum and HF183II) during the annual cycle (Jan 2014–Jan 2015) at the three selected Danube stations downstream from Vienna, downstream from Budapest and downstream from Belgrade. Significant differences between the left, middle and right side samples are marked with asterisks: *p ≤ 0.05; **p ≤ 0.01, ***p ≤ 0.001.
Table 1
Spearman rank correlation coefficients (rho) and p-values for correlations between the faecal pollution parameters in the Danube (upper panel) and the tributaries (lower panel).

|                  | Danube (n = 160) | Tributaries (n = 26) |
|------------------|------------------|----------------------|
|                  | ENT   | BacHum | HF183II | ENT   | BacHum | HF183II |
| **E. coli**      |       |        |         |       |        |         |
|                   | rho   | 0.685  | 0.776   | 0.848 | 0.596  | 0.465   | 0.483   |
|                   | p-value | 0.000  | 0.000   | 0.000 | 0.001  | 0.017   | 0.020   |
| **ENT**          |       |        |         |       |        |         |
|                   | rho   | 0.492  | 0.561   | 0.894 | 0.338  | 0.326   |         |
|                   | p-value | 0.000  | 0.000   | 0.000 | 0.091  | 0.129   |         |
| **BacHum**       |       |        |         |       |        |         |
|                   | rho   | 0.894  | 0.903   | 0.894 |        |         |         |
|                   | p-value | 0.000  |         | 0.000 | 0.000  |         |         |
### Table 2
Component matrix of the principal component analysis.

| Variables                        | Component 1 | Component 2 | Component 3 | Component 4 | Component 5 |
|----------------------------------|-------------|-------------|-------------|-------------|-------------|
| HF183 [ME/100 ml]                | 0.91        | −0.14       | 0.11        | <0.1        | <0.1        |
| BacHum [ME/100 ml]               | 0.91        | −0.16       | 0.13        | −0.11       | <0.1        |
| *E. coli* [MPN/100 ml]          | 0.84        | −0.23       | −0.23       | −0.14       | 0.11        |
| Enterococci [MPN/100 ml]        | 0.65        | 0.02        | −0.45       | <0.1        | <0.1        |
| Bacterial production [μgC/L/h]   | 0.54        | 0.30        | <0.1        | <0.1        | 0.42        |
| Oxygen [mg/L]                    | −0.22       | 0.85        | <0.1        | −0.11       | −0.20       |
| pH                              | −0.26       | 0.83        | <0.1        | <0.1        | −0.19       |
| Chlorophyll a [μg/L]             | 0.03        | 0.81        | 0.16        | 0.13        | 0.17        |
| Total nitrogen [mg/L]            | −0.08       | 0.09        | 0.82        | −0.18       | 0.25        |
| Discharge [m³/sec]               | 0.11        | −0.08       | −0.74       | <0.1        | <0.1        |
| Total phosphorus [μg/L]          | 0.14        | 0.14        | 0.68        | 0.24        | −0.18       |
| Large cell numbers [cells/ml]    | 0.10        | 0.25        | <0.1        | 0.81        | <0.1        |
| Total cell numbers [cells/ml]    | −0.09       | −0.07       | −0.39       | 0.79        | <0.1        |
| Acesulfame [μg/L]                | −0.16       | −0.27       | 0.27        | 0.56        | 0.26        |
| Conductivity [μS/cm]             | −0.04       | −0.08       | 0.36        | 0.56        | <0.1        |
| Carbamazepine [μg/L]             | −0.06       | −0.17       | 0.20        | 0.17        | 0.78        |
| Temperature [°C]                 | 0.16        | 0.23        | <0.1        | <0.1        | 0.58        |
| Caffeine [μg/L]                  | 0.09        | −0.38       | −0.41       | <0.1        | 0.57        |
| Dissolved organic carbon (mg/L)  | −0.02       | 0.08        | <0.1        | 0.15        | <0.1        |
| % explained variance             | 22.4        | 12.7        | 11.8        | 10.6        | 7.0         |
Table 3

Prevalence and concentrations of the host associated genetic faecal markers BacHum, HF183II, BacR and Pig2Bac [ME/100 ml] in the Danube and the tributaries during Joint Danube Survey 2013.

| Sample number | % positive | Median | Min   | Max   | 5% percentile | 95% percentile |
|---------------|------------|--------|-------|-------|---------------|----------------|
| Danube total  |            |        |       |       |               |                |
| BacHum        | 159        | 92.4   | 6.6 x 10^1 | 2.5 x 10^2 | 1.3 x 10^6  | 5.8 x 10^2  | 1.5 x 10^5 |
| HF183II       | 164        | 82.3   | 1.3 x 10^3 | 5.3 x 10^4 | 4.0 x 10^5  | 1.3 x 10^2  | 4.3 x 10^4 |
| BacR          | 164        | 7.3    | 3.3 x 10^2 | 5.3 x 10^3 | 2.8 x 10^5  | 5.3 x 10^3  | 1.6 x 10^1 |
| Pig2Bac       | 164        | 8.5    | 9.6 x 10^2 | 1.8 x 10^3 | 6.9 x 10^3  | 2.1 x 10^2  | 6.9 x 10^3 |
| Danube left   |            |        |       |       |               |                |
| BacHum        | 55         | 90.9   | 5.8 x 10^1 | 5.6 x 10^2 | 1.3 x 10^6  | 7.9 x 10^2  | 2.0 x 10^5 |
| HF183II       | 55         | 89.1   | 1.3 x 10^3 | 7.5 x 10^1 | 4.0 x 10^5  | 1.1 x 10^2  | 3.7 x 10^4 |
| BacR          | 56         | 8.9    | 2.7 x 10^3 | 7.5 x 10^1 | 2.8 x 10^3  | 1.0 x 10^2  | 2.4 x 10^3 |
| Pig2Bac       | 56         | 8.9    | 1.9 x 10^2 | 2.3 x 10^2 | 6.9 x 10^3  | 3.7 x 10^2  | 6.0 x 10^3 |
| Danube middle |            |        |       |       |               |                |
| BacHum        | 54         | 90.7   | 4.4 x 10^3 | 2.5 x 10^2 | 3.1 x 10^5  | 4.3 x 10^2  | 7.0 x 10^4 |
| HF183II       | 54         | 79.6   | 1.1 x 10^3 | 5.3 x 10^3 | 9.1 x 10^4  | 1.8 x 10^3  | 1.8 x 10^4 |
| BacR          | 54         | 3.7    | 4.2 x 10^2 | 3.7 x 10^2 | 4.7 x 10^2  | 3.7 x 10^2  | 4.7 x 10^2 |
| Pig2Bac       | 54         | 9.3    | 8.8 x 10^2 | 6.1 x 10^2 | 1.4 x 10^3  | 6.3 x 10^2  | 1.3 x 10^2 |
| Danube right  |            |        |       |       |               |                |
| BacHum        | 50         | 96.0   | 7.1 x 10^3 | 5.8 x 10^2 | 5.8 x 10^3  | 8.6 x 10^2  | 1.4 x 10^5 |
| HF183II       | 55         | 78.2   | 1.5 x 10^3 | 7.5 x 10^1 | 9.9 x 10^4  | 2.1 x 10^2  | 5.4 x 10^4 |
| BacR          | 54         | 9.3    | 3.2 x 10^3 | 5.3 x 10^1 | 6.2 x 10^2  | 5.3 x 10^1  | 5.7 x 10^2 |
| Pig2Bac       | 54         | 7.4    | 7.9 x 10^3 | 1.8 x 10^2 | 6.8 x 10^3  | 2.1 x 10^2  | 6.0 x 10^3 |
| Tributaries   |            |        |       |       |               |                |
| BacHum        | 22         | 100    | 2.4 x 10^3 | 2.6 x 10^2 | 4.6 x 10^6  | 2.9 x 10^2  | 7.2 x 10^5 |
| HF183II       | 22         | 59.1   | 2.8 x 10^3 | 5.8 x 10^1 | 1.5 x 10^6  | 1.2 x 10^2  | 6.5 x 10^5 |
| BacR          | 25         | 8.0    | 1.6 x 10^3 | 3.4 x 10^2 | 2.9 x 10^3  | 4.7 x 10^2  | 2.8 x 10^3 |
| Pig2Bac       | 25         | 4.0    | 9.5 x 10^2 | –         | –           | –           | –           |