

Elucidating fecal pollution patterns in alluvial water resources by linking standard fecal indicator bacteria to river connectivity and genetic microbial source tracking

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ABSTRACT

A novel concept for fecal pollution analysis was applied at alluvial water resources to substantially extend the information provided by fecal indicator bacteria (FIB). FIB data were linked to river connectivity and genetic microbial source tracking (MST). The concept was demonstrated at the Danube River and its associated backwater area downstream of the city of Vienna, using a comprehensive 3-year data set (10 selected sites, $n = 317$ samples). Enumeration of *Escherichia coli* (ISO 16649-2), intestinal enterococci (ISO 7899-2) and *Clostridium perfringens* (ISO 14189) revealed a patchy distribution for the investigation area. Based on these parameters alone a clear interpretation of the observed fecal contamination patterns was not possible. Comparison of FIB concentrations to river connectivity allowed defining sites with dominating versus rare fecal pollution influence from the River Danube. A strong connectivity gradient at the selected backwater sites became obvious by 2D hydrodynamic surface water modeling, ranging from 278 days (25%) down to 5 days (<1%) of hydraulic connectivity to the River Danube within the 3-year study period. Human sewage pollution could be identified as the dominating fecal source at the highly connected sites by adding information from MST analysis. In contrast, animal fecal pollution proved to be dominating in areas with low river connectivity. The selection of genetic MST markers was focusing on potentially important pollution sources in the backwater area, using human (BacHum, HF183II), ruminant (BacR) and pig (Pig2Bac) -associated quantitative PCR assays. The presented approach is assumed to be useful to characterize alluvial water resources for water safety management throughout the globe, by allocating fecal pollution to autochthonous, allochthonous, human or animal contamination components.

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The established river connectivity metric is not limited to bacterial fecal pollution, but can be applied to any type of chemical and microbiological contamination.

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1. Introduction

The socioeconomic importance of riverine floodplains is well recognized. Besides flood water retention, the drinking water supply and space provided for recreational activities play important roles (Baart et al., 2013). Fecal pollution is a substantial hazard to these water resources, especially in terms of water supply. For alluvial backwater systems, two potential pollution sources must be considered: i) allochthonous sources from the main river channel and ii) autochthonous sources from the floodplain itself. In addition, both sources can be impacted by humans or animals or by a combination of both. Routine surface water quality monitoring is mainly based on the cultivation of *Escherichia coli* (*E. coli*) and intestinal enterococci. Recently, it was demonstrated that these fecal indicator bacteria (FIB) can sensitively detect fecal pollution from mammal, bird and human (individual or sewage) sources equally in such systems (Frick et al., 2018). In addition, fecal emissions from poikilothermic animals, such as snails, frogs, and fish, may also contribute to the observed FIB levels in backwater systems to some extent (Frick et al., 2018). But allocating fecal contamination to their sources of origin is not possible by relying on FIB monitoring data alone.

For the studied backwater area of the Danube River in the east of Austria, we speculated that two specific contamination types would influence the water quality of the floodplain area. First, for the allochthonous contamination input into the backwater, human fecal sources should dominate, as the water quality of the Danube River is mainly determined by large capitals, tributaries and branches that receive municipal (human) wastewater (Kirschner et al. 2008, 2017). The extent of fecal contamination from those sources in the backwater area should depend on the level of human fecal pollution in the river and the connectivity to the concerned backwater sites. At locations with higher river connectivity, increased allochthonous human fecal input can be expected compared to sites with lower connectivity. As a second and likewise important fecal source, autochthonous fecal material from wild animals has to be considered for the concerning water resource. The studied alluvial backwater is an important wilderness area that harbors numerous species of wild animals (Weigelhofer et al., 2013). A pollution source profile of the study area identified birds, deer and wild boar as the most important fecal sources from homeothermic animals (Frick et al., 2018). The area and its surroundings are also popular for recreational activities such as bathing, sports fishing or cycling (Arnberger et al., 2009). Thus, human visitors, in the absence of appropriate sanitary installation, could also contribute to the fecal input. However, compared to the reported numbers of animals in this area, humans should play a minor role as a fecal pollution source (Frick et al., 2018).

Except for basic hydrological data, such as precipitation or river discharge, there are only a few published studies available that combined information from microbial source tracking (MST) with hydrological models or hydrological metrics derived from it. To identify sources of water compartments that were mainly responsible for bacterial loadings within the Yarra estuary, McCarthy et al. (2017) implemented MST and evaluated this method using a 3-dimensional hydrodynamic model. Sokolova et al. (2012) combined MST markers with a 3-dimensional coupled hydrodynamic

model to investigate the contribution from different contamination sources in a Swedish lake. In addition, Derx et al. (2016) used a human-associated MST marker to calibrate a hydrological process-based microbial fate and transport model (QMRacatch) for a river and backwater river system for microbial risk assessment. To the best of our knowledge, detailed hydrological characterization or modeling in combination with the use of MST markers to explain patterns and sources of FIB in riverine floodplains has not been reported in the literature so far.

The aim of this study was to characterize patterns, temporal variation and sources of fecal pollution in a typical backwater area of the Danube River. A new concept was applied by extending the observed FIB data with information on river connectivity and genetic MST marker levels. River connectivity was calculated by a detailed 2D hydrodynamic surface water model. To date, the term hydrological connectivity has been used in an ecological context to refer to water-mediated transfer of matter, energy and/or organisms within or between elements of riverine corridors (Tockner et al., 2000; Hein et al. 2003, 2016; Pringle, 2003; Reckendorfer et al., 2013; Weigelhofer et al., 2015; Mayr et al., 2020). In this study, river connectivity was used in a new context to support improved data interpretation guiding pollution and health-related water quality management. For this survey, connectivity was defined as the degree of hydraulic connection between the River Danube and the sampling locations in the backwater area. It is given as relative connectivity (in %) calculated for each sampling site as the number of connected days during the total investigation period (3 years). We hypothesized that the proposed concept is able to allocate the observed fecal pollution levels to their allochthonous and autochthonous sources of human or animal origin in the backwater area. To support the evaluation of the new approach, the investigation design covered a pronounced connectivity gradient from the river to the backwater environment, as well as significant variations in the expected animal abundance to cover sites with both low and high animal impact.

2. Material and methods

2.1. Study site

The investigated alluvial backwater area, called porous aquifer (PA), is a typical urban riverine wetland whose dynamic character was fundamentally changed by the regulation of the Danube River in the 19th century (Weigelhofer et al., 2013). It is located on the north side of the Danube River at the eastern border of Vienna, Austria (Fig. 1) and covers an area of approximately 12 km². Due to the regulation of the Danube, and the construction of a flood protection dam alongside the Danube, the PA backwater was almost completely disconnected from the main stream. The backwater presently consists of a river channel network, whose main side arm has a surface connection with the Danube River at its lowermost end through a levee opening ('backflow connection'). Numerous partly disconnected backwaters and isolated ponds are also present. The PA backwater is subdivided into 6 basins by check dams and natural fords. Floods that enter the floodplain via the backflow connection move upstream along the main side arm, creating a distinct gradient in hydrological connectivity in the various

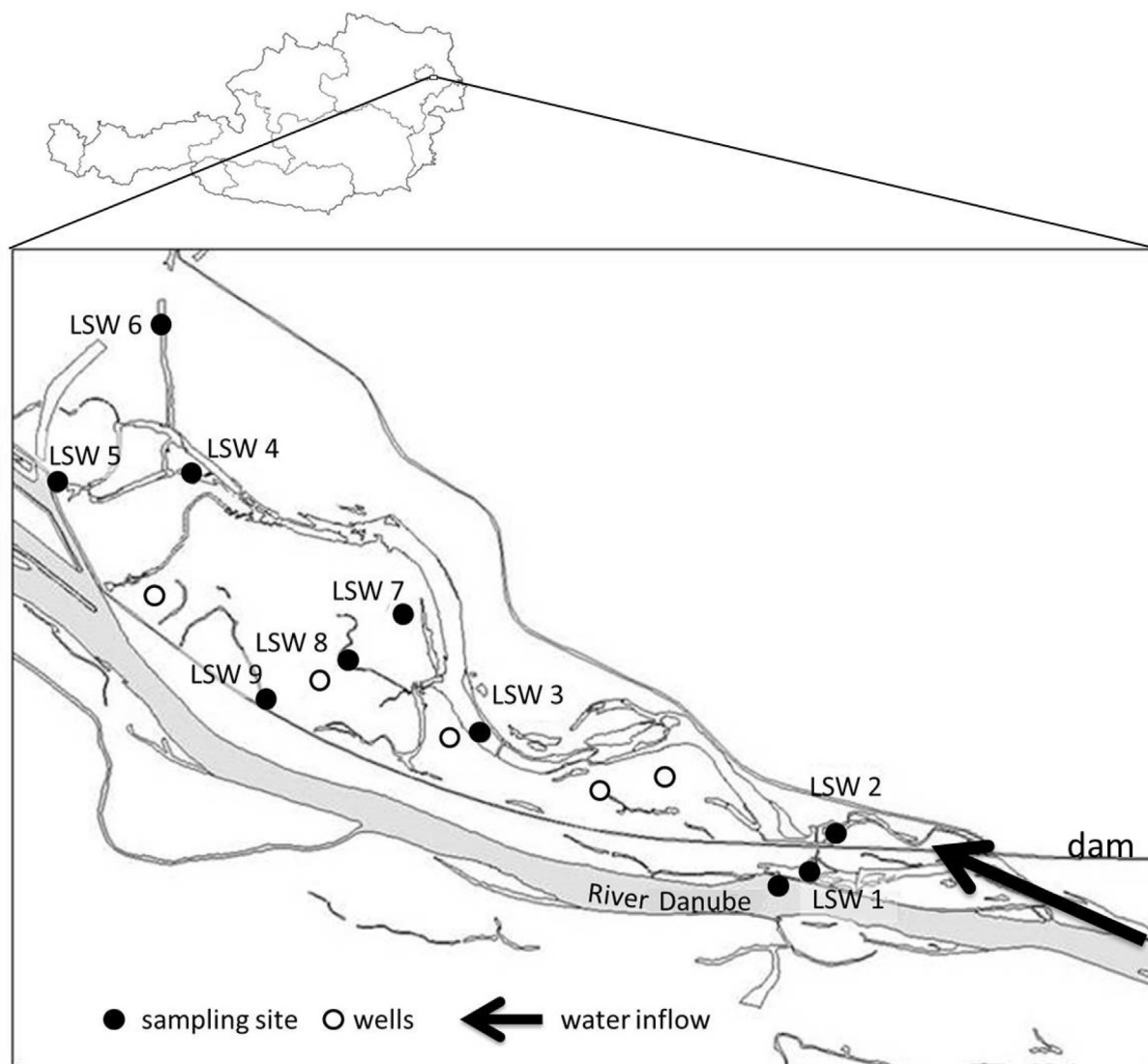


Fig. 1. Study site in the east of Austria with one sampling site at the Danube River and nine sampling sites in the backwater area (LSW1 - LSW9). The area is usually disconnected from the river by a dam, and five drinking water wells are located inside the protected area. Depending on the Danube water level, water can flow into the backwater area. The point where the water flows into the study site is indicated by a black arrow.

waterbodies with distance to the inflow (Reckendorfer et al., 2013).

The PA backwater is an important water resource for Vienna's drinking water supply (Hein et al., 2006), and five drinking water wells (riverbank filtrate) are situated in the floodplain area. It is also part of a national park that plays a strategic role as a wilderness area and for recreation (Arnberger et al., 2009). There are no settlements or livestock in the considered backwater area, but there is a considerable population of wild animals, such as ruminants, wild boars and birds (Frühauß and Sabathy, 2006, b, Parz-Gollner, 2006, Arnberger et al., 2009). In addition, 600,000 people visit the national park area every year (Hinterberger et al., 2000). Bathing is prohibited within the considered backwater area, and visitors may only use selected paths for walking and cycling. Therefore, the possibility for human fecal input from visitors is considered to be low (Frick et al., 2018).

2.2. Sampling

Surface water samples were taken monthly at the Danube River site and at nine representative backwater locations LSW1 - LSW9

(Fig. 1) at different sections of the backwater area (Gabriel et al., 2014) from May 2010 to June 2013. The sampling location LSW1 was situated in a side arm outside the flood-protected area delineated by the dam and therefore represented waterbodies with high connectivity to the Danube River. The locations LSW2 and LSW3 represent waterbodies at the main side arm, with a decreasing gradient of connectivity, and average water depths of approx. 110 cm and 170 cm, respectively. The sites LSW4, LSW5 and LSW6 represent side ditches with very low connectivity at the western end of the study area. The water depth at LSW4 was approx. 30–50 cm, and it once completely dried out by the end of 2012. At this location, animal traces (e.g., traces or fecal material from ruminants) could be repeatedly detected in the waterbody. LSW5 was located in a side ditch with a water depth of approx. 110 cm on average. The depth at LSW6 was approx. 100 cm. Three sampling sites (LSW7, LSW8, LSW9) were chosen in disconnected, shallow backwaters that repeatedly completely dried out. LSW7 was located in a small pond with a water depth of approx. 30 cm. At LSW7, a range of animal traces (footprints and wallows of wild boars) were repeatedly detected, so a high animal abundance could

be expected. LSW8 was in a very shallow side ditch (approx. 20–40 cm water depth) near a feeding lot for game and was frequently used by wild animals (e.g., wild boar for wallowing). In 2010 and 2011, locations LSW7 and LSW8 were sampled alternately (each month LSW7 or LSW8 was sampled). LSW9 was situated in a shallow side ditch (water depth of approx. 40–60 cm) right beside a frequented path for visitors; therefore, the abundance of wild animals was expected to be low.

Water samples were taken approx. 15 cm below the water surface. If the water depth was lower than approx. 30 cm, samples were taken very carefully to avoid contamination from the surface layer or from whirled-up sediments. Samples for microbiological analysis were collected in sterile 2 l plastic bottles. Samples for chemical analysis were collected in prewashed polyethylene bottles. The samples were transported in dark cool boxes to the laboratory within 4 h. During sampling, relevant additional information (i.e., numbers of visible animals, animal traces in soil and sediments, wallows, amount of visible fecal pats) on animal abundance was gathered.

2.3. Standard fecal indicator bacteria

Bacteriological analysis of surface water samples was performed according to established ISO standards based on membrane filtration and cultivation. One hundred milliliters (or an appropriate dilution) of each water sample was filtered through a cellulose nitrate filter (pore size 0.45 μm). *E. coli* was investigated on TBX agar (44 ± 0.5 °C, 44 ± 4 h) according to ISO 16649-2 (International Organisation for Standardisation, 2001). Intestinal enterococci were enumerated on Slanetz and Bartley agar (36 ± 2 °C, 44 ± 4 h) and confirmed on bile aesculin agar (44 ± 0.5 °C, 2 h) according to ISO 7899-2 (International Organisation for Standardisation, 2000). *C. perfringens* was investigated in accordance with ISO 14189 (International Organisation for Standardisation, 2013) on TSC agar (44 ± 1 °C, 21 ± 3 h) and confirmed by testing for acid phosphatase. Water samples for the detection of *C. perfringens* were pasteurized before filtration (15 ± 1 min, 60 ± 2 °C) to detect spores only. For quality control, the following type strains were used: *E. coli*, NCTC 9001; *Enterococcus faecalis*, NCTC 775; and *Clostridium perfringens*, NCTC 8237. The threshold of detection was 1 or 10 Colony Forming Units (CFU) per 100 ml depending on the sample volume analyzed.

2.4. Genetic source tracking markers

For the analysis of microbial source tracking (MST) markers, samples from the Danube River (Danube) and from five representative sampling locations (LSW1, LSW3, LSW4, LSW7 and LSW8) were selected. In the main side arm, LSW1 represents a location with high connectivity, and LSW3 represents a location with low connectivity. LSW4 was chosen as a side ditch closely connected to the main side arm but with low connectivity to the River Danube, and furthermore a high animal abundance. LSW7 and LSW8 represent more disconnected ditches with anticipated, significant animal abundance. Water samples (200 ml until July 2011, afterwards 600 ml) were filtered through 0.2 μm polycarbonate filters (Merck Millipore, USA). If the filter clogged before the intended volume was filtered, filtration was terminated after 1 h. The filtered volume was determined as a weight difference by comparing the start and end weights of the bottles. DNA extraction was performed using a bead-beating and phenol/chloroform based DNA extraction protocol as described previously (Griffiths et al., 2000). The extracted DNA was eluted in TRIS buffer (pH 8.0) and stored at -80 °C until further analysis.

Prior to the qPCR assays, the concentration of the DNA extracts was quantified with a NanoDrop 1000 Spectrophotometer (Thermo

Fisher Scientific, United Kingdom). A universal *Bacteroidetes* marker, BacDick (Dick and Field, 2004), was used as a quality control to assess the ability to amplify DNA in the samples and to rule out the presence of DNA-inhibiting substances in the DNA extracts (Reischer et al., 2008; Mayer et al., 2018). Furthermore, all qPCR assays (except for BacDick) were run with the *ntb2* fragment as an internal amplification control in duplex (IAC, noncompetitive) to monitor for qPCR amplification inhibition (Anderson et al., 2011).

All sample DNAs in the qPCR assays detecting host-associated fecal pollution from humans, BacHum (Kildare et al., 2007) and HF183II (Green et al., 2014), ruminants, BacR (Reischer et al., 2006), and pigs/wild baors, Pig2Bac (Mieszkin et al., 2009), were measured in duplicate. Quality assessment of qPCR data was performed as previously described in detail (Reischer et al. 2006, 2011; Mayer et al., 2018). All controls and no-template controls were consistently negative (i.e., the fluorescence never exceeded the threshold). qPCR standard dilutions ranging from 10^1 to 10^6 targets per reaction were used in a linear regression model to calculate the qPCR calibration curve. The results are reported as marker equivalents per filtered water volume (ME vol^{-1}) as previously described (Reischer et al., 2006). The filtration volume of the sample, the analyzed volume for qPCR analysis, and the minimal detectable marker concentration per PCR defined the threshold of detection (Reischer et al., 2011; Mayer et al., 2018). The threshold of detection of the data set presented herein ranged from 11 to 222 marker equivalents per analyzed water sample (see supplemental material, data).

The BacDick assay was run on a MasterCycler RealPlex PCR Machine (Eppendorf Germany) in a total reaction volume of 25 μl with 2.5 μl diluted sample DNA (1:4, 1:16, and if necessary 1:64). All other qPCR assays were run on a Rotor-Gene Q thermocycler in a total reaction volume of 15 μl with 2.5 μl diluted sample DNA (1:4 or 1:16).

To ensure the applicability of the genetic MST markers, their performance needs to be tested for the individual catchment and fecal sources (Reischer et al., 2013; Mayer et al., 2018). All MST assays applied, the BacHum, the HF183II, the Pig2Bac and the BacR assay, were tested for their level of specificity and sensitivity for the respective fecal source group(s) on samples from the study area.

Further details on molecular methods, quality assurance and marker performance are presented as supplemental material.

2.5. Chemical and chemophysical analysis

Water temperature was measured in situ with portable meters at each site (Hach-Lange, Sension 156). Total phosphor (P-tot in $\mu\text{g/l}$) was measured photometrically ($\lambda = 885$ nm) as reduced molybdenum phosphoric acid in the unfiltered sample after HNO_3 microwave digestion (reducer, ascorbic acid). Phosphate (PO_4 in $\mu\text{g/l}$) was measured photometrically ($\lambda = 880$ nm) as reduced molybdenum phosphoric acid in the filtrate (reducer, ascorbic acid). Total suspended solids (TSS in mg/l) were measured as the dry weight of the particulate fraction. Samples were filtered through GF/F glass microfiber filters and dried for 48 h at 80 °C. Relative particulate organic matter (relPOM in %) is the relative part of the organic fraction of TSS. To calculate the relative particulate organic matter, the inorganic fraction was measured as ash weight after 4 h at 450 °C. An overview of the measured values (range) is given in the supplemental material (Table S6).

2.6. Hydrodynamic modeling and calculating river connectivity

The connection of the selected sampling sites along the floodplain river to the Danube River was determined based on a 2D hydrodynamic surface water model (CCHE2D Version 2.0, National

Center for Computational Hydroscience and Engineering, University of Mississippi).

The model is based on the solution of the two-dimensional formulation of the shallow water equations and has been successfully applied for the analysis of river flows (Chao et al. 2015, 2016). The model uses depth integrated Reynolds equations. The spatial discretization of the geometry and the related processes is based on the Efficient Element Method (Wang and Hu, 1992). For temporal discretization, the implicit first order Euler's method was implemented and was able to simulate subcritical and supercritical flow conditions. The model domain covered an area of approximately 22 km² (Fig. S1). The terrain and riverbed elevation in the model grid cells were a product from a digital terrain model (DTM). The DTM was based on laser scan data, which were complemented with terrestrial surveys along the side-arm channel (Gschöpf and Blaschke, 2009). The spatial distribution of the Manning roughness values were based on a detailed land use and vegetation classification (43 classes) that was mapped into 12 different roughness classes (Hein et al., 2008). During model calibration the Manning roughness values were fine adjusted to floods of the River Danube during August 2008, June 2009 and June 2011, ranging from 0.024 to 0.125 s m^{-0.3} within the model domain. The spatial resolution of the rectangular model grid varied between 0.1 m and 20 m, depending on local topographic characteristics. The total number of calculation nodes was 396,000. As the upstream model boundary (B_u) the observed hourly discharges at the Danube stream gauge in Fischamend were used. The downstream boundary (B_d) condition was given by the observed water level at the eastern model boundary at river station 1911 km (Fig. S1). The initial flow conditions at the calculated nodes were based on steady flow simulations for typical flow conditions in the Danube River at Fischamend. Depending on the temporal dynamics of the input hydrographs, calculation time step lengths between 30 s and 1 h were used.

For calculating the river connectivity the 2D surface water flow velocities of the floodplain river were simulated on an hourly basis during a flood event in January 2011 with an approximate 10-year return period. The floodplain river is connected to the Danube River if the flow discharge exceeds the mean flow discharge of 1910 m³ s⁻¹. In this case, the Danube River water flows into the floodplain river during the rising limb of a flood event. When the flood peak is reached, the flow direction reverses, and the water flows back toward the Danube River. A specific sampling site was considered to be connected as soon as the inflowing Danube River water reached up to this point during the rising limb of the flood event. This criterion was fulfilled if the simulated 2D flow velocities in the floodplain river were larger than 0.1 m s⁻¹ from the point of entry towards the sampling site (Fig. S3). In this way, a threshold water level at the Danube River was determined for each sampling site. If the Danube river water level was higher or equal to this threshold level, a sampling site was considered to be connected. The connectivity of the floodplain river was then defined for each sampling site as the time span during which the site was connected. The connectivity of each sampling site was determined based on the threshold water levels (Fig. S2) and the water level duration curves of the gauge station for the observation period from 2010 to 2013. The connectivity in % was calculated for each sampling site as the number of connected days in the observation period. Furthermore, the time span since the last surface water connection to the Danube River was calculated for each sampling site and date.

2.7. Statistics

For further analysis, all values under the threshold of detection were set to zero. Statistics were calculated in IBM SPSS version 23

and SigmaPlot version 13.0. For correlation analysis, Spearman's rank correlation and the Kruskal-Wallis test, for the comparison of groups, were applied (Kruskal and Wallis, 1952). For significance testing, multiple correlations were adjusted according to Bonferroni correction (Bonferroni, 1936).

3. Results

3.1. Establishing basic knowledge on standard fecal indicator bacteria (FIB)

From 2010 to 2013, the selected sites in the backwater area and the Danube River (10 sites, n = 317 samples) were investigated for the occurrence and temporal variation of fecal pollution by enumerating the FIB (Table 1). The results on the occurrence of the FIB *E. coli*, intestinal enterococci and *C. perfringens* spores at the sampling sites showed a patchy distribution, with a median concentration range from 0.3 to 2.1 log₁₀ CFU 100 ml⁻¹. No clear spatial trend of fecal contamination was obvious from the FIB data alone.

The highest median concentrations of *E. coli* and enterococci were detected at the Danube River and the side ditches LSW4, LSW7 and LSW8 (1.72–2.01 log₁₀ CFU 100 ml⁻¹ and 1.28 - 2.14 log₁₀ CFU 100 ml⁻¹, respectively). The lowest concentrations were found in the main side arm at LSW3 and at the side ditches LSW5 and LSW9 (0.78–1.04 log₁₀ CFU 100 ml⁻¹ and 0.60 - 0.85 log₁₀ CFU 100 ml⁻¹, respectively). The concentrations of *C. perfringens* spores were highest at the Danube River, the locations in the main side arm LSW1 and LSW2, and in the side ditches LSW7 and LSW8 (1.32–1.72 log₁₀ CFU 100 ml⁻¹). The lowest concentrations were detected at LSW3 and the side ditches LSW5 and LSW9 (0.30–0.98 log₁₀ CFU 100 ml⁻¹).

3.2. River connectivity and FIB concentrations in context to the connectivity gradient

The determined river connectivity values of the sampling sites to the Danube River were expressed as days and percentage of days within a 3-year period (2010–2013). The selected sampling sites showed a strong connectivity gradient from 1050 days at the Danube River (100%) down to 278 days at LSW 1 (25%), 30 days at LSW 2 (3%), 20 days at LSW 3 (2%), 9 days at LSW4 to LSW7 (<1%) and 5 days at LSW 8 and 9 (<1%) (Fig. 2). The investigated backwater locations could thus be separated into two sections. The first is a somewhat more frequently river connected section (FCS), covering the locations from the Danube River along the main side

Table 1

Results for *E. coli*, enterococci and *C. perfringens* spores at the Danube and the nine backwater sampling sites (LSW) are given as log₁₀ (x+1) CFU/100 ml. Samples (number = n) were taken between May 2010 and June 2013.

| Site | n | <i>E. coli</i> | | | enterococci | | | <i>C. perfringens</i> | | |
|--------|----|----------------|-----|-------------|-------------|-----|-------------|-----------------------|-----|-------------|
| | | median | | percentiles | median | | percentiles | median | | percentiles |
| | | | | 5% | 95% | 5% | 95% | 5% | 95% | |
| Danube | 36 | 1.8 | 1.3 | 2.5 | 1.3 | 0.6 | 2.1 | 1.7 | 1.1 | 2.3 |
| LSW1 | 37 | 1.2 | 0.0 | 2.6 | 0.9 | 0.2 | 2.3 | 1.3 | 0.7 | 2.3 |
| LSW2 | 37 | 1.1 | 0.0 | 1.9 | 0.9 | 0.0 | 1.9 | 1.3 | 0.0 | 1.9 |
| LSW3 | 37 | 0.8 | 0.0 | 1.7 | 0.6 | 0.0 | 1.5 | 0.9 | 0.0 | 1.5 |
| LSW4 | 31 | 2.0 | 1.4 | 3.0 | 1.7 | 0.8 | 2.8 | 1.1 | 0.0 | 1.8 |
| LSW5 | 34 | 1.0 | 0.4 | 1.9 | 0.7 | 0.0 | 1.3 | 0.3 | 0.0 | 0.9 |
| LSW6 | 34 | 1.2 | 0.2 | 1.8 | 1.0 | 0.0 | 1.9 | 0.8 | 0.0 | 1.6 |
| LSW7 | 13 | 1.7 | 0.5 | 3.5 | 1.8 | 0.2 | 3.0 | 1.4 | 0.0 | 3.2 |
| LSW8 | 26 | 1.9 | 1.2 | 3.3 | 2.1 | 0.7 | 3.2 | 1.3 | 0.0 | 3.0 |
| LSW9 | 32 | 0.8 | 0.0 | 1.5 | 0.9 | 0.0 | 1.5 | 1.0 | 0.0 | 2.1 |

arm (LSW1, LSW2). LSW3 is located in a transition area to the second, rarely river connected section (RCS), including the rest of the sampling locations from LSW4 to LSW9 (Fig. 2).

At the FCS locations, within the course of the main backwater branch, the declining connectivity gradient from 100% to 3% was also clearly reflected by a decreasing trend in FIB concentrations (Table 1, Fig. 2). This trend could be consistently observed for *E. coli* (Kruskal-Wallis test, $n = 145$, $p < 0.001$), enterococci (Kruskal-Wallis test, $n = 148$, $p < 0.001$) and *C. perfringens* spores (Kruskal-Wallis test, $n = 145$, $p < 0.001$), indicating a dominating and frequent influence of allochthonous sewage pollution from the Danube River into the backwater system, in accordance with the hydrological link of the investigated systems.

In contrast, in the RCS (LSW3 - LSW9), the observed patchy distribution of fecal pollution, as indicated by *E. coli*, enterococci and *C. perfringens* spores, was not related to river connectivity at all (Table 1). For example, the strong variation in fecal pollution at the RCS, irrespective of the situation at the Danube River, is impressively demonstrated by the observed *E. coli* concentrations (Fig. 2). Concentration ranges from very low *E. coli* concentrations up to levels even higher than those detected at the Danube River. An extremely fluctuating situation was observed at LSW7, ranging from not detectable up to $3.5 \log_{10}$ CFU 100 ml^{-1} (Fig. 2). The situation at the RCS regarding the detected FIB levels suggested animal fecal pollution sources of autochthonous origin from the backwater area. To test this hypothesis, genetic MST markers were applied to generate information on the origin of observed fecal pollution levels.

3.3. Complementing the data set with information on pollution sources (MST)

From 2010 to 2013, a number of 174 water samples were also analyzed for human, ruminant and pig-associated genetic MST

markers from six selected locations (Danube River, LSW1, LSW3, LSW4, LSW7, LSW8) to conduct fecal source allocation (Fig. 3).

For the Danube River samples, human-associated MST markers (BacHum, HF183II) were the dominant markers, proving that this river environment is indeed mainly impacted by municipal wastewater. The median values for the human-associated fecal markers BacHum and HF183II were 3.6 and $2.7 \log_{10}$ ME 100 ml^{-1} , respectively. In contrast, the median concentrations for ruminant (BacR) and pig-associated (Pig2Bac) markers were below the detection limits at the Danube River (Fig. 3).

At LSW1, a backwater river site with 25% river connectivity within the FCS, the concentrations of the human-associated MST markers were considerably lower compared to the Danube River. Nonetheless, concentrations were still detectable at the 75th percentiles, with BacHum and HF183II levels higher than $2.0 \log_{10}$ ME 100 ml^{-1} , demonstrating the impact of human fecal pollution events from the Danube River. Except for a few cases of very low qPCR determinations, ruminant- and pig-associated MST markers could not be detected in any of the LSW1 samples (Fig. 3).

At LSW3, a backwater river location with low connectivity (2%) at the transition area between the FCS and RCS, BacHum could be found in only 2 (7%) of the samples, indicating a sporadic impact by human fecal pollution (Fig. 3). The human-associated marker HF183II was not detectable in any of the samples. Ruminant- (BacR) and pig- (Pig2Bac) associated fecal markers were detectable in 45% and 16% of all samples, with 90th percentiles at $2.9 \log_{10}$ and $2.5 \log_{10}$ ME 100 ml^{-1} , respectively, showing that wildlife had a detectable influence.

At LSW4, LSW7 and LSW8 within the RCS, locations in side ditches with very low connectivity (<1%) to the Danube River, wildlife, especially ruminant populations, exhibited a strong and obvious influence. At LSW4, the ruminant-associated MST marker BacR was detected in 90% of the samples with a median

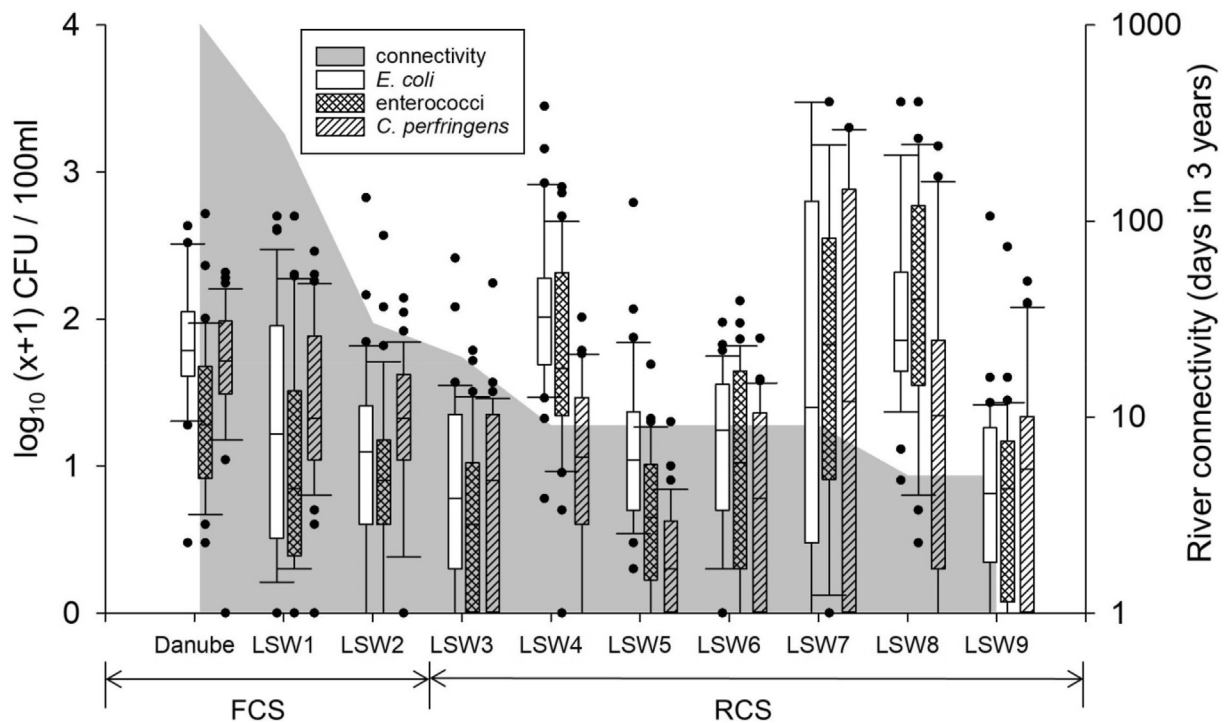


Fig. 2. *E. coli*, enterococci, and *C. perfringens* (spores) concentrations are given in $\log_{10}(x+1)$ CFU/100 ml in box plots. CFU = colony forming units. Box plots indicate the median, 25th and 75th percentile (box), 10th and 90th percentile (whiskers) and outliers (dots). The mean connectivity of the sampling sites to the Danube River is given as days in three years (gray background). The sampling sites are situated in two different sections of the PA area: a more frequently river connected section (FCS) and a rarely river connected section (RCS).

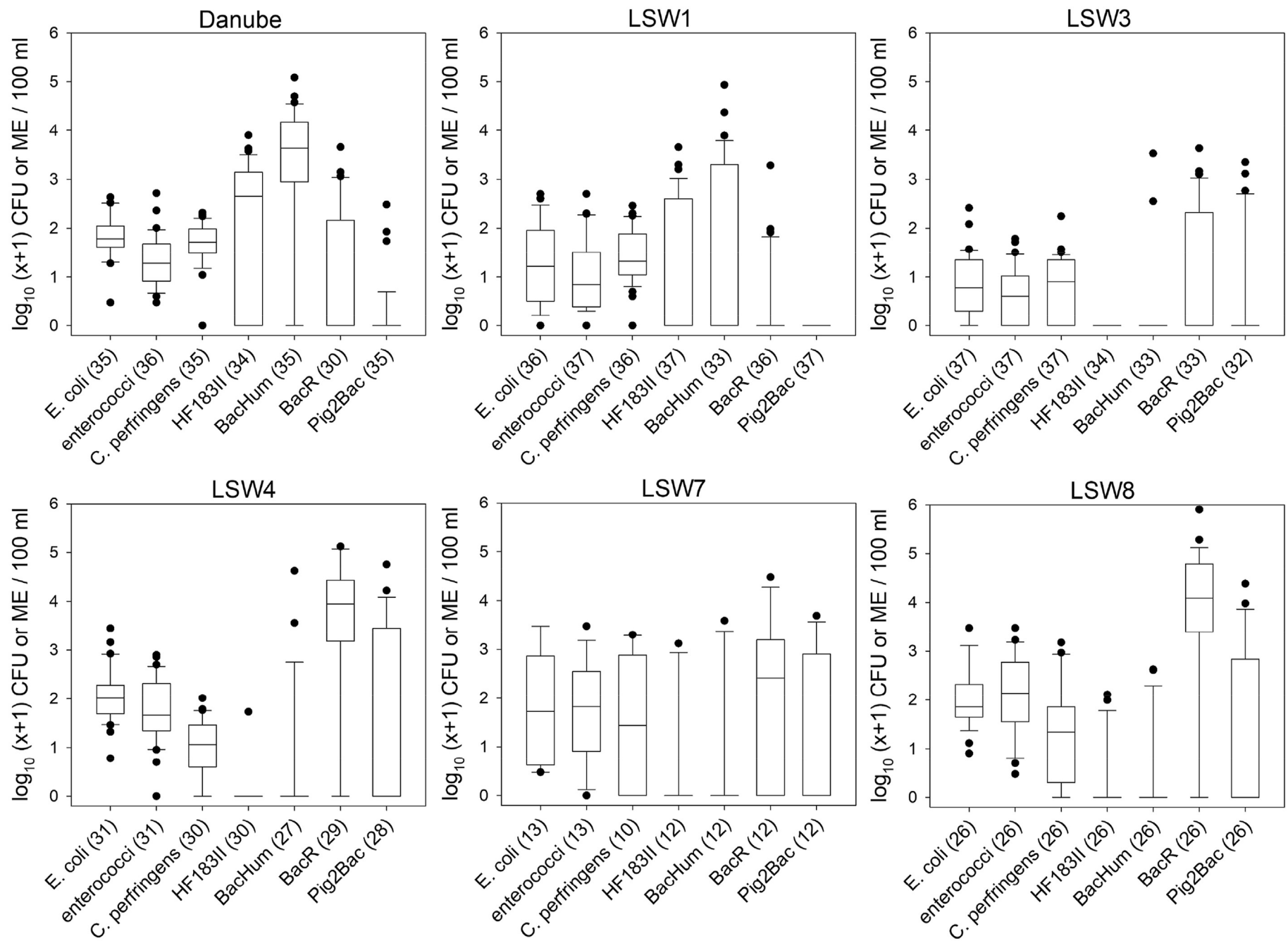


Fig. 3. Concentrations of the FIB *E. coli*, enterococci, and *C. perfringens* (spores), and of the genetic MST markers HF183II, BacHum, BacR and Pig2Bac at the Danube, the main side arm of the backwater (LSW1, LSW3) and in the side ditches (LSW4, LSW7 and LSW8). Sample sizes are given in brackets. CFU = colony forming units, ME = marker equivalents. Box plots indicate the median, 25th and 75th percentiles (box), 10th and 90th percentiles (whiskers) and outliers (dots).

concentration of $4.0 \log_{10}$ ME 100 ml^{-1} and a 90th percentile of $5.0 \log_{10}$ ME 100 ml^{-1} . The pig-associated marker Pig2Bac was detectable in 43% of the samples at lower concentrations, with a 90th percentile of $3.7 \log_{10}$ ME 100 ml^{-1} . BacR could be found in 58% (90th percentile of $3.8 \log_{10}$ ME 100 ml^{-1}), Pig2Bac in 42% (90th percentile of $3.2 \log_{10}$ ME 100 ml^{-1}) of the samples from LSW7. At LSW8, BacR was detected in 88% of the samples with a median concentration of $4.1 \log_{10}$ ME 100 ml^{-1} and a 90th percentile of $5.0 \log_{10}$ ME 100 ml^{-1} . Pig2Bac was found in 35% of the samples with a 90th percentile of $3.7 \log_{10}$ ME 100 ml^{-1} . Human-associated markers (BacHum, HF183II) indicated only a sporadic impact by human fecal pollution (Fig. 3) at those locations.

Human-associated markers decreased with declining connectivity. This effect was most distinct for BacHum (Fig. 4), showing a strong decreasing median concentration gradient from high to low river connectivity.

3.4. Highlighting habitat differences by statistical analysis

The results of a statistical correlation analysis for the selected locations (Danube River, LSW1, LSW3, LSW4, LSW8) with additional MST information further supported the different pollution characteristics between frequently and rarely river connected locations (LSW7 was omitted because of low sample size, $n = 12$).

At the Danube River site, significant correlations were detectable between all analyzed FIB and many chemophysical parameters (Table S1, supplemental material). *E. coli* correlated significantly with the human-associated fecal markers HF183II and BacHum, whereas no significant correlations with the animal-associated markers were detectable (Table 2). Similar to the Danube River location, significant correlations were also detectable between all analyzed FIB and many chemophysical parameters for the sampling site LSW1 (Table S2, supplemental material). *E. coli* correlated significantly with the human-associated MST markers HF183II and BacHum (Table 2). The highest correlation between FIB and human-associated MST markers was observed for the combination of HF183II and *C. perfringens* spores ($r = 0.73$, $p < 0.01$, $n = 32$; see supplemental material). *C. perfringens* was recently suggested as a human-associated sewage tracer (Vierheilig et al., 2013). Very remarkably, for LSW1, a clear dependence for all FIB parameters and human-associated MST marker concentrations on the time

span since the last hydrological connection to the Danube River became obvious ($r = -0.50$ to -0.61 , $p < 0.05$, $n = 33-37$, Table S2). In contrast, no significant correlations to the animal-associated MST markers were observed (Table 2). Taken together, human-derived sewage had a strong governing effect on the observed fecal pollution levels for the FCS.

Statistical correlation outcomes from LSW3, located in the transition area between the FCS and RCS, and LSW4 as well as LSW8, located in the RCS, exhibited contrasting results from the FCS. Hardly any correlations could be detected among the analyzed microbiological, chemophysical and chemical data sets (Tables S3, S4, and S5, supplemental material). A significant correlation between the FIB fractions could be detected at LSW3 and LSW8 for *E. coli* vs. enterococci (Table 2).

4. Discussion

4.1. Successful realization of the new approach

The newly established approach was able to characterize the observed fecal pollution patterns in the backwater area in detail. FIB data alone only provided the basis for a highly speculative interpretation of the nature of the observed fecal pollution. Combining the information from the FIB with river connectivity and genetic MST marker concentrations gave very useful information on two potential pollution aspects of the selected water resource. On the one hand, information on the allochthonous (i.e., externally from the main river channel) vs. the autochthonous (i.e., internally from the backwater area) natures of fecal pollution could be gathered. On the other hand, information on the host origin was also generated (i.e., human vs. animal). By combining both aspects, a congruent and precise picture of the characteristics of the observed fecal pollution at the backwater locations could successfully be drawn. It could be demonstrated that linking host-associated genetic fecal markers with river connectivity data can add valuable spatial information on MST efforts. Information on MST marker occurrence per se only provides an indication of the responsibility of host groups for fecal pollution events (e.g., pigs vs. ruminants vs. pets, humans vs. animals) but does not give any information on its spatial origin. If required, information on the spatial origin of MST markers can be generated by laborious hydraulic transport modeling efforts (Sokolova et al., 2012). However, in this study, we demonstrated that combining microbiological and hydrological connectivity data provides a very straightforward way to obtain information on the spatial component of fecal contamination in backwater resources. As seasonal river fluctuations determined the hydrological connectivity in the investigated area, this data also provides information on the temporal component of fecal contamination (compare Fig. S3, supplemental material). The suggested approach may not be limited to alluvial backwater resources but is also likely useful for other types of water resources, where different water compartments interact, such as in estuaries and marshes (Colón-Rivera et al., 2012; Wild-Allen and Andrewartha, 2016; Raimonet and Cloern, 2017). In addition, this study provides the first clear evidence that hydrological river connectivity is not only a very useful metric in river ecology (Tockner et al., 1999; Hein et al., 2003; Reckendorfer et al., 2013; Weigelhofer et al., 2015) but can efficiently be applied for user- and health-related alluvial water quality investigations, too.

4.2. Significance of results for water resource management

Alluvial backwater systems are essential water resources throughout the world (Tockner and Stanford, 2002; Filippini et al., 2015; Bonsor et al., 2017; Martinez et al., 2017), often used not only

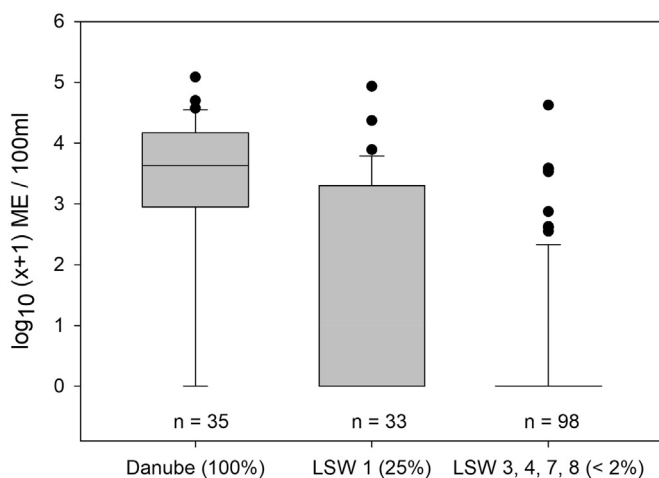


Fig. 4. Concentrations of the genetic MST marker BacHum at the River Danube, the frequently connected (25% of days) main side arm of the backwater (LSW1) and in the rarely connected (<2% of days) sampling locations (LSW3, LSW4, LSW7 and LSW8). ME = marker equivalents. Box plots indicate the median, 25th and 75th percentiles (box), 10th and 90th percentiles (whiskers) and outliers (dots).

Table 2

Statistical association of *E. coli* with other microbiological and chemophysical parameters as calculated by Spearman's rank correlation (ρ) for the Danube River and the four backwater sites LSW1, LSW3, LSW4 and LSW8. Enterococci (ENT), *C. perfringens* spores (CP), human associated fecal markers HF183II and BacHum, ruminant associated marker BacR, pig associated marker Pig2Bac, time since the last surface water connection to the Danube River (TLC), dissolved phosphorus (PO_4), total phosphorus (P-tot), total suspended solids (TSS), relative particulate organic matter (relPOM).

| | | | ENT | CP | HF183II | BacHum | BacR | Pig2Bac | TLC | PO_4 | P-tot | TSS | relPOM |
|---------------|-----------------------|--------|---------------|--------------|--------------|--------------|------|--------------|--------------|--------------|--------------|--------------|---------------|
| Danube | <i>E. coli</i> | ρ | .783* | .546* | .476* | .540* | .204 | -.098 | — | .413 | .605* | .446 | -.482 |
| | | N | 35 | 35 | 33 | 34 | 29 | 34 | — | 32 | 32 | 32 | 32 |
| LSW1 | <i>E. coli</i> | ρ | .821* | .729* | .699* | .616* | .376 | ^a | .571* | .626* | .437 | .773* | -.815* |
| | | N | 36 | 36 | 36 | 32 | 35 | — | 36 | 33 | 33 | 33 | 33 |
| LSW3 | <i>E. coli</i> | ρ | .526* | .294 | ^a | -.062 | .086 | .321 | ^b | .092 | .618* | .389 | -.511* |
| | | N | 37 | 37 | — | 33 | 33 | 32 | — | 34 | 34 | 33 | 33 |
| LSW4 | <i>E. coli</i> | ρ | .397 | .067 | -.182 | .123 | .036 | .125 | ^b | .377 | .233 | .283 | -.353 |
| | | N | 31 | 30 | 30 | 27 | 29 | 28 | — | 31 | 31 | 31 | 31 |
| LSW8 | <i>E. coli</i> | ρ | .731** | -.007 | .060 | .240 | .126 | .230 | ^b | .034 | .136 | .303 | -.060 |
| | | N | 26 | 26 | 26 | 26 | 26 | 26 | — | 24 | 24 | 22 | 22 |

**Significant at the 0.01 level (all values Bonferroni corrected).

*Significant at the 0.05 level (all values Bonferroni corrected).

^a Too few positive results, correlation analysis could not be calculated.

^b Mean connectivity was considered to be too low (3 to less than 1%) to influence results; therefore, this parameter was not included in the correlation matrix at these sampling sites.

for drinking water supply and recreational activities but also for aquaculture (Handisyde et al., 2014; Kumar et al., 2016; Bayazid et al., 2019). The new approach is able to realize improved fecal pollution monitoring practices to support target-oriented water quality management in backwater areas all over the globe. Different methods for the determination of river connectivity matrices are available, and it is not a must to rely on hydrodynamic modeling, as provided in this study (Tritthart et al., 2011; Shen et al., 2015; Stone et al., 2017; Diaz-Redondo et al., 2018). The suggested approach is not limited to fecal hazard characterization and improved backwater catchment protection (i.e., minimization of fecal pollution events) but is also able to essentially support microbial risk assessment activities (QMRA). It can be further used to provide information on the relevant fecal pollution sources to develop sound QMRA modeling scenarios for the backwater area of consideration (Derx et al., 2016). The river connectivity approach can be extended to any reference pathogen (Global Water Pathogen Project, 2019) or chemical pollutant of interest (Islam et al., 2015; Hai et al., 2018; van Driezum et al., 2019).

For the investigated backwater area downstream of Vienna, it was demonstrated that two different pollution sources have to be regarded when talking about fecal contamination: i) human fecal pollution from municipal wastewater as the dominating allochthonous component by the Danube River (Kirschner et al., 2017) and ii) wildlife fecal pollution as an important autochthonous source directly situated in the backwater. This aspect is important for target-oriented quality management of the PA backwater because just until recently, the Danube River was considered the only fecal pollution source for the PA drinking water resources. However, other backwaters may show completely different pollution patterns. For example, autochthonous pollution from human activities (settlements, leaking sewage pipes, pit latrines) and agriculture and livestock can be of significant relevance for many other floodplain areas (Tockner and Stanford, 2002). Because the PA backwater is managed as a nature conservation area where no livestock farming or human settlements are allowed, it can be considered a kind of "natural" fecal pollution reference area for the Central European region, with wild boars and deer as the major managed populations of the hunting ground (Arnberger et al., 2009). Furthermore, the main river, in contrast to the Danube River, may also show significant pollution with nonhuman fecal sources. Recent studies revealed the adverse effect of grazing livestock on river water quality (Jabbar and Grote, 2019) and identified direct defecation to the stream by wading livestock and the release of FIB from cowpats

by surface runoff from the adjacent pastures as very important transmission routes for FIB to the river (Kay et al., 2018).

4.3. Applicability of genetic MST markers in backwaters

The application of genetic *Bacteroidetes* MST markers is at least partly driven by the availability of appropriately performing genetic markers according to certain MST quality criteria, such as pollution source sensitivity and specificity. For the Austrian PA backwater area, the selection of this set of markers, focusing on human, ruminant and pig fecal pollution, was scientifically justified, as the pollution source profile (PSP) indicated humans, ruminants and pigs as potential emission sources with the highest relevance (Frick et al., 2018). Interestingly, the investigated PA area inhabits approximately twice as many ruminants as wild boar (Arnberger et al., 2009), apparently explaining the higher concentrations of ruminant-associated markers compared to pig markers at the investigated sampling locations (Fig. 3). However, the local animal distribution and abundance seems to be an important factor for fecal pollution levels as detected by FIB concentrations.

The PSP also indicated that birds may play a role as fecal sources in the PA backwater area (Frick et al., 2018). Unfortunately, a comprehensive genetic MST marker assay covering all avian populations of potential interest is currently not available for the PA area. In addition, pollution source profiling (PSP) linked to GIS modeling indicated that poikilothermic species, i.e., animals whose body temperature depends on the temperature of the environment, such as fish, frogs and snails, may further contribute relevant amounts of FIB to the PA backwater (Frick et al., 2018). No genetic MST marker assay exists for poikilotherms so far, as these animals were not regarded as relevant sources until very recently. Complementing MST by genetic qPCR targets for birds and poikilothermic animals would add more details about the nature of the observed wildlife pollution source in the future. Nonetheless, extending the MST marker set would not change the main outcome of this study regarding the identified main drivers of the autochthonous (i.e., human sewage) and allochthonous (i.e., wildlife feces) fecal pollution sources at the PA backwater.

MST efforts during this study focused on bacterial genetic markers to complement the FIB data. The sampling technique (frequency, required volumes) as well as the processing of samples (e.g., filtration) can easily be extended to support robust interpretation for the bacterial compartment (Mayer et al. 2015, 2016; Savio et al., 2018). However, extending MST for backwater resources

towards the viral compartment can be highly attractive for several reasons (e.g., high specificity characteristics for the targeted fecal source). Many human, animal and bacterial (phage) viral MST targets for qPCR exist (Ahmed and Harwood 2017; Edwards et al., 2019) and can be linked with the suggested river connectivity approach.

5. Conclusions

On the example of the Austrian porous aquifer backwater area, it was demonstrated that the combination of river connectivity and genetic MST markers could explain the fecal pollution patterns revealed by standard fecal indicator bacteria monitoring, whereas FIB data alone could not. It was demonstrated that allochthonous and autochthonous sources can be important for alluvial backwaters. In the study area, the relevance of external sources from the river, dominated by human fecal sources from municipal sewage disposal systems, is depending on the connectivity of the concerned sites. Internal fecal sources from wild animals are not distributed homogeneously but depend on local animal abundance, which is determined by specific habitat preferences. The results are a valuable input for sustainable and target-oriented water source management and for microbial risk assessment. The presented approach is assumed to be useful for characterizing alluvial water resources for water safety management over the globe.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.watres.2020.116132>.

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