



## Probabilistic fecal pollution source profiling and microbial source tracking for an urban river catchment



Julia Derx<sup>a,\*</sup>, H. Seda Kılıç<sup>a,1,2</sup>, Rita Linke<sup>b,1</sup>, Sílvia Cervero-Aragó<sup>c,1</sup>, Christina Frick<sup>d</sup>, Jack Schijven<sup>e,f</sup>, Alexander K.T. Kirschner<sup>c,g,1</sup>, Gerhard Lindner<sup>a,1</sup>, Julia Walochnik<sup>h</sup>, Gabrielle Stalder<sup>i</sup>, Regina Sommer<sup>c,1</sup>, Ernis Saracevic<sup>j</sup>, Matthias Zessner<sup>j</sup>, Alfred P. Blaschke<sup>a,1</sup>, Andreas H. Farnleitner<sup>b,g,1</sup>

<sup>a</sup> Institute of Hydraulic Engineering and Water Resources Management, TU Wien, Austria

<sup>b</sup> Institute of Chemical, Environmental and Bioscience Engineering, Research Group Microbiology and Molecular Diagnostics 166/5/3, TU Wien, Austria.

<sup>c</sup> Institute for Hygiene and Applied Immunology, Center for Pathophysiology, Infectiology and Immunology, Medical University of Vienna, Austria

<sup>d</sup> Vienna City Administration, Municipal Department 39, Division of Hygiene, Vienna, Austria

<sup>e</sup> Utrecht University, Faculty of Geosciences, Department of Earth Sciences, Utrecht, the Netherlands

<sup>f</sup> National Institute for Public Health and the Environment, Department of Statistics, Informatics and Modelling, Bilthoven, the Netherlands

<sup>g</sup> Division Water Quality and Health, Department of Pharmacology, Physiology, and Microbiology, Karl Landsteiner University of Health Sciences, Krems an der Donau, Austria

<sup>h</sup> Institute of Specific Prophylaxis and Tropical Medicine, Center for Pathophysiology, Infectiology and Immunology, Medical University of Vienna, Austria

<sup>i</sup> Research Institute of Wildlife Ecology, Department of Interdisciplinary Life Sciences, University of Veterinary Medicine Vienna, Vienna, Austria

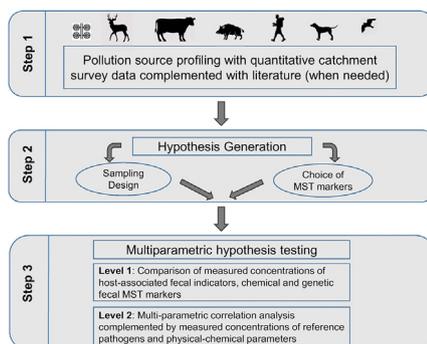
<sup>j</sup> Institute for Water Quality and Resource Management, TU Wien, Vienna, Austria

### HIGHLIGHTS

- Integrated approach for characterizing fecal pollution in urban river catchments
- Probabilistic estimates of DPIPN based on catchment and literature surveys
- Verification by MST and pathogen monitoring at point of use
- Multiple tiers support recreational water safety planning and risk assessment.
- Human wastewater identified as main source of pollution at urban study site.

### GRAPHICAL ABSTRACT

Multi-tiered approach for estimating the extent of fecal pollution sources in urban river catchments with mixed land uses. Adapted from (Linke et al., 2019).



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\* Corresponding author.

E-mail address: [julia.derx@tuwien.ac.at](mailto:julia.derx@tuwien.ac.at) (J. Derx).

<sup>1</sup> Interuniversity Cooperation Centre Water & Health ([www.waterandhealth.at](http://www.waterandhealth.at)).

<sup>2</sup> Authors contributed equally.

### ABSTRACT

We developed an innovative approach to estimate the occurrence and extent of fecal pollution sources for urban river catchments. The methodology consists of 1) catchment surveys complemented by literature data where needed for probabilistic estimates of daily produced fecal indicator (FIBs, *E. coli*, enterococci) and zoonotic reference pathogen numbers (*Campylobacter*, *Cryptosporidium* and *Giardia*) excreted by human and animal sources in a river catchment, 2) generating a hypothesis about the dominant sources of fecal pollution and selecting a source targeted monitoring design, and 3) verifying the results by comparing measured concentrations of the informed choice of parameters (i.e. chemical tracers, *C. perfringens* spores, and host-associated genetic microbial source tracking (MST) markers) in the river, and by multi-parametric correlation analysis. We tested the approach at a study area in Vienna, Austria.

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Probabilistic modelling  
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The daily produced microbial particle numbers according to the probabilistic estimates indicated that, for the dry weather scenario, the discharge of treated wastewater (WWTP) was the primary contributor to fecal pollution. For the wet weather scenario, 80–99 % of the daily produced FIBs and pathogens resulted from combined sewer overflows (CSOs) according to the probabilistic estimates. When testing our hypothesis in the river, the measured concentrations of the human genetic fecal marker were  $\log_{10}$  4 higher than for selected animal genetic fecal markers. Our analyses showed for the first-time statistical relationships between *C. perfringens* spores (used as conservative microbial tracer for communal sewage) and a human genetic fecal marker (i.e. HF183/BacR287) with the reference pathogen *Giardia* in river water (Spearman rank correlation: 0.78–0.83,  $p < 0.05$ ). The developed approach facilitates urban water safety management and provides a robust basis for microbial fate and transport models and microbial infection risk assessment.

## 1. Introduction

Urban surface water bodies are important resources for drinking water production, irrigation and recreational water use. Fecal pollution of surface waters with waterborne pathogens can lead to potential infection risks and waterborne disease outbreaks (Donald et al., 2013; Fewtrell and Kay, 2015; Roberts et al., 2007). The potential sources of fecal pollution are discharges from WWTPs and CSOs, and fecal deposits of domestic animals, livestock and wildlife that can be transferred to water resources via overland transport (Cox et al., 2005; Farnleitner et al., 2010; Sterk et al., 2016a). More extreme precipitation events (Myhre et al., 2019), and high variations in river flow regimes at local and global scales (e.g. Blöschl et al., 2018, 2019) are likely to increase the risk of infectious diseases caused by water-transmissible pathogens (Baumgartner, 2021).

Traditionally, the monitoring of microbiological water quality is based on the application of standardized methods for the enumeration of fecal indicator bacteria (FIB), mostly *E. coli* and enterococci. However, detection of these bacteria does not provide information about the origin of pollution sources (EU, 2015; WHO, 2009) due to their ubiquity in human and animal fecal sources. For identifying the fecal pollution sources, molecular techniques, known as microbial source tracking (MST), have been developed in recent years. These are targeting genes, most commonly the 16S rRNA gene, of host-associated bacteria that are specific for the gastrointestinal tract of the host species (Reischer et al., 2008; Nguyen et al., 2018). Besides methods targeting bacteria, also methods targeting host-associated viruses, as well as ones targeting chemical substances and micropollutants are used for the detection of various pollution sources. Lee et al. (2014) monitored *E. coli* and MST marker concentrations to accurately identify the dominant fecal pollution sources in the Grand River Watershed, Canada. More recent studies have shown that a combination of chemical, isotope, genetic sequencing data, and genetic fecal marker data can be used for identifying the predominant fecal pollution sources in rivers with high confidence (e.g. Duvert et al., 2019; Li et al., 2022). Especially, adding *C. perfringens* to the MST toolbox might be valuable. *C. perfringens* is an endospore forming anaerobic, fastidious, pathogenic bacterium. The spores are conservative tracers for human wastewater, as demonstrated e.g. by Vierheilig et al. (2013).

Probabilistic methods were developed for quantifying the fecal pollution of water resources (Arnöne and Walling, 2006; Gerba, 2000; Sanders et al., 2013). Dorner et al. (2004) used a probabilistic approach for quantifying the daily produced numbers of *Campylobacter* and *Cryptosporidium* based on the animal fecal shedding rates and pathogen source concentrations for the same study area as in Lee et al. (2014). Sterk et al. (2016a) later used a similar approach as Dorner et al. (2004) for estimating the pathogen loads from sewer systems. These previous studies resulted in a very high variability of the estimated microbial source loads. The accuracy of the predictions may be questioned, as they could not further verify the hypothesis about the main sources of fecal pollution. Reischer et al. (2011) and Farnleitner et al. (2011) developed a multi-tiered approach for pristine, alpine catchments consisting of 1) “pollution source profiling” (PSP), i.e. catchment surveys and statistical analysis of the produced numbers of *E. coli* for potential sources of fecal pollution, 2) a host targeted MST study design based on the PSP and 3) analysis of the MST marker

concentrations in spring water and a statistical analysis with *E. coli* and other physical-chemical parameters. Frick et al. (2018) later extended the PSP approach for an urban floodplain area impacted by autochthonous fecal sources from various types of animals. To date, accurate source identification concepts are missing for urban rivers which are often impacted by direct and indirect sources.

This study aims to develop a new approach for estimating the extent of fecal pollution sources in urban recreational rivers impacted by direct (human wastewater) and indirect fecal sources (from livestock, urban and truly native wildlife species) during different hydrological situations. The approach consists of 1) catchment surveys complemented by literature data where needed for establishing probabilistic distributions and estimating the daily produced FIB and zoonotic reference pathogen numbers (DPIP) for dry and wet weather scenarios; 2) generating a hypothesis about the dominant sources and selecting a source targeted study design, and 3) supporting the hypothesis by comparing measured concentrations of the informed choice of parameters (i.e. host-associated fecal indicators, chemical and genetic fecal MST markers) in river water and CSOs during base flow and heavy rainfall events. The data is then used for a multi-parametric correlation analysis by further integrating measured concentrations of the reference pathogens (*Cryptosporidium* and *Giardia*) and physical-chemical parameters. To support robust and fecal-sensitive testing of our hypothesis, we select a multi-metric parameter-set with complementing indicator performance characteristics that have been tested for the area (a combination of host-associated genetic MST markers for the selected targets in our environment, as well as the chemical MST marker carbamazepine and the fecal indicator *Clostridium perfringens* spores, both conservative human sewage tracers). Our approach is a substantial extension of the approaches by Reischer et al. (2011), Farnleitner et al. (2011), and Frick et al. (2018) who limited the PSP to *E. coli* and enterococci, and indirect sources (from livestock and wildlife species) of fecal pollution.

## 2. Methods

### 2.1. Study area

The study area is a densely populated river catchment in Vienna, Austria (Fig. 1). The catchment area is 177 km<sup>2</sup> in size from which 86 % is forested area with abundant wildlife, a small number of livestock species, and no arable farming. The observed annual precipitation in the area is approximately 770 mm (during 2012–2017, Austrian Meteorology Survey). The river flow discharges range from 0.1 to 60 m<sup>3</sup>/s with a mean of 1.8 m<sup>3</sup>/s. The river levels respond quickly during storm events with a response time of <30 min. The river is fed primarily from urban runoff and runoff from forested flysch soil areas. The selected point of potential exposure (PE, Fig. 1) is situated at the downstream end of a river section that people use for recreational activities. PE was thus considered relevant for the identification of fecal pollution and selected as sampling point for water quality monitoring (Section 2.3). The river originates west of Vienna and receives discharges from six tributaries and a WWTP (WWTP 1) upstream of PE, which is located 26 km catchment downstream (Fig. 1). WWTP 1 disposes the municipal wastewater of approximately 19,000 habitants in total. Several CSO outfalls are further located upstream of PE. These are

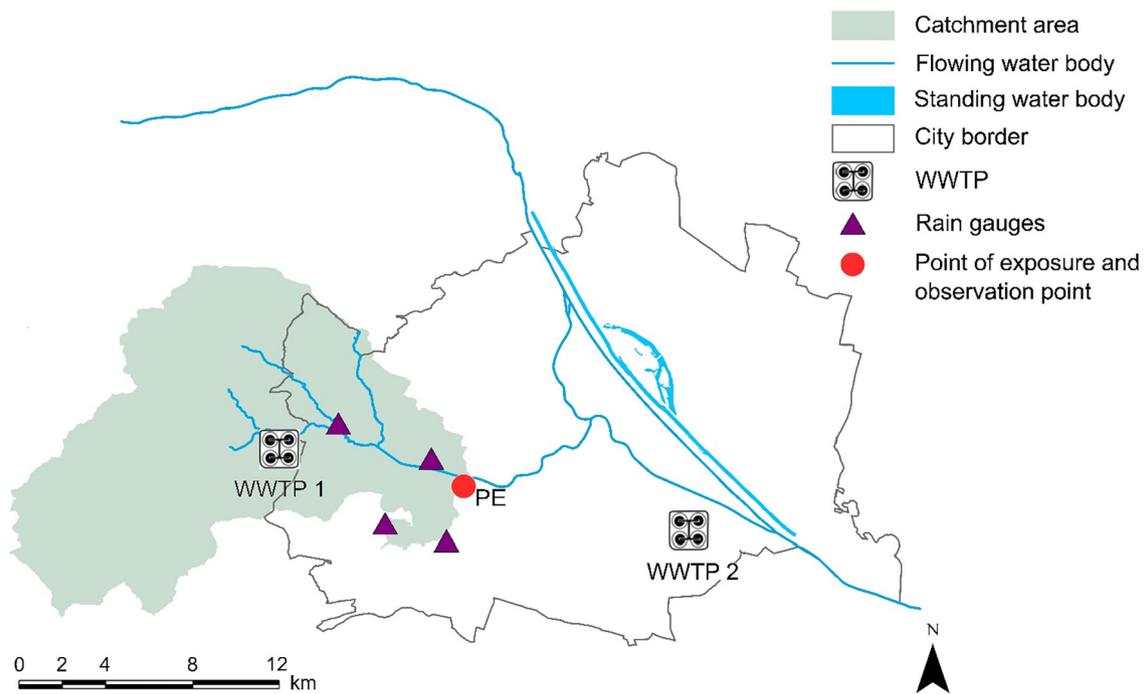


Fig. 1. Study area.

connected to the city's sewer system upstream of WWTP 2. CSOs occur during times when the amount of rainfall and wastewater exceeds the storage capacity. Further direct sources of fecal pollution upstream of PE are from dogs that can have body contact with the river water e.g. during swimming and birds at the study site. Abundant wildlife (birds, ruminants and wild boar), livestock (cattle, sheep), horses, dogs and dog owners are further potential indirect sources of fecal pollution during heavy rainfalls.

2.2. Pollution source profiling (PSP) for urban environments

For the PSP, we estimated the daily produced fecal indicator and pathogen numbers (DPIPn) for the different sources of fecal pollution in our study area. For that, we conducted quantitative catchment surveys complemented by literature data where needed. During a survey in August 2018, we counted dogs, birds, hikers and bathers along a stretch of the river from 1 km upstream towards PE (Fig. 1). During a second survey in October

Table 1  
Input data for the PSP for estimating DPIPn from solid fecal sources (Eq. (3)).

Parameter	Dimension	Details	Distribution	Value	References		
Population P Mean (min – max)	N	Deer	Uniform	(766–2300)	Vienna Forestry Authority (personal communication, 2019)		
		Wild boar	Uniform	(920–1073)			
		Sheep	–	135			
		Cattle	–	176			
		Horses	–	61			
		Birds (indirect sources)	Uniform	(3500–5000)			
		Birds (direct sources)	Uniform	(0–75)		Catchment survey (this study)	
		Dogs (indirect sources)	Poisson	20		Vienna Forestry Authority (personal communication, 2019)	
		Dogs (direct sources)	Uniform	(0–40)		Catchment survey (this study)	
		Humans (indirect sources)	Poisson	150		Vienna Forestry Authority (personal communication, 2019)	
		Defecation rate D Mean values (min – max)	g/d/individual	Deer		–	$1.13 \times 10^3$
Wild boar	–			$1.45 \times 10^3$	(Frick et al., 2018; Hamilton et al., 1997)		
Sheep	–			$1.0 \times 10^3$	(Moriarty and Gilpin, 2014)		
Cattle	–			$2.48 \times 10^4$	(Moriarty et al., 2015; Muirhead et al., 2011)		
Horses	Uniform			$(1.25–2.1) \times 10^4$	(Lawrence et al., 2003; Moriarty et al., 2015)		
Birds	Uniform			10–24	(Hahn et al., 2007)		
Dogs	Uniform			7.6–52	(Wright et al., 2009)		
Humans (indirect sources)	–			$1.5 \times 10^2$	(Reischer et al., 2011; Geldreich, 1978)		
Environmental availability EA	%			Deer	–	100	Assumed based on local observations Assumed based on accessible infrastructure (Vienna Forestry Authority, personal communication, 2019)
				Wild boar	–	100	
		Sheep	–	100			
		Cattle	–	100			
		Horses	–	1			
		Birds	–	100			
		Dogs	–	50			
		Humans	–	3			

2018, we counted dogs and dog owners and hikers along one 10 km hiking path in the forested area (Fig. 1). We then extrapolated the resulting population numbers for the total respective land use areas (Table 1).

For estimating the DPIPN, we selected the fecal indicators *E. coli* and enterococci, and the zoonotic reference pathogens *Campylobacter*, *Cryptosporidium* and *Giardia*. We conducted a literature survey on the source concentrations, defecation rates and pathogen prevalence rates. To select the most representative data for our study area, we consulted studies from Germany and Austria for the concentrations of fecal indicators and reference pathogens in treated wastewater and CSOs (Gallas-Lindemann et al., 2013; Mayer et al., 2016; Rechenburg and Kistemann, 2009; Tondera et al., 2016). For fecal indicator concentrations in wildlife, livestock and birds, data were retrieved from studies conducted in the same province as our study area (Farnleitner et al., 2010; Frick et al., 2018). For *Giardia* and *Cryptosporidium* concentrations in dog, deer, wild boar and sheep feces, and *Campylobacter* concentrations in pig, cattle, sheep and bird feces, studies were consulted that were conducted in Central European countries (Castro-Hermida et al., 2011; Castro-Hermida et al., 2007; Garcia-Preseido et al., 2013b; Jensen et al., 2006; Kostopoulou et al., 2017; Ogden et al., 2009). In few cases, such as for *Giardia* and *Cryptosporidium* concentrations in CSO, cattle, horse and bird feces, and *Campylobacter* concentrations in dog, deer and horse feces, data were retrieved from other developed regions with comparable climates, i.e. in the US, New Zealand and Australia (Arnone and Walling, 2006; Cox et al., 2005; Graczyk et al., 1998; Pattis et al., 2017; Pintar et al., 2015). For the data on pathogen prevalence in animal feces, studies were consulted exclusively from European countries (Table 4). Differences in enumeration methods and types of pathogen species were not considered due to the insufficient number of studies containing this information. The direct sources, i.e. WWTPs and CSOs contribute during a dry weather and a wet weather scenario, respectively. For these, DPIPN are estimated according to

$$DPIPN = Q_{CSO|WWTP} \times C_{CSO|WWTP}, \tag{1}$$

where  $Q_{CSO|WWTP}$  is the mean daily discharge from WWTP 1 or CSOs [ $m^3/day$ ], respectively, and  $C_{CSO|WWTP}$  is the microbial concentrations in treated wastewater and CSO water [number/L], respectively.  $Q_{CSO}$  were estimated according to

$$Q_{CSO} = (R \times A) + E - I - S, \tag{2}$$

where R [mm/d] is the daily rainfall, A [ $m^2$ ] is the size of the paved area, E [ $m^3/d$ ] is the average daily dry weather sewage influent at WWTP 2, I [ $m^3/d$ ] is the daily observed sewage influent at WWTP 2, and S [ $m^3/d$ ] is the storage capacity of the combined sewer system for the catchment (data provided by the Vienna Sewer Administration in 2019). For R, E and I, we used observed data from 2012 to 2017 at the rainfall gauges (Fig. 1) and at

WWTP2, respectively. A and S were assumed based on local reports (Table S1, Vienna Sewer Administration, 2019).

Direct and indirect fecal shedding sources contribute to fecal pollution during a dry weather and a wet weather scenario, respectively. DPIPN shed by hikers or animals are estimated according to

$$DPIPN = D \times P \times EA \times C_{Shed} \times prev, \tag{3}$$

where D is the animal defecation rate [g/individual/day], P is the animal population number [N], EA is the environmental availability [–],  $C_{Shed}$  is the concentration of microorganisms in feces [N/g], and prev is the pathogen prevalence rate in the considered animal populations [–].

Outdoor defecation of hikers was assumed with an environmental availability (EA in Eq. (1)) of 3 % based on the number of accessible sanitary infrastructures in the study area (Table 1). As poop-bag-dispensers in the city are easily reachable, but not always used according to local observations, we assumed an EA of 50 % for dog feces. Horses are located in fenced off pasture areas or stables, and a value of 1 % was assumed for EA. For all other fecal shedding sources, an EA of 100 % was assumed. The animal abundances and defecation rates were described by uniform distributions (Table 1). For hikers, bathers and dogs, the abundances were described by Poisson distributions to reflect the seasonal variations. The microbial source concentrations were described by gamma distributions. The parameters of the gamma distributions were estimated based on the mean and 95th percentile values (Tables 2 and 3). Random values were drawn from the respective probability density functions repeatedly for  $10^6$  iterations, following a Monte Carlo framework using Python 3.7.3. The model parameters are listed in Tables 1–4 and S1.

### 2.3. Monitoring of the microbiological and chemical water quality

#### 2.3.1. Water sampling

Basic monitoring was conducted by taking 5–20 L of grab samples at PE from the river during base flow conditions between June 2018 and September 2020 ( $n = 26$ , Fig. 1). The grab samples were filtered immediately on site using a Cellulose Nitrate Filter (11303-142), Sartorius Stedim Biotech GmbH, Goettingen, Germany (pore size 1.2  $\mu m$ ; diameter 142 mm), and were then analyzed in the lab for *Cryptosporidium* and *Giardia*. In addition, 2 L grab samples were taken for the determination of concentrations of fecal indicators (*E. coli*, enterococci, *C. perfringens* spores), the selected genetic fecal MST markers (Section 3.1), as well as the standard parameters total organic carbon (TOC), the electric conductivity, turbidity, pH and water temperature. As chemical MST parameter, we further analyzed the wastewater micropollutant carbamazepine in 100 mL samples of river water from June 2019 to September 2020 ( $n = 13$ ). Rainfall event monitoring was conducted by taking 2 L of grab samples from the river at PE ( $n = 22$ ) and from a CSO outfall located immediately upstream of PE ( $n = 9$ ). This sample volume was sufficient for analyzing all parameters due to the in

**Table 2**  
Input data for the PSP for estimating DPIPN from wastewater sources (Eqs. 1 and 2).

Parameter	Dimension	Details	Gamma $\alpha, \beta$	Microorganism	Value (Mean, 95th percentile)	References
CSO discharge $Q_{CSO}$	$m^3/d$	Mean of daily values from 2012 to 2017			0.6	Eq. (2), this paper
WWTP 1 discharge $Q_{WWTP}$	$m^3/d$	Annual mean			0.02	Data provided by Vienna authority MA45 (2019)
Concentrations in CSO water $C_{CSO}$	part/L		0.13, $3.1 \times 10^{-8}$	<i>E. coli</i>	$1.3 \times 10^7, 2.0 \times 10^7$	(Tondera et al., 2016)
			0.13, $2.0 \times 10^{-6}$	Enterococci	$2.0 \times 10^6, 3.2 \times 10^6$	(Tondera et al., 2016)
			0.1, 0.01	<i>Giardia</i>	$1.0 \times 10^0, 3.0 \times 10^2$	(Arnone and Walling, 2006)
			n.a.	<i>Cryptosporidium</i>	$\leq 1.0 \times 10^0$	(Arnone and Walling, 2006)
			0.1, $1.0 \times 10^{-6}$	<i>Campylobacter</i>	$1.0 \times 10^5, 1.0 \times 10^6$	(Rechenburg and Kistemann, 2009)
Concentrations in treated wastewater $C_{WWTP}$	part/L		0.3, $1.8 \times 10^{-6}$	<i>E. coli</i>	$1.6 \times 10^5, 5.0 \times 10^5$	(Mayer et al., 2016)
			0.13, $2.0 \times 10^{-6}$	Enterococci	$5.0 \times 10^4, 5.1 \times 10^5$	(Mayer et al., 2016)
			0.5, 0.1	<i>Giardia</i>	$5.3, 1.9 \times 10^1$	(Gallas-Lindemann et al., 2013)
			0.1, 0.2	<i>Cryptosporidium</i>	0.5, 2.0	(Gallas-Lindemann et al., 2013)
			0.1, $5.0 \times 10^{-5}$	<i>Campylobacter</i>	$1.0 \times 10^3, 1.0 \times 10^5$	(Rechenburg and Kistemann, 2009)

**Table 3**

Input concentration of microorganisms in feces ( $C_{shed}$  [part/g]) for the PSP as obtained from the literature survey for estimating DPIPN (Eq. 3).

Source	Microorganism	Gamma $\alpha, \beta$	Mean, 95th percentile	References
Deer	<i>E. coli</i>	0.2, $1.0 \times 10^{-7}$	$1.0 \times 10^5$ , $2.5 \times 10^7$	(Frick et al., 2018)
	Enterococci	0.1, $2.5 \times 10^{-7}$	$4.0 \times 10^4$ , $2.5 \times 10^6$	(Frick et al., 2018)
	<i>Giardia</i>	0.6, $6.7 \times 10^{-3}$	88.9 <sup>a</sup> , 320	(Garcia-Preseido et al., 2013a)
	<i>Cryptosporidium</i>	0.3, $1.0 \times 10^{-3}$	103 <sup>a</sup> , 225	(Garcia-Preseido et al., 2013a)
Wild boar	<i>Campylobacter</i>	0.12, $3.5 \times 10^{-5}$	$3.28 \times 10^3$ SD: $9.35 \times 10^3$	(Pattis et al., 2017)
	<i>E. coli</i>	0.1, $3.0 \times 10^{-8}$	$4.0 \times 10^6$ , $2.5 \times 10^8$	(Frick et al., 2018)
	Enterococci	0.3, $1.0 \times 10^{-6}$	$1.0 \times 10^5$ , $5.0 \times 10^6$	(Frick et al., 2018)
	<i>Giardia</i>	3.5, 0.7	7.5 <sup>a</sup> , 10.0	(Castro-Hermida et al., 2011)
	<i>Cryptosporidium</i>	0.4, $6.0 \times 10^{-3}$	69 <sup>a</sup> , 133.0	(Castro-Hermida et al., 2011)
Sheep	<i>Campylobacter</i>	0.3, $1.3 \times 10^{-6}$	$2.3 \times 10^5$ , $1.0 \times 10^7$	(Jensen et al., 2006)
	<i>E. coli</i>	3.5, $1.4 \times 10^{-8}$	$2.5 \times 10^8$ , $5.0 \times 10^8$	(Farnleitner et al., 2010)
	Enterococci	0.01, $6.8 \times 10^{-10}$	$2.0 \times 10^7$ , $2.0 \times 10^7$	(Farnleitner et al., 2010)
	<i>Giardia</i>	0.1, $6.0 \times 10^{-4}$	177, 4319	(Castro-Hermida et al., 2007)
Cattle	<i>Cryptosporidium</i>	0.1, $1.5 \times 10^{-3}$	69, 1067	(Castro-Hermida et al., 2007)
	<i>Campylobacter</i>	3.6, $1.8 \times 10^{-5}$	$2.0 \times 10^5$ , $4 \times 10^5$	(Ogden et al., 2009)
	<i>E. coli</i>	0.1, $1.0 \times 10^{-8}$	$1.0 \times 10^7$ , $6.3 \times 10^7$	(Farnleitner et al., 2010)
	Enterococci	0.1, $3.3 \times 10^{-7}$	$3.1 \times 10^5$ , $2.0 \times 10^6$	(Farnleitner et al., 2010)
	<i>Giardia</i>	3.5, $2.4 \times 10^{-2}$	Min – max: 0–293	(Cox et al., 2005)
Horses	<i>Cryptosporidium</i>	3.6, 0.7	Min – max: 0–10	(Cox et al., 2005)
	<i>Campylobacter</i>	1.4, $4.7 \times 10^{-5}$	$3.0 \times 10^4$ , $8 \times 10^4$	(Ogden et al., 2009)
	<i>E. coli</i>	0.2, $2.1 \times 10^{-8}$	$8.0 \times 10^7$ , $2.0 \times 10^8$	(Unpublished data from this study area)
	Enterococci	1.0, $1.2 \times 10^{-6}$	$4.0 \times 10^6$ , $9.0 \times 10^6$	(Unpublished data from this study area)
	<i>Giardia</i>	3.6, 0.9	Min – max: 0–8	(Cox et al., 2005)
Birds	<i>Cryptosporidium</i>	3.6, 1.4	Min – max: 0–5	(Cox et al., 2005)
	<i>Campylobacter</i>	2.0, 0.2	13.0, 23.0	(Moriarty et al., 2015)
	<i>E. coli</i>	0.1, $1.0 \times 10^{-6}$	$1.0 \times 10^5$ , $3.2 \times 10^8$	(Frick et al., 2018)
	Enterococci	0.2, $1.6 \times 10^{-7}$	$1.3 \times 10^6$ , $1.0 \times 10^9$	(Frick et al., 2018)
	<i>Giardia</i>	0.4, $9.8 \times 10^{-4}$	405, 786	(Graczyk et al., 1998)
Dogs	<i>Cryptosporidium</i>	1.0, $3.4 \times 10^{-3}$	370, 686	(Graczyk et al., 1998)
	<i>Campylobacter</i>	0.2, $3.0 \times 10^{-5}$	$7.0 \times 10^3$ , $2.0 \times 10^4$	(Ogden et al., 2009)
	<i>E. coli</i>	0.1, $1.4 \times 10^{-9}$	$1.0 \times 10^7$ , $2.5 \times 10^9$	(Frick et al., 2018)
	Enterococci	0.1, $8.4 \times 10^{-9}$	$1.2 \times 10^5$ , $7.9 \times 10^8$	(Frick et al., 2018)
	<i>Giardia</i>	0.1, $2.3 \times 10^{-5}$	$4.5 \times 10^3$ , $2.2 \times 10^5$	(Kostopoulou et al., 2017)
Humans	<i>Cryptosporidium</i>	0.1, $5.0 \times 10^{-4}$	$2.0 \times 10^2$ , $1.4 \times 10^3$	(Kostopoulou et al., 2017)
	<i>Campylobacter</i>	0.2, $7.0 \times 10^{-7}$	$2.9 \times 10^5$	(Pintar et al., 2015)
	<i>E. coli</i>	1.0, $1.0 \times 10^{-9}$	$1.0 \times 10^8$ , $7.9 \times 10^8$	(Farnleitner et al., 2010)
	Enterococci	0.1, $4.0 \times 10^{-8}$	$2.5 \times 10^6$ , $2.5 \times 10^7$	(Farnleitner et al., 2010)
	<i>Giardia</i>	0.15, 0.04	2.6, 64	(Daniels et al., 2015)
	<i>Cryptosporidium</i>	0.1, $3.1 \times 10^{-3}$	32, 490	(Daniels et al., 2015)
	<i>Campylobacter</i>	0.2, $7.2 \times 10^{-7}$	$6.0 \times 10^5$ , SD: $8 \times 10^5$	(Lin et al., 2008; Sterk et al., 2016a)

<sup>a</sup> Mean of maximum and minimum values.

**Table 4**

Input data for the PSP on pathogen prevalence rates (prev [–]) as obtained from the literature survey for estimating DPIPN (Eq. (1)).

Source	Microorganism	Value	References
Deer	<i>Giardia</i>	0.1	(Trogu, 2015)
	<i>Cryptosporidium</i>	0.03	(Trogu, 2015)
	<i>Campylobacter</i>	0.01	(Atanassova et al., 2008)
Wild boar	<i>Giardia</i>	0.15	(Stojecki et al., 2015a)
	<i>Cryptosporidium</i>	0.13	(Němejc et al., 2013)
	<i>Campylobacter</i>	0.01	(Atanassova et al., 2008)
Sheep	<i>Giardia</i>	0.3	(Castro-Hermida et al., 2007)
	<i>Cryptosporidium</i>	0.05	(Castro-Hermida et al., 2007)
	<i>Campylobacter</i>	0.25	(Ogden et al., 2009)
Cattle	<i>Giardia</i>	0.2	(Stojecki et al., 2015b)
	<i>Cryptosporidium</i>	0.2	(Kváč et al., 2011)
	<i>Campylobacter</i>	0.2	(Ogden et al., 2009)
Horses	<i>Giardia</i>	0.2	(Cox et al., 2005)
	<i>Cryptosporidium</i>	0.1	(Cox et al., 2005)
	<i>Campylobacter</i>	0.03	(Moriarty et al., 2015)
Birds	<i>Giardia</i>	0.15	(Majewska et al., 2009; Plutzer and Tomor, 2009)
	<i>Cryptosporidium</i>	0.07	(Majewska et al., 2009; Plutzer and Tomor, 2009)
	<i>Campylobacter</i>	0.13	(Konicek et al., 2016; Silva, 2014)
Dogs	<i>Giardia</i>	0.1	(Guest et al., 2007; Hinney et al., 2017; Overgaauw et al., 2009)
	<i>Cryptosporidium</i>	0.1	(Overgaauw et al., 2009)
	<i>Campylobacter</i>	0.2	(Parsons et al., 2011)
Humans	<i>Giardia</i>	0.09	(Plutzer et al., 2010)
	<i>Cryptosporidium</i>	0.03	(Dong et al., 2020, for Europe in Fig. 3)
	<i>Campylobacter</i>	0.02	(Havelaar et al., 2012)

general higher microbial concentrations during rainfall events than during basic monitoring. In contrast to the basic monitoring, the grab samples during rainfall event monitoring were filtered in the lab, using between 2 and 3 cellulose acetate filters per sample with pore size 1.2  $\mu\text{m}$ . We further analyzed 100 mL samples of river water ( $n = 14$ ) and CSO water ( $n = 2$ ) for the wastewater micropollutant carbamazepine during events from June 2019 to September 2020.

**2.3.2. Fecal indicator and reference pathogen analysis**

*Giardia* spp. and *Cryptosporidium* spp. (oo)cysts were enumerated in the water samples following the flat membrane method as described in ISO 15553 (2006) and the methodological steps described by Cervero-Aragó et al. (2021). Briefly, after filtration on site, the cellulose acetate membranes were placed in Stomacher Bags (Seward Ltd., Bedfordshire, UK) and transported to the lab. Particles on the membranes were scraped with a cell scraper and recovered using 50 mL of Glycine 1 M (Sigma Aldrich, Steinheim, Germany) buffer at pH 5.5, followed by an incubation of 10 min in the Stomacher Lab-Blender 400 and 5 min in an ultrasound bath. The contents of the bags were then placed into 50 mL tubes and centrifuged at  $1550 \times g$  for 15 min. Supernatants were discarded and pellets were resuspended in 2 mL of ultrapure water. One milliliter of the suspension was used for immunomagnetic separation of *Giardia* and *Cryptosporidium* using the Dynabeads GC Combo kit (Life Technologies, Oslo, Norway). Concentrates were stained with the EasyStain kit (Biopoint Pty. Ltd., Belrose, Australia) and quantified as described by Stevenson et al. (2015), see Supplementary Section S2 for microscopic images. After

implementation, tests were performed to determine the recovery efficiency of the used enumeration method when spiking surface water samples as described by Cervero-Aragó et al. (2021). For that purpose, reference materials *G. muris* H3 and *C. parvum* (Waterborne Inc., New Orleans, LA, USA) were used. As water matrixes we used several samples from surface waters with turbidity ranging from 1.7 to 70 NTUs. Recovery efficiencies were  $47 \pm 27\%$  and  $58 \pm 28\%$  for *Cryptosporidium* oocysts and *Giardia* cysts, respectively. The theoretical limit of detection of the flat membrane method varied according to the volume of water analyzed (i.e. sample limit of detection, SLOD). The SLODs ranged from 0.2 to 2.5 [(oo)cysts/L] during base flow monitoring, and from 3 to 80 [(oo)cysts/L] during rain-fall event monitoring.

Cultivation-based enumeration of *E. coli*, enterococci and *C. perfringens* spores was performed in the frame of an ISO 17025 accreditation. For membrane filtration, appropriate dilutions were performed (Farnleitner et al., 2010; Vierheilig et al., 2013). Water samples were filtered over Cellulose Nitrate Filter (11406-50), Sartorius Stedim Biotech GmbH, Goettingen, Germany (pore size 0.45  $\mu\text{m}$ ; diameter 50 mm). Enumeration of *E. coli* was based on the ISO standard 16649-1 (2001), using the chromogenic TBX agar (Oxoid, Thermo Fisher Scientific Inc., United Kingdom) and incubation at  $44 \pm 2^\circ\text{C}$  for  $24 \pm 0.5$  h. Enumeration of enterococci was based on the ISO standard 7899-2 (2000), using Slanetz–Bartley medium (Oxoid) and incubation at  $36 \pm 2^\circ\text{C}$  for  $44 \pm 4$  h, confirmation was performed using Bile-Esculin-Acid agar ( $44 \pm 0.5^\circ\text{C}$  for  $120 \pm 10$  min). For quantification of *C. perfringens* spores, samples were pasteurized at  $60 \pm 2^\circ\text{C}$  for 15 min. *C. perfringens* was analyzed according to ISO standard 14189 (2013), based on selective cultivation on TSC agar (Scharlau, Spain) at  $44 \pm 2^\circ\text{C}$  for  $21 \pm 3$  h and subsequent identification of colonies by acid phosphatase reaction (Ryzinska-Paier et al., 2011). For quality assurance (media control as well as performance control of the methods), the reference strains *E. coli* NCTC 9001, *Enterococcus faecalis* NCTC 775 and *C. perfringens* NCTC 8237 were used.

### 2.3.3. Genetic fecal MST marker analysis

For determining the concentrations of the selected genetic fecal MST markers, water samples were filtered over 0.2  $\mu\text{m}$  polycarbonate filters (Millipore, Bedford, MA; Section 3.3). DNA extraction was performed by applying a bead-beating and phenol/chloroform based method as described previously (Griffiths et al., 2000; Linke et al., 2021). In brief, cell lysis was attained by the addition of CTAB buffer and glass beads in a FastPrep 24 benchtop homogenizer for cell lysis (MP Biomedicals Inc., Irvine, CA) at speed setting of  $6\text{ m s}^{-1}$  for 30 s. Polycarbonate filters were completely dissolved at this step and the DNA was subsequently purified. Precipitation of the DNA was achieved by addition of isopropanol. The extracted DNA was eluted in 100  $\mu\text{L}$  10 mmol  $\text{L}^{-1}$  TRIS buffer (pH 8.0) and stored at  $-80^\circ\text{C}$  until further analysis.

For the present study investigating an urban environment, we selected a human associated (HF183/BacR287, Green et al., 2014a) and a dog associated marker (DG72, Green et al., 2014b) as well as a ruminant- (BacR, Reischer et al., 2006) and a pig-associated (Pig2Bac, Mieszkin et al., 2009) assay. The selection of markers based on the probabilistic PSP is described in Section 3.1. All qPCR assays were performed in duplicate in a 15  $\mu\text{L}$  reaction volume on a Rotor-Gene Q thermocycler (Qiagen, Hilden, Germany) each containing 2.5  $\mu\text{L}$  sample DNA dilution (1:4). The composition of the respective reaction mixtures and cycling parameters were used as described previously (Linke et al., 2021; Mayer et al., 2018; Reischer et al., 2013).

Quality assessment of qPCR data was done as previously described (Mayer et al., 2018; Reischer et al., 2006, 2011). The reaction efficiency of all qPCR runs ranged from 90 to 105%. All negative controls and no-template controls were continuously negative (i.e., fluorescence never exceeded the threshold). Before applying the host-associated MST assays, all samples were measured in duplicate in two 4-fold DNA dilution steps with the AllBac assay (Layton et al., 2006), a general *Bacteroidetes* marker. Samples with matching concentrations (e.g., the ratio [(concentration 1:16):4]/[(concentration 1:4)] was between 0.5 and 2) in the 1:4 and 1:16

dilutions were judged free of PCR inhibiting substances in the 1:4 dilution. This dilution was then used for all further measurements. qPCR standard dilutions (plasmid DNA carrying the respective target sequence for the different assays) ranging from  $10^1$  to  $10^6$  targets per reaction were used in a linear regression model for calculation of the qPCR calibration curve.

As a robust approximation for the SLOD, which can only be determined by elaborate spiking processes to determine sample processing efficiencies on a sample-to-sample basis (filtration- and extraction efficiencies with representative MST mock communities), we applied the established threshold of detection (TOD) concept for MST field applications Reischer et al. (2007, 2008). The filtration volume (200–300 mL), the use of 2.5 mL of diluted DNA extract in qPCR and the minimal amount of detectable targets per PCR reaction defines the detection threshold (Reischer et al., 2006, 2007). The quantitative microbial source tracking results were then expressed as marker equivalents per 100 mL [ $\log_{10}(\text{ME} + 1)/100\text{ mL}$ ] The TOD in this sample set ranged from  $\log_{10} 1.7\text{--}2.5(\text{ME} + 1)/100\text{ mL}$ . The TOD covers sampling and sample processing and also the efficiency of qPCR analysis.

All MST assays used in this study have been vigorously evaluated in the lab and tested in the area. They are routinely applied in this region (e.g., Reischer et al., 2006, 2013; Kirschner et al., 2017; Frick et al., 2020; Steinbacher et al., 2021). Binary sensitivity and specificity for the genetic fecal MST markers were determined using fecal samples from the study region by Steinbacher et al. (2021) and Reischer et al. (2011). Briefly summarized, the respective values for fecal specificity and sensitivity were as follows: 78% and 90% for HF183/BacR287 (human), 86–100%, and 100% for BacR (ruminant), and 91–100% and 100% for Pig-2-Bac (wild boar). The dog-associated marker was evaluated on a different fecal sample set which also originated from the study area. Here, values of 100% were obtained for both binary sensitivity and specificity (Linke, unpublished data). For calculation of the binary sensitivity and specificity, we used only individual fecal samples of the sources, and no pooled fecal samples or wastewater samples for evaluation of the human-associated assay (HF183/BacR287). This is a very conservative (worst case) approach, likely decreasing the estimated binary fecal specificity as compared to real field applications, where fecal source-specificity is based on pooled contaminations sources, resulting in significant higher binary fecal-specificity values.

Quantitative marker abundance obtained from fecal samples (median values) were  $\log_{10} 5.6$  [(ME + 1)/g feces] for HF183/BacR287 ( $n = 10$ ),  $\log_{10} 9.1$  [(ME + 1)/g feces] for BacR ( $n = 13$ ), and  $\log_{10} 9.2$  [(ME + 1)/g feces] for Pig-2-Bac ( $n = 12$ ) (Steinbacher et al., 2021). Quantitative marker abundance for the DG72 marker was 6.18  $\log_{10}$  [(ME + 1)/g feces] ( $n = 13$ ; Linke unpublished data). The data per gram of feces refer to the fresh weight which was used for DNA extraction. The DNA extraction from all fecal samples was performed using the MoBio Power Soil Kit according to the manufacturer's instructions (MoBio Laboratories, Carlsbad, CA).

### 2.3.4. Chemical MST marker and standard parameter analysis

As chemical MST marker, the wastewater micropollutant carbamazepine was selected and analyzed using an automated online solid phase extraction coupled with LCMSMS analysis. For automated online solid phase extraction (online SPE) a Phenomenex Strata X on-Line extraction cartridge ( $20 \times 2.0\text{ mm}$ ;  $25\text{ }\mu\text{m}$ ) and injection volumes of 10 mL with a CTC PAL autosampler (CTC Analytics AG Industriestrasse 20 CH-4222 Zwingen Switzerland) were used. For high performance liquid chromatograph (HPLC) separation we used an analytical column (Phenomenex Luna C-18;  $150 \times 3.0\text{ mm}$ ;  $5\text{ }\mu\text{m}$ ) and Phenomenex C18-Security guard cartridges ( $40 \times 3.0\text{ mm}$ ) with an Agilent System (5301 Stevens Creek Blvd. Santa Clara, CA 95051 United States) consisting of two binary pumps. Injection volumes of 10 mL of Sample were used for the automated online SPE. HPLC separation with eluent 0.1% acetic acid solution in de-ionized water (A) and 0.1% acetic acid in Acetonitrile solution (B) were performed in gradient mode. The program for online SPE and HPLC separation can be seen in Supplementary Table S2. For quantitative analyses, an MSMS system was used consisting of a hybrid triple quadrupole linear ion

trap tandem mass spectrometer Q Trap 6500 from Sciex (500 Old Connecticut Path Framingham, MA 01701 U.S.A.). We further used MRM analysis with electro spray ionization mode (MRM ESI) by 500 °C and nitrogen collision gas. The SLOD for the LCMS analysis were 0.05 ng/L, which was 3 times the standard deviation of the signal to noise ratio (S/N) (Kromidas, 2011), and were determined using the LCMSMS chromatogram for the standard solution.

TOC was measured according to DIN EN 1484:2019-04 (2019). Briefly, acidification of the sample was subsequently followed by oxidation with peroxi-disulphate and UV light to CO<sub>2</sub>, measured by means of a non-dispersive infrared detector (Phoenix 8000, Tekmar Dohrmann, Mason, USA). Electric conductivity (20 °C) was analyzed according to DIN EN 27888:1993-11, 1993, (Cond Level 2, WTW, Weilheim, Germany), turbidity according to DIN EN ISO 7027-1, 2016, (TURB 430 IR, WTW, Weilheim, Germany) and pH according to DIN EN ISO, 2012, pH7310P, WTW, Weilheim, Germany).

2.4. Hypothesis generation and multiparametric hypothesis testing

The first step of the multi-tiered approach (i.e. PSP according to Section 2.2, see Graphical Abstract) provided the needed information to generate a hypothesis about the main fecal pollution sources. For this step, we distinguished between direct (i.e. direct input into river water) and indirect (i.e. indirect input from catchment via surface run-off) fecal sources. Direct sources contribute to fecal pollution during dry weather (WWTPs, animal shedding) or wet weather (CSOs), while indirect sources contribute only during wet weather. Based on this first step, we selected the microbial targets for monitoring and designed the monitoring campaign at the PE in the river (Step 2 in the Graphical Abstract). Basic monthly monitoring allowed investigating the impact from WWTP effluents and continuous direct animal sources. The monitoring during heavy rainfall events served for investigating the impact of CSOs and indirect sources which were transported towards PE. CSOs are short-duration events over several minutes to hours, which we sampled on every possible occasion during the sampling period. For the multiparametric hypothesis testing level 1, we compared the monitored concentrations of the host-associated fecal indicators, chemical and genetic fecal MST markers during base flow and heavy rainfall events (Step 3 in the Graphical Abstract). For level 2 testing, we used loads of all the MST markers in the river and included measured concentrations of the reference pathogens *Cryptosporidium* and *Giardia* and physical-chemical data, i.e. the electric conductivity (EC), the turbidity, the total organic carbon (TOC) for a multi-parametric correlation analysis, using the Spearman rank correlation method. The *p*-values were corrected using the Benjamini-Hochberg method (Benjamini and Hochberg, 1995). Computations were performed using Python 3.7.3. Non-detects of all measured parameters were treated as zero values.

3. Results

3.1. Estimating the extent of fecal pollution sources in the catchment and selection of MST markers

To estimate the extent of fecal pollution sources using the PSP, we estimated the DPIPN within the catchment of the point of exposure for a dry and a wet weather scenario (PE, Fig. 1). For the dry weather scenario, discharges from the WWTP 1, dogs and birds at the river are the direct contributors to fecal pollution of the river. According to the probabilistic estimates the discharges from WWTP 1 resulted in the highest DPIPN (Figs. 2 and 3). For the wet weather scenario, 80–99 % of the estimated DPIPN resulted from CSO discharges in the study area (Figs. 2 and 3). For *E. coli* and enterococci, the estimated mean daily produced numbers from CSOs (log<sub>10</sub> 14 and log<sub>10</sub> 13 [numbers/d]) were log<sub>10</sub> 2–2.5 higher than from wastewater effluent, log<sub>10</sub> 1–2 higher than sources from ruminants, and wild boar, and log<sub>10</sub> 0.3–6 higher than from all other sources (Kruskal-Wallis, *p* < 0.05, Fig. 2). 90–99 % of the estimated daily produced numbers of *Campylobacter* and *Giardia* were from CSOs (mean values: log<sub>10</sub> 12.7 and log<sub>10</sub> 8.5 [numbers/d], Fig. 3). The estimated mean daily produced numbers of *Campylobacter* and *Giardia* from CSOs were log<sub>10</sub> 1–5 higher than from wastewater effluent, ruminants, wild boar and indirect bird sources with a proportional contribution of <4 % each (Kruskal-Wallis, *p* < 0.05, Fig. 3). The major fraction of the estimated mean daily produced numbers of *Cryptosporidium* resulted from ruminants and wild boar for the wet weather scenario (92 % contribution in total, i.e. log<sub>10</sub> 7.2 [numbers/d] each), followed by WWTP 1 for the dry weather scenario and indirect bird sources for the wet weather scenario, which contributed 3 % in total (log<sub>10</sub> 6.0 [numbers/d] each, Fig. 3). The 25–75 % ranges according to the probabilistic estimates of DPIPN varied over log<sub>10</sub> 1–4 [numbers/d] for *E. coli* and enterococci from wastewater effluent and CSOs, and up to log<sub>10</sub> 5 [numbers/d] for *E. coli* from wild boar and birds (Fig. 2). From wastewater effluent and CSO sources, the 25–75 % ranges of estimated DPIPN varied over log<sub>10</sub> 4–8 [numbers/d], and over log<sub>10</sub> 0–4 [numbers/d] from indirect and direct animal sources (Fig. 3).

Based on the probabilistic estimates, we selected a human-associated genetic fecal MST marker (HF183/Bac R287) and two conservative tracers for communal sewage pollution, including *C. perfringens* spores (as demonstrated by Vierheilg et al. (2013) for the study region) and carbamazepine, supporting robust and sensitive detection of human fecal pollution and waste water. To discriminate between the animal fecal pollution sources we selected a ruminant (BacR), a pig (Pig-2-Bac) and a dog genetic fecal marker (DG72). We refer to Section 2.3.3 for the analytical methods and the binary specificity and sensitivity values for the respective genetic fecal markers for our study region, and to Section 4.1 for a discussion on the performance characteristics of the MST markers. Despite of a considerable contribution of horses according to the probabilistic estimates of

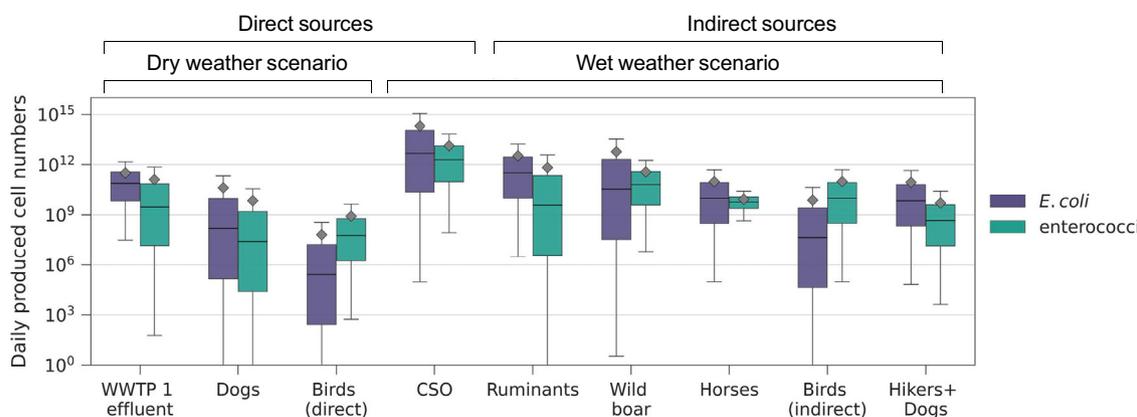


Fig. 2. Pollution source profiling for estimating daily produced numbers of *E. coli* and enterococci using Eqs. (1) and (2) for direct and indirect fecal sources (Fig. 1). Grey diamonds show the mean, boxes the 25th and 75th percentile, whiskers the 5th and 95th percentile, and dark grey lines the median values.

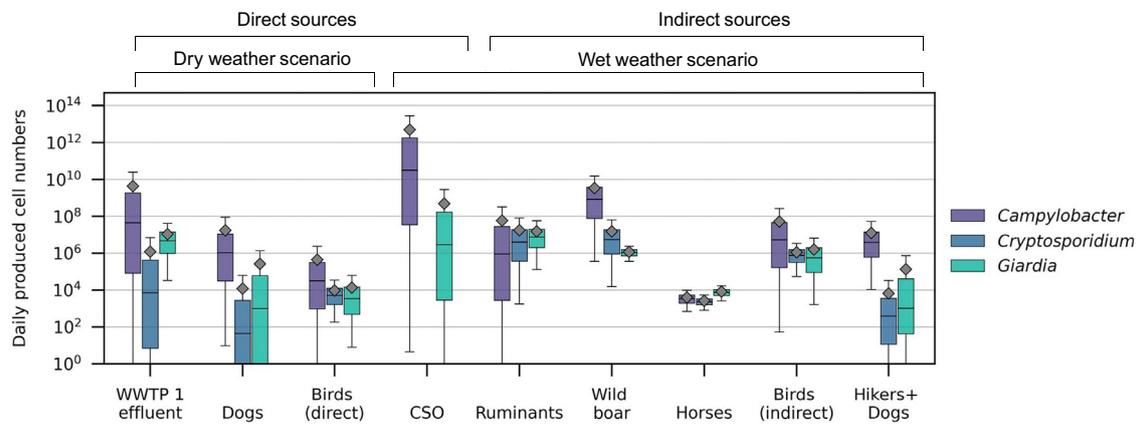


Fig. 3. Pollution source profiling for estimating daily produced numbers of *Campylobacter*, *Cryptosporidium* and *Giardia* using Eqs. (1) and (2) for direct and indirect fecal sources (Fig. 1). Grey diamonds show the mean, boxes the 25th and 75th percentile, whiskers the 5th and 95th percentile, and dark grey lines the median values.

DPIPN for *E. coli* and enterococci, the estimated daily produced pathogen numbers were low (Figs. 2 and 3). We therefore did not include a horse MST marker in our monitoring design.

The hypothesis for the subsequent monitoring at PE in the river and CSO water was that the discharges from WWTP 1 were the main source of fecal contamination during base flow conditions, and CSOs discharges were the main source of fecal contamination during heavy rainfall events. Consequently, we expected (i) highest concentrations of the human-associated fecal indicators (*C. perfringens* spores), chemical (carbamazepine), and genetic fecal MST markers (HF183/BacR287) in river water, (ii) a significant correlation between FIBs and the human-associated MST marker, sewage tracers and human reference pathogens, (iii) a weak correlation between FIBs and ruminant- and pig-associated MST markers, and non-human types of reference pathogens.

### 3.2. Concentrations of the host-associated fecal indicators, chemical and genetic fecal MST markers

In order to test our hypothesis that the main source of fecal pollution was human wastewater, we compared the measured concentrations of the

host-associated fecal indicators, chemical and genetic fecal MST markers (*C. perfringens* spores, carbamazepine, human genetic marker, Section 2.3) at PE (Fig. 1). During base flow, the highest concentrations in river water were found for the human genetic marker (mean value: log<sub>10</sub> 6.4 [(ME + 1)/100 mL]), followed by the ruminant, pig and dog genetic markers with mean values of log<sub>10</sub> 2.1–2.8 [(ME + 1)/100 mL] (Fig. 4). Likewise, during heavy rain events, the highest concentrations in river water were found for the human genetic marker with log<sub>10</sub> 1.3 [(ME + 1)/100 mL] higher mean values than during base flow, followed by the ruminant, pig and dog markers with log<sub>10</sub> 1.3–2.2 [(ME + 1)/100 mL] higher mean values than during base flow (Fig. 4). During base flow and heavy rain events, the mean concentrations of *C. perfringens* spores in river water were log<sub>10</sub> 2.4 and log<sub>10</sub> 3.8 [CFU/100 mL], respectively. During base flow and heavy rain events, the mean concentrations of carbamazepine in river water were log<sub>10</sub> 0.5 and log<sub>10</sub> 0.3 [ng/100 mL], respectively. The concentrations in CSO water, available only during heavy rain events, were slightly higher than in river water for *C. perfringens* and the human genetic marker (Kruskal-Wallis test, *p* < 0.05), while they were not significantly different in CSO and in river water for the dog MST marker (Kruskal-Wallis test, *p* > 0.05).

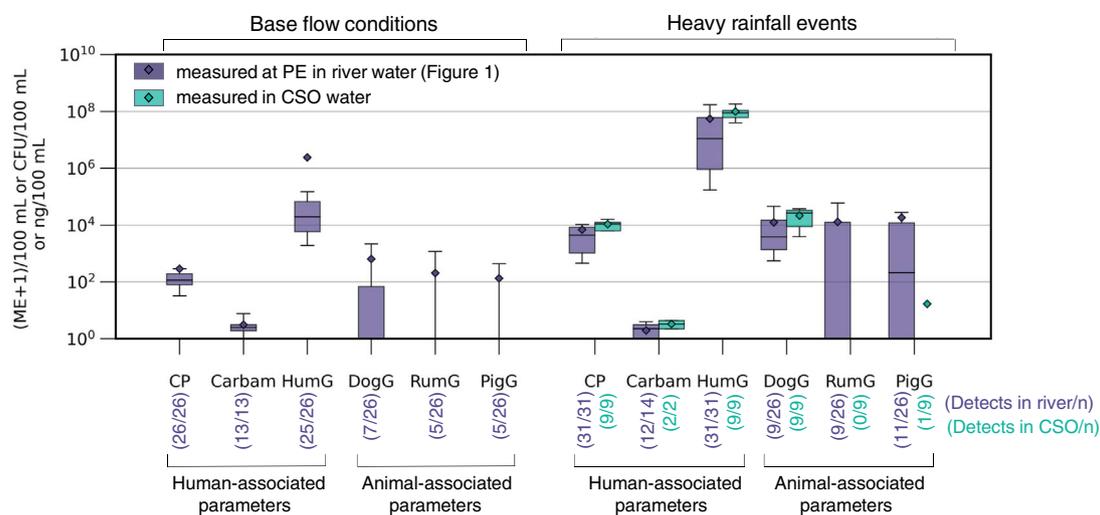


Fig. 4. Measured concentrations of the human-associated fecal indicators, chemical tracers and genetic fecal MST markers (*C. perfringens* spores [CP], carbamazepine [Carbam], the human genetic fecal marker [HumG]), and the dog- (DogG), ruminant- (RumG), and pig-associated genetic fecal markers (PigG) in the river at PE (Fig. 1). Values are shown during base flow in the river (left, blue) and during heavy rainfall event in the river (right, blue) and in CSO water (right, green). Diamonds show the mean, boxes the 25th and 75th percentile, whiskers the 5th and 95th percentile, and dark grey lines the median values. ME: marker equivalent. Non-detects were treated as zero values (Section 2.2).

### 3.3. Multi-parametric correlation analysis

The correlation analysis supported our hypothesis that the sources of fecal pollution at PE (Fig. 1) are mainly human. For both *E. coli* and enterococci loads, there was a strong and significant correlation with the loads of the human-associated fecal indicators, chemical and genetic MST markers (i.e. *C. perfringens* spores, the wastewater micropollutant carbamazepine, and the human genetic MST marker) during both base flow and heavy rain events (Spearman rank coefficients: 0.67–0.97,  $p < 0.05$ , Supplementary Table S3). We further found a strong and significant correlation between loads of *E. coli* and *Giardia* during base flow and heavy rain events (Spearman rank coefficients: 0.64–0.66,  $p < 0.05$ ), and between enterococci and *Giardia* during heavy rain events (Spearman rank coefficient: 0.85,  $p < 0.05$ , Supplementary Table S3). Pictures of *Giardia* and *Cryptosporidium* from the fluorescent microscope are provided in Supplementary S3. For *E. coli* loads, the correlation analysis further showed strong and significant correlations with the dog MST marker loads during heavy rain events (Spearman rank correlation: 0.8,  $p < 0.05$ ).

## 4. Discussion

### 4.1. Advanced approach for estimating the extent of direct and indirect fecal pollution sources in urban river catchments

This paper describes an innovative multi-tiered approach to estimate the extent of fecal pollution sources in mixed land-use urban river catchments. This aim was in particular challenging because estimated DPIP from direct and indirect fecal sources are not directly comparable at the water resource. In contrast to direct sources, complex fate and transport processes are involved during the mobilization of microbial particles from indirect fecal sources, such as attachment or detachment to or from the soil, and inactivation. To allow for a precise identification, we significantly extended the probabilistic source profiling approach by Farnleitner et al. (2011) and Reischer et al. (2011) by estimating the daily produced numbers for *E. coli*, and additionally for enterococci, *Campylobacter*, *Cryptosporidium*, and *Giardia* in animal and human sources. Studies have previously used similar probabilistic approaches but only for either direct or indirect sources (Dorner et al., 2004; Sanders et al., 2013; Sterk et al., 2016b).

#### 4.1.1. Multi-parametric detection of the dominating source of fecal pollution

According to the PSP results, human waste water turned out to be the main source of fecal pollution (>90 %) for both the dry weather- and wet weather-scenarios of the PSP. To support robust and fecal-sensitive testing of this hypothesis for different situations and conditions (e.g. direct and indirect sources), we quantified a combination of 1) highly abundant and sensitive prokaryotic human genetic MST marker (i.e. HF183/BacR287, as the best performing for our environment, see Section 2.3.3) supported by 2) a chemical source tracking marker (carbamazepine, specifically found in our human waste water sources, Fenz et al., 2005), and 3) *Clostridium perfringens* spores (conservative human sewage marker, as demonstrated by Vierheilig et al., 2013 for our area) in the river. This multi-metric parameter set with complementing indicator performance characteristics is highly suitable to robustly and sensitively detect microbial fecal pollution from various human communal waste water resources and conditions, as given in our investigated catchment.

It is important to note that the chosen prokaryotic human HF183/BacR287 MST-marker ensures also sufficient fecal-specificity. According to our previous work (Reischer et al., 2011, based on conditional detection probabilities using Bayes' theorem of true positive MST results) only moderate fecal-specificity levels (i.e. required binary fecal-specificity  $\geq 0.5$ ) are required to ensure specific fecal pollution identification with high confidence (i.e.  $\geq 90$  % probability of correct detection of human waste water). Thus, it was more efficient to invest in a multi-parametric fecal indicator data set (see above), as compared to a second human genetic MST marker, which was not required for high-confidence human fecal pollution detection.

### 4.1.2. Detecting pollution signatures from lower abundant fecal sources

To support also statistical comparisons (e.g. correlation analysis) between dominating human waste water vs. lower abundant, but potentially still detectable animal sources, abundant prokaryotic MST markers were selected. According to the PSP profiles potential ruminant, wild-boar and also dog sources for genetic MST were selected. For ruminant (i.e. BacR), wild-boar (Pig2bac), and dog (DG72) highly abundant qPCR system of excellent binary fecal sensitivity and fecal specificity are available for the catchment (Section 2.3.3). It is important to note that non-dominating fecal pollution portions in mixtures of dominating fecal pollution require high fecal specificity levels. According to our previous investigations (Reischer et al., 2011, as described above) high binary fecal-specificity levels (i.e. required binary fecal-specificity  $\geq 0.9$ ) are required to enable sensible detection of spurious fecal pollution aliquots. The genetic ruminant, pig and dog MST markers were earlier selected as the best suited ones for our study area (Reischer et al., 2006, 2013; Kirschner et al., 2017; Frick et al., 2020; Steinbacher et al., 2021, see Section 2.3.3) and fulfill the required performance criteria.

To complement the multi-parametric statistical analysis, we additionally quantified the reference pathogens *Cryptosporidium* and *Giardia*, and several standard physical-chemical parameters in the river (Section 2.3). We measured all parameters at the PE in the river during base flow and heavy rainfall, and in CSO water during heavy rainfall, sampled from an outfall point directly upstream the PE.

The presented probabilistic approach demonstrates also the involved intrinsic variability of the fecal microbial source loads. For our study area, the PSP results indicated an extremely high intrinsic variability of DPIP due to the high variability of the reported source concentrations (Section 3.1, Tables 2–3, Supplementary Section S1). The probabilistic approach can help to evaluate whether it is possible to increase the confidence in the predictions e.g. by improved MST assays. As human fecal sources contribute 80–95 % of the DPIP according to the probabilistic estimates the performance of the human-associated genetic fecal marker in our study region is more than sufficient (Section 2.3.3). On the contrary, the ruminant, pig, and dog sources with a low proportional contribution (<50 %) require MST assay specificity levels higher than 90 % and a sufficient sensitivity according to Reischer et al. (2011).

### 4.2. Estimation of the extent of fecal pollution sources

The probabilistic approach of this paper for the dry and a wet weather scenario indicated that regular discharges of treated human wastewater and CSOs are the main sources of fecal pollution in the investigated catchment, respectively. This hypothesis was later supported by steps 2 and 3 of our developed approach. The probabilistic approach further demonstrated an impressively high variability of estimated DPIP, even more so for pathogens than for the fecal indicators (Figs. 2 and 3). From the three reference pathogens, *Campylobacter* resulted in the highest estimated daily produced numbers. As *Campylobacter* are less persistent during higher temperatures in comparison to the other two reference pathogens, their impact may be lower during bathing seasons (Sterk et al., 2016a; Strathmann et al., 2016; Thomas et al., 1999). While our study focused on direct and indirect fecal sources contributing to dry and wet weather scenarios, seasonal components could be further considered in future studies.

Our analyses at PE in the river showed statistical relationships between *E. coli*, *C. perfringens* spores and the human genetic fecal marker (i.e. HF183/BacR287) with pathogens (i.e. *Giardia*) in fresh (river) water. According to a recent review by Korajkic et al. (2018), this has not shown before except for *E. coli*. In our study, this was shown by strong, significant correlations of the respective loads during base flow and/or heavy rain events (Supplementary Table S3, Spearman rank correlation: 0.78–0.83,  $p < 0.05$ ). We further found a significant correlation between the loads of enterococci with *Giardia* and *Cryptosporidium*, and between the loads of *C. perfringens* spores and carbamazepine with *Cryptosporidium* loads during heavy rainfall (Supplementary Table S3,  $p < 0.05$ ). In summary, the correlation results indicate an association of *Giardia* with WWTP 1 (Fig. 1) and

CSOs, and of *Cryptosporidium* with CSOs, supporting the results of the PSP and our hypothesis. This finding was further supported by the genotyping of *Giardia* isolates which were found to be human-associated (data not shown, Derx et al., in preparation). Previous reports, however, found no significant association or very low concentrations of *Cryptosporidium* in CSO water (Arnone and Walling, 2006; Kistemann et al., 2012; Schreiber et al., 2019; Tondera et al., 2016). This may be ascribed to the fact that pathogen concentrations in CSO water are still largely underreported. Ruminant and pigs may be alternative sources of *Cryptosporidium*, as suggested by our PSP results and e.g. by Santin (2020). The presence of the ruminant and pig MST marker in the river, albeit at low concentrations (Fig. 4), indicate a small contribution of wildlife via direct shedding into smaller tributaries upstream of PE (Fig. 1). More intense rainfall events may lead to an increased importance of these sources for the microbiological river water quality in the future.

#### 4.3. Implications of the results of this study for recreational water quality management

The results of this study demonstrated the need for fecal pollution protection measures at the studied river site used for recreation. For that, we can consider dry weather and wet weather scenarios. To increase the pollution protection during dry weather, improving the performance of WWTPs by implementing disinfection processes would be a suitable measure. To prevent human infections through contaminated water during wet weather events, suitable measures would be to raise the awareness of potential hazards, e.g. by putting up information signs for the public, or to implement measures to avoid CSOs (e.g. by increasing the sewage reservoirs or reducing the sealing of urban surfaces, etc.). Our results showed that dogs and wildlife cannot be disregarded as sources of fecal pollution. The presented approach is also useful to provide best-available quantitative information as input for microbial fate and transport and QMRA models. Using such models for investigating the present and future scenarios can aid in defining long term treatment performance targets and supports water safety management according to the WHO (2017).

## 5. Conclusion

We developed an innovative approach for estimating the extent of fecal pollution sources in mixed land-used urban river catchments. The probabilistic estimates of DPIP for *E. coli*, enterococci, *Campylobacter*, *Cryptosporidium* and *Giardia* indicated that direct sources from CSOs, and WWTPs are the largest contributors to fecal pollution at the studied site. Our hypothesis was supported by comparing measured concentrations of FIBs (*E. coli*, enterococci), and an informed choice of communal sewage-associated fecal indicators (genetic human MST marker, conservative sewage tracers *C. perfringens* spores and carbamazepine) and animal MST markers in the river at the PE of our study site. If present, the measured MST marker concentrations in the river were highest for the human, followed by the dog, ruminant and the pig MST markers. The concentrations of all MST markers were  $\log_{10}$  1–2 higher during heavy rain events than during base flow indicating the strong impact of CSOs. The correlation analysis complemented by reference pathogen data (*Cryptosporidium* and *Giardia*) and chemical-physical data in the river further supported our hypothesis, e.g. showing a strong significant correlation between *E. coli* and the human-associated fecal indicators, chemical and genetic fecal markers (*C. perfringens* spores, carbamazepine, HF183/BacR287), and *Giardia* (Spearman rank correlation: 0.64–0.97,  $p < 0.05$ ). The approach supports recreational water safety management and can be implemented in other urban areas, provided that catchment-specific data regarding the potential fecal sources and hydrology are available.

#### CRedit authorship contribution statement

**Julia Derx:** Funding acquisition; Supervision; Investigation; Formal analysis; Data curation; Writing – review & editing; **H. Seda Kılıç:**

Data curation; Formal analysis; Writing – original draft; **Rita Linke:** Data curation; Methodology, Writing – review & editing; Validation; **Silvia Cervero-Aragó:** Funding acquisition; Investigation; Data curation; **Christina Frick:** Methodology, Writing – review & editing, **Jack Schijven:** Conceptualization; Supervision; **Alexander K.T. Kirschner:** Writing – review & editing; **Gerhard Lindner:** Investigation; **Julia Walochnik:** Data curation; Writing – review & editing; **Gabrielle Stalder:** Data curation; **Regina Sommer:** Methodology, Investigation; Data curation; Writing – review & editing, **Ernis Saracevic:** Methodology; Investigation; Data curation; (chemical sewage tracer); **Matthias Zessner:** Methodology; Data curation; Supervision (analysis of chemical sewage tracer); **Alfred P. Blaschke:** Conceptualization; Supervision; and **Andreas H. Farnleitner:** Conceptualization; Methodology, Supervision.

#### Data availability

The data that has been used is confidential.

#### 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.scitotenv.2022.159533>.

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