Exploring the full information content of genetic faecal markers for next generation water safety management

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Abstract

Faecal contamination of water is the primary origin of waterborne diseases. After well over a century of dominance of cultivation-based parameters, genetic methods allow now new opportunities to detect and analyse faecal contamination in water. The aim of the thesis was to explore how genetic faecal markers may support next generation water safety management, and in particular, how they may be incorporated into health risk assessment. Additionally, it also aimed to assess the faecal indication capacity and field applicability of near-real time β-D-glucuronidase (GLUC) activity measurements for online faecal pollution detection.

The first study provides a systematic analysis of application areas, key research questions and study designs in the scientific field of genetic faecal markers based on over 1000 publications. The scientific field was shown to grow and the focus has shifted from method establishment to their implementation in field research. Faecal pollution detection and microbial source tracking were identified as the current core areas of application. Emerging areas of application include health and infection risk assessment, (waste)water treatment evaluation and a support role in wastewater surveillance, among others. Genetic faecal parameters are often combined with cultivation-based FIOs, pathogens and environmental parameters, in a toolbox approach. Nucleic acid extracts may be stored (biobanking), allowing retrospective measurements.

The second study characterises the microbial water quality of the Danube River and its floodplains at Vienna, Austria, using a multi-parametric and multiyear monitoring dataset, including FIOs, viral pathogens and host-associated genetic faecal markers. The results indicate that the Danube River is primarily impacted by human faecal pollution, which, in this catchment, is urban wastewater. In contrast, the floodplains were found to be impacted by mixed sources: ruminant, pig, duck and human sources were detected, and the level of faecal pollution is low.

In the third study, genetic faecal markers were employed to guide the setup and to calibrate a combined catchment microbial fate and transport and QMRA model, to assess future faecal pollution scenarios and their effect on drinking water safety. The study site was the Danube River at Vienna, where the study assumed water abstraction from the river for drinking water supply. The outcomes of the second study guided the setup of the model: urban wastewater sources were incorporated into the catchment model, which was calibrated using human-associated genetic faecal marker and human infectious enterovirus data, and the QMRA was set up for human pathogens. The calibrated model was used to simulate future scenarios of climatic and demographic, as well as wastewater infrastructure changes. According to the outcomes, climatic and demographic changes had little impact on drinking water treatment requirements for safe supply in the scenario where 98 % of the pathogen loads stemmed from WWTP discharges. Strong climate change effects were shown in the scenario with enhanced WWTP treatment, where CSOs were the main faecal pollution sources. In the investigation of wastewater infrastructure upgrades, the combination of enhanced wastewater treatment preventing CSOs had the most significant positive effect on the on drinking water treatment requirements.

The fourth study evaluated the fluorescence-based detection of GLUC activity measurements for automated, online faecal pollution detection, based on peer-reviewed literature. New technological adaptations enable now its automated, near-real-time measurement in a robust and analytically precise manner. Large datasets of high temporal or spatial resolution have been reported from a variety of freshwater resources, demonstrating the great potential of this automated method. However, the faecal indication capacity of GLUC activity and the potential link to health risk is still unclear.

In summary, genetic faecal markers constitute a new era in water quality assessment. In combination with environmental and other microbiological parameters in a tailored investigation design, they allow deciphering complex faecal pollution patterns for targeted measures and guide and support health and infection risk assessment, among many other roles in water safety assessment and management. This thesis contributed to our understanding of these roles through a systematic analysis of the literature and two case studies. Additionally, rapid microbiology using online GLUC-based determination is an established method with open questions regarding its interpretation, as the last chapter of this thesis has shown.

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1 Introduction

1.1 Genetic faecal markers for microbial water quality assessment

Water is essential for life. However, it is also a vehicle for the transmission of infectious agents, such as pathogenic viruses, bacteria or protozoa. In terms of contact with water, the greatest infection risk is associated with the ingestion of water contaminated with faecal matter (WHO 2017b). Recent decades have seen a considerable progress in drinking water, sanitation and hygiene globally. For example, the proportion of the global population using an improved drinking water source increased between from 76% in 1990 to 91% in 2015 (United Nations 2015). Despite these successes, the World Health Organisation estimates that 829,000 people still die annually from diarrhoea as a result of contact with unsafe waters, including 297,000 children under five (WHO 2019).

The detection of all relevant intestinal pathogens is not possible for routine monitoring of water resources due to their great diversity, low environmental concentration and laborious detection methods. Therefore, for well over 100 years, faecal pollution assessment through the microbiological analysis of water has relied on the cultivation-based detection of facultative anaerobic commensals of the animal and human gut, so-called faecal indicator organisms (FIO), such as *Escherichia coli* (*E. coli*) and intestinal enterococci. While these FIOs have revolutionized public health protection, they are unable to differentiate between faecal pollution sources (i.e., human, cattle, pig, etc.), making the deciphering of complex faecal contamination patterns difficult or even impossible. Cultivation-based FIOs also require more than one working day to produce results, which means that the results of a pollution event are only available retrospectively. This highlights the need for more comprehensive, informative and rapid microbiological assessment approaches.

The past 20-30 years have seen the development and fast spreading of genetic detection methods for faecal pollution analysis. With the advent of polymerase chain reaction (PCR) in the 1990's, the first assays were developed for the genetic detection of bacterial and viral targets for water quality monitoring (Bej et al. 1990, Bernhard and Field 2000, Puig et al. 1994). These genetic faecal markers target, on the one hand, FIOs detecting faecal pollution in general. On the other hand, host-associated commensal anaerobic bacteria are targeted (e.g., (Bernhard and Field 2000, Reischer et al. 2006, Shanks et al. 2008)) which are also called microbial source tracking markers, since they allow tracking the biological source of faecal pollution. Today, a large range of general and host-associated assays are available for quantitative PCR and increasingly also for digital PCR, allowing the targeted detection of many animal groups, such as humans, ruminants, pigs, dogs, gulls, etc. (e.g., (Lee et al. 2013, Mieszkin et al. 2009, Reischer et al. 2006)). Besides anaerobic bacteria, their phages (Stachler et al. 2017), viruses present with food (Rosario et al. 2009) as well as mitochondrial DNA of host cells (Martellini et al. 2005) have also been suggested as genetic faecal markers. High-throughput DNA sequencing (HTS) may also be used in microbial source tracking. This approach relies on pre-defined faecal reference sequence libraries and sophisticated machine learning algorithms for data analysis and interpretation (Mathai et al. 2020, Raza et al. 2021, Tan et al. 2015, Unno et al. 2018). To sum up, novel genetic tools offer fascinating new ways to analyse and track faecal microorganisms or viruses in water, considerably increasing the amount of faecal-pollution-related information retrievable from a water sample and guiding site- and problem-directed measures.

The primary application area of host-associated genetic faecal markers is the identification of faecal pollution sources. However, the most relevant information for human health is the health risk associated with the ingestion of the polluted water. Linking host-associated genetic faecal markers with health risk, and thereby deriving the health risk attributable to selected pollution sources is a relatively new field. Three main routes have been described so far: (i) measuring the health risk by including genetic faecal markers into epidemiological studies (Napier et al. 2017); (ii) estimating the health risk using quantitative microbial risk assessment (QMRA), a mathematical approach relying on selected pathogens, and applying pathogen to marker ratios (Boehm et al. 2018, Brown et al. 2017, Schoen et al. 2020), and finally (iii) estimating the health risk by calibrating a microbial

fate and transport model for host-associated genetic faecal markers, and running scenario simulations for selected pathogens, coupled to QMRA (Schijven et al. 2015). While the first and second approaches were predominantly used to derive recreational water quality criteria (Boehm et al. 2018, Brown et al. 2017, Napier et al. 2017, Schoen et al. 2020), the second approach was employed in scenario analyses for drinking water safety planning (Demeter et al. 2021, Derx et al. 2021).

Genetic faecal marker detection using qPCR requires 4 to 5 hours, and thus provides same-day results, which is significantly faster than the standardised, cultivation-based detection of FIOs. Nonetheless, it is still too slow for real-time measurements, for example for near-real-time recreational water quality monitoring. Rapid microbiology for health-related water assessments is an emerging field that provide near-real time water quality information. Three major approaches have been described: cell counts using flow cytometry or solid phase cytometry with or without specific staining (Besmer et al. 2014, Hammes et al. 2008), enzymatic measurements (Koschelnik et al. 2015, Ryzinska-Paier et al. 2014) and metabolic activity assessment using ATP measurements (Berney et al. 2008, Hammes et al. 2010). While ATP measurements and cell counting methods (unless using taxon-specific staining) provide information on the entire aquatic microbiome, activity measurement of certain bacterial enzymes, such as β-D-glucuronidase and β-D-galactosidase provide information on faecal pollution (Demeter et al. 2020, Fiksdal and Tryland 2008). Given the great interest of the water sector in online monitoring tools in an era of increasing digitalisation, several commercial products have appeared in the last few years.

1.2 Aims and structure of the thesis

The primary objective of the thesis was to explore ways how genetic faecal markers may support next generation water safety management, and in particular, how they may be incorporated into health risk assessment. The secondary objective of the thesis was to assess the faecal indication capacity and field applicability of near-real time β-D-glucuronidase activity measurements for online faecal pollution detection.

The first aim was to provide an analysis of the scientific field of genetic faecal markers spanning all water-related applications and the entire period since their appearance, based on published literature. While the specificities of genetic faecal markers have been reviewed regularly as the methodological advances have progressed, so far there has been no assessment of the application types of these genetic methods. The aim was to fill this gap by conducting a systematic analysis of the peer-reviewed literature to identify application areas, key research questions and study designs from over 1000 publications identified through tailored database searches and manual screening. A combination of statistical analyses and case study descriptions, followed by a critical discussion should paint an unbiased and comprehensive picture (Chapter 2).

The second aim was to characterise the microbial water quality of the Danube River and its backwaters at Vienna, Austria, by investigating the extent and sources of faecal pollution in these water bodies. This study was part of a large-scale project to assess present and future faecal-pollution-related impacts on the near-river groundwater body where water is abstracted from for the drinking water supply of Vienna. The Danube River catchment upstream is large (850 river km), with a multitude of potential faecal pollution pressures. To characterise this complex pattern and thereby reach the second aim of the thesis, a thorough, multi-parametric and many years long monitoring dataset, including FIOs, viral pathogens and host-associated genetic faecal markers, should be collected and analysed (Chapter 3).

The third aim was to incorporate the information gained through Chapter 3 into an integrative model of microbial fate and transport and QMRA and thereby assess present and future faecal-pollution-related impacts on the near-river groundwater body. The aim was to achieve this through an analysis of the required log reductions of selected pathogens for safe drinking water supply in various pollution scenarios. Based on the outcomes of Chapter 3, two separate models were built with differing aims. The Danube study aimed to assess the future changes that may

affect human faecal pollution sources in the upstream catchment of the Danube River: climate change, population growth as well as infrastructural changes at wastewater treatment plants and combined sewer overflows (Chapter 4). The floodplain study aimed to decipher the role that the three main pathways of faecal pollution play in the floodplain area: (i) faecal pollution brought by floodwater, (ii) the resuspension of faecal microorganisms from animal faecal deposits in inundated areas, and (iii) the release of microorganisms from animal faecal deposits after rainfall (due to the candidate not being the lead author, the resulting publication is given in Annex III: Published co-authored article).

Finally, the fourth aim was to provide a critical review of the fluorescence-based, automated detection of the enzymatic activity of beta-D-glucuronidase as a rapid method to monitor faecal pollution. The analysis should be based on peer-reviewed literature and should consider the technical-analytical performance of these measurements, give an overview of field studies and critically discuss the faecal indication capacity of β-D-glucuronidase measurements (Chapter 5).

The last, 'Conclusions' section discusses the outcomes of the individual chapters in relation to the primary and secondary objectives of the thesis.

2 Have genetic targets for faecal pollution diagnostics and source tracking revolutionised water quality analysis?

Glossary

General terms

Genetic (method, detection, target, etc.): nucleic-acid-based

Microbial source tracking: Methods to discriminate between human and various non-human sources of faecal contamination based on molecular characteristics of microorganisms.

Terms describing indicator types

General faecal indicator organism: An intestinal microorganism whose presence in the environment indicates the presence of faecal matter.

Host-associated faecal indicator: An intestinal microorganism that is strongly associated with its particular host species or range of host species. Its presence provides information about the faecal pollution sources in the environment.

Index organism: A microorganism that is indicative of the presence of a certain pathogen (group) and is a measure of faecal pollution.

Risk indicator: A risk indicator is a faecal indicator where the correlation to waterborne disease has been clearly demonstrated and quantified. Threshold values are then derived, where a certain concentration of the risk indicator corresponds to a given health risk (rate of the selected waterborne disease).

Treatment indicator: A microorganism indicative of the behaviour of a certain pathogen (group) in wastewater treatment and disinfection processes.

Transport surrogate: A microorganism mimicking the behaviour of a certain pathogen (group) in surface and subsurface microbial fate and transport.

Terms related to genetic methods for faecal pollution detection

General faecal marker: A nucleic acid target indicative of total faecal pollution, including the genetic detection of traditional faecal indicator organisms (*E. coli*, enterococci) and general *Bacteroidetes* markers.

Host-associated faecal marker or MST marker: A nucleic acid target strongly associated with a particular host species or range of host species. Its presence in water provides information about the faecal pollution source(s) in the environment.

Genetic faecal pollution diagnostics: Any methodology that relies on detection and/or quantification of nucleic acid-based targets to achieve faecal pollution characterization in a sample.

Abbreviations

CSO – combined sewer overflow
FIO – faecal indicator organism
GFPD – genetic faecal pollution diagnostics
HRWM – health-related water microbiology
HTS – high-throughput sequencing
MST – microbial source tracking
mtDNA – (host) mitochondrial DNA
PCR – polymerase chain reaction
dPCR – digital polymerase chain reaction
qPCR – quantitative polymerase chain reaction
WASH – drinking water, sanitation and hygiene
WWTP – wastewater treatment plant
16 AmpSeq – 16S rRNA gene amplicon sequencing

2.1 Introduction

Safe drinking water, sanitation and hygiene (WASH) are a prerequisite to good health and well-being. Despite considerable progress globally in the past decades, around 850,000 people still die each year from diarrheal disease, principally through faecal-oral pathways, as a result of unsafe WASH practices (World Health Organisation 2019). While there is clear evidence that safely managed water resources, water supply and adequate sanitation reduce the health risk related to water exposure and consumption (drinking, recreational activities, household exposure as well as transmission through irrigation, aquaculture, etc.), there is a constant, urgent need for more comprehensive, informative and rapid microbiological assessment approaches to elucidate intricate WASH-related questions and to clarify complex faecal contamination patterns.

For well over 100 years, faecal pollution assessment through the microbiological analysis of water has relied on the cultivation-based detection of facultative anaerobic bacterial colonisers of the animal and human gut, e.g., total coliforms, faecal coliforms, *Escherichia coli* (*E. coli*) and intestinal enterococci. Recent advances in nucleic acid sequencing methods and bioinformatics revealed the immense richness and diversity of gut microbiota, opening unprecedented possibilities to develop new microbiological assessment approaches. Given the great diversity of assessment types made possible by genetic detection and analysis methods, we introduce the new term of *´genetic faecal pollution diagnostics'* to cover the entirety of this field, wherein 'genetic' means 'nucleic-acid-based'.

Gut microbiotas are profoundly different from free-living microbial communities (e.g., (Chen et al. 2018)) across the biosphere (Ley et al. 2008). The Human Microbiome Project revealed *Bacteroidota* and *Firmicutes* to be the dominant phyla in the human gut, however, with substantial variability among individuals (The Human Microbiome Project Consortium 2012). The microbiome of municipal wastewater provides a community fingerprint that captures this diversity, with a significantly lower community-level variability compared to individuals (Newton et al. 2015). In addition to faecal taxa, the wastewater microbiome also harbours a large proportion of wastewater infrastructure-related microorganisms (Shanks et al. 2013). The within-species variability in the human gut proves to be minor in comparison to the stark differences among other animal species, where both host phylogeny and diet are key drivers (Ley et al. 2008, Mallott and Amato 2021, Youngblut et al. 2021, Youngblut et al. 2019). In addition to the prokaryotic community, the gut also harbours a great diversity of viruses (bacteriophages, viruses of archaea and of human cells as well as viruses transiently present in food, (Liang and Bushman 2021)). Novel molecular biological and genetic tools offer fascinating new ways to analyse

and track faecal microorganisms or viruses in water. To date, these opportunities have only partially been exploited, and future research is poised to further the discovery and impact of the genetic faecal pollution diagnostics field.

The aim of this work is to assess the impact of nucleic acid-based methods on faecal pollution detection and analysis in the field of health-related water microbiology (HRWM). For the first time, this review provides a critical analysis of the new possibilities that state-of-the-art genetic methods have opened in a great diversity of application areas. This is accomplished via a systematic literature approach to identify genetic faecal pollution diagnostic application areas, key research questions and study designs from more than 1,000 publications, since the very beginning of using such molecular biological techniques in the environmental water compartment. The review focuses on genetic targets and parameters that take a faecal indication role; therefore, specific pathogen detection is only included if the indicator role is explicitly stated. Furthermore, description of the various methodological developments of molecular methods and their evaluation is outside the scope of this effort (please find a selection of methodological review articles in Section *'Background information on genetic targets and methods: a historical overview'*). The outcomes of the systematic literature review include trend analyses of relevant scientific literature (Section *'Outcomes of the systematic study design analysis'*), followed by the analysis and discussion of seven identified application areas in HRWM (Section *'In-depth review of each genetic faecal pollution diagnostic 'application' type'*). The review concludes with a critical discussion on the benefits and limitations of genetic faecal pollution diagnostics in health-related water quality and management research.

2.2 Background information on genetic targets and methods: a historical overview

2.2.1 Cultivation-based methods for faecal pollution detection: where it all began

The first routine bacteriological analyses of drinking water were initiated by Percy and Grace Frankland in London in 1885, building on the seminal work of Robert Koch and colleagues regarding microbiological media for detecting bacteria (Koch 1881). Around this time, Escherich described the bacterium that was later renamed *Escherichia coli,* in the faeces of breast-fed children (Castellani and Chalmers 1919, Escherich 1886). *E. coli* is currently one of the most widely used faecal indicator organisms (FIO, see *'Glossary'*) of water quality testing (Geldreich 1966, Levine 1921, Perry and Bayliss 1936), together with intestinal enterococci (Geldreich and Kenner 1969, Kjellander 1960) and their phages, such as somatic coliphages and F-specific RNA bacteriophages (Grabow 2001, Jofre et al. 2016).

These standardised, cultivation-based FIO parameters have found their entrance into regulations all over the world and are still the gold standard for monitoring general faecal pollution in most kinds of water resources. While these FIO revolutionized water quality testing and public health protection at the end of the 19th century, they also have multiple limitations. For example, most protocols require more than one working day to produce results and these FIOs are unable to differentiate between faecal pollution sources (i.e., human, bird, cattle, etc.). It has to be mentioned that host-associated cultivable enteric microorganisms, such as human-associated sorbitol fermenting bifidobacteria were known (Mara and Oragui 1983, Mushi et al. 2010) and paved the way for the field of microbial source tracking (MST, see *'Glossary'*). However, advances in molecular biology offered an unprecedented range of new opportunities to develop genetic technologies that can provide same-day water quality results and characterize key sources of faecal pollution.

2.2.2 The early days of genetic methods for faecal pollution diagnostics

Faecal indicator bacteria often show tremendous genotypic sub-species variation. MST studies in the early 2000s attempted intensively to exploit this strain-level diversity by genetic fingerprinting and -typing methods (e.g. repetitive element PCR, ribotyping, amplified fragment length polymorphism, pulsed-field gel electrophoresis) to

track the origin of *E. coli* and enterococci isolates (Mott and Smith 2011). Huge isolate libraries, covering faecal pollution sources and polluted water bodies in a given catchment of interest, were typed and band-patterns statistically analysed to account for the high spatial and temporal variation (*classical library-based MST*, (Domingo et al. 2007, Mott and Smith 2011)). Such library-based genotyping strategies were also used to evaluate the general faecal indication capacity of faecal indicator bacteria (Ishii et al. 2006, Ishii and Sadowsky 2008).

2.2.3 Detection and quantification of genetic markers for faecal pollution diagnostics

Genetic characterization led to the identification of key genes with host-specificity for a key pollution source (Bernhard and Field 2000). With the advent of conventional end-point PCR in the 1990's, the first studies appeared on targeted detection of host-associated genetic bacterial and viral targets for water quality monitoring ((Bej et al. 1990, Bernhard and Field 2000, Puig et al. 1994), reviewed in (Noble and Weisberg 2005, Scott et al. 2002)), which were later adapted to quantitative real-time PCR (qPCR, (Seurinck et al. 2005)).

The use of conventional PCR for quantification of targets has many limitations, thus qPCR began to dominate the field of genetic faecal pollution diagnostics since the early 2000s and became the most wide-spread culture-independent technology (Jofre and Blanch 2010). Today, there are numerous qPCR assays for a wide variety of bacterial and viral targets, such as enterococci (USEPA 2012b, 2013), *E. coli* (Sivaganesan et al. 2019), human- and other animal-associated bacterial markers [original works: (Mieszkin et al. 2009, Reischer et al. 2006, Shanks et al. 2008), large-scale evaluations: (Layton et al. 2013, Mayer et al. 2018b, Reischer et al. 2013), reviews: (García-Aljaro et al. 2018, Wuertz et al. 2011)], viral MST markers including crAssphage [(García-Aljaro et al. 2017, Stachler et al. 2017) reviewed in (Bivins et al. 2020)] and pepper mild mottle virus [PMMoV, (Rosario et al. 2009), reviewed in (Kitajima et al. 2018, Symonds et al. 2018)] or human enteroviruses [reviewd in (Farkas et al. 2020)]. Mitochondrial DNA targets have also been proposed as a host-associated MST tools (Malla and Haramoto 2020, Martellini et al. 2005, Schill and Mathes 2008). Interestingly, intestinal fungi have not been targeted yet. A good overview of the most useful indicators and MST markers for which qPCR assays are available is provided in the online book of the Global Water Pathogens Project (GWPP) for bacterial (Harwood et al. 2018) and viral indicators of faecal pollution (Ahmed and Harwood 2017) or in a recent review article (Li et al. 2021a). Many of these methods have been subjected to multiple laboratory performance assessments and shown to be highly reproducible when standardized protocols are used (Ebentier et al. 2013, Shanks et al. 2016). Some human-associated qPCR assays are even available as government agency standardized protocols (USEPA 2019a, b) with certified companion reference materials (Kralj et al. 2021, Sivaganesan et al. 2022, Willis et al. 2022).

More recent research areas of genetic quantification methods include ease of use, rapid field-testing and more sensitive and reproducible methods. For example, isothermal amplification assays such as LAMP (loop-mediated isothermal amplification; Martzy, *et al.*, 2017) or HDA (helicase dependent amplification; (Kolm et al. 2017)) have been developed for rapid enterococci detection in environmental waters; an overview can be found in (Nieuwkerk et al. 2020).

In contrast to qPCR, where quantification of target genes relies on a calibration model, digital PCR (dPCR) allows quantification based on Poisson statistics of presence/absence results from thousands to millions of reaction mixture droplets per sample. Advances in microfabrication technologies in the 2010s allowed the development of commercial dPCR platforms making it an emerging and highly promising technology for the genetic faecal pollution diagnostics field (Tiwari, *et al.*, 2022).

2.2.4 High-throughput DNA sequencing for genetic faecal pollution diagnostics

With the advent of high-throughput DNA sequencing (HTS) in the 2010s, whole community profiling revolutionized gut microbiome research. This, in turn has enabled the identification of new host-associated and general faecal pollution targets followed by the development of new qPCR assays (Bibby et al. 2019, McLellan and

Eren 2014). Applying HTS to environmental samples stimulated the development of entirely new concepts for the genetic faecal pollution diagnostics field. HTS-based approaches have evolved rapidly, concomitant with rising capabilities in computing and bioinformatics (Garner et al. 2021). Currently, the two most widely used methods are 16S rRNA gene amplicon sequencing (16S AmpSeq) providing taxonomic information and whole metagenome sequencing allowing, in addition to taxonomic profiling, the identification of functional genes, such as virulence or antibiotic resistance genes (Chan et al. 2019). There are two strategies to use HTS for faecal pollution analysis in aquatic environments. One approach works by identifying gut-associated taxa within the complex aquatic microbiome signal and thus identifying the presence of faecal pollution (e.g., (Ulrich et al. 2016a)). The other approach relies on pre-defined faecal reference sequence libraries, based on a local sample collection and public sequence databases with the aim to identify specific sources of faecal pollution. Sophisticated machine learning algorithms such as SourceTracker, FEAST or FORENSIC, are then required for data analysis and interpretation (Mathai et al. 2020, Raza et al. 2021, Tan et al. 2015, Unno et al. 2018). HTS, as currently applied for most applications in microbiomics, only provides relative quantification within the sequence pool recovered (percent of target sequences within total recovered sequences). The resolution depends on the applied sequencing depth (i.e., number of total sequence reads per sample). It does not provide quantitative information on the analysed sequences in relation to their occurrence in the water sample (see Section *'Sensitivity of environmental detection of nucleic acid targets'*).

2.3 Methods of the systematic study design analysis

2.3.1 Literature database searches

The literature databases Scopus and Web of Science / Core collection were searched for studies on genetic methods to detect microbial faecal pollution in water. In both cases, the query included the following building blocks: 'genetic methods' AND 'faeces' AND 'water quality', with a suite of related words for each term. 'Genetic methods': (genetic OR qPCR OR ddPCR OR PCR OR ribotyp* OR DGGE OR metagenomics OR "microbial communit*" OR "bacterial communit*" OR "microbial diversity" OR (source AND track*)); 'faeces': (feces OR faeces OR fecal OR faecal OR wastewater OR sewage OR enteric OR intestinal); 'water quality': ((water* OR freshwater OR seawater) AND (quality OR pollution OR contamination)). Each of the blocks was searched in the title, the abstract and the author keyword fields. The document type was restricted to research articles. All years were included. The queries can be accessed through the following links: Web of Science, Scopus. The resulting list included 2977 articles from Web of Science / Core Collection and 3348 articles from Scopus (as of 06/07/2022). After removing duplicates and articles with no DOI, the combined list contained 3224 articles (Figure 2.1).

2.3.2 Article screening

Next, the combined list (titles and abstracts) was screened manually to remove off-topic studies. Only articles that explicitly stated the use of at least one genetic microbial parameter as indicator for faecal pollution diagnostics (but not if used as e.g., enteric pathogen) were retained. Studies developing and evaluating new methods for genetic faecal pollution diagnostics as well as their field application were both retained. A total of 1091 articles fulfilled these criteria ('all genetic studies', Figure 2.1).

Table 2.1 Systematic study design analysis. Each article in the 'application studies' pool was assessed for each study element (columns), with a single or multiple choices from the categories (rows).

Genetic faecal parameters				Other types of parameters	Data analysis approach	Sample		Application area
Class	Target organism	Host	Method	Class		Sample type	Use type	
general faecal marker, traditional (e.g., *E. coli*, enterococci)	prokaryotes	general faecal	PCR	culture-based FIO	Summary statistics, qualitative data	freshwater	recreational	detection of total pollution
general faecal marker, new (eg. general Bacteroidetes)	viruses	human or sewage	qPCR/ dPCR	culture-based MST	Summary statistics, quantitative data	seawater	irrigation	source tracking: single source
microbial source tracking marker (MST marker)	host cell mitochondrial DNA (mtDNA)	non-human	sequencing	pathogen	Correlations, hypothesis tests or simple bioinformatics	estuary	drinking	source tracking: multiple sources
microbial source tracking, other approaches (MST other)	other	multiple hosts	other	epidemiology	Multivariate statistics or advanced bioinformatics	domestic water	shellfish-growing	evaluation of treatment processes
community analysis			not defined	chemical tracers	QMRA, fate & transport modelling	groundwater	other	health and infection risk assessment
other				physicochemistry and nutrients	other data analyses	rainwater	n/a	outbreak tracing and wastewater surveillance
				antibiotic resistance		faeces		other
				hydrology		sewage		
				meteorology		stormwater, CSO		
				land use		sludge		
				other		soil		
						sediment & sand		
						microcosm or spiked water		
						other		

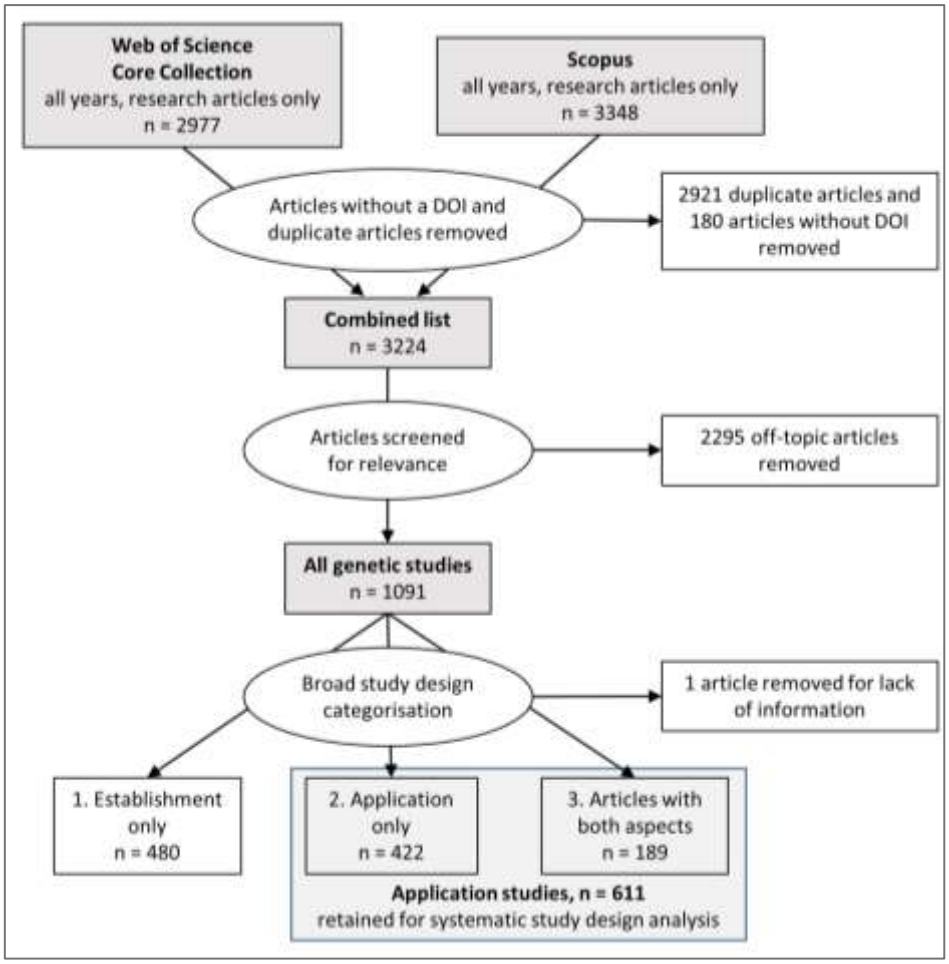


Figure 2.1 Methodology of the systematic literature analysis.

2.3.3 *Broad categorisation of 'all genetic studies'*

The 1091 articles in the 'all genetic studies' pool were then categorised based on their *broad study aim*. (1) *Method establishment* articles: the research question relates to method development and evaluation/validation (sensitivity/specificity, persistence, resistance, etc.). (2) *Application* articles: the research question relates to the environment, and the genetic parameter is assumed to have been previously validated. Studies on, e.g., the detection and source tracking of faecal pollution, or the estimation of the associated health risk, belong to this category. (3) *Both*: articles having both method establishment and application aspects (Figure 2.1). Since the review aims to assess application areas, articles from (2) and (3) were retained for detailed analysis ('application studies', n = 611, Figure 2.1).

2.3.4 *Systematic analysis of the 'application studies'*

Titles and abstracts from all application studies (n = 611, Figure 2.1) were reviewed to extract information for five study elements including: (i) genetic faecal parameters, (ii) other types of parameters, (iii) sample type and use, (iv) data analysis approach and (v) application area. The following section and Table 2.1 describe study element definitions.

(i) Genetic faecal parameters: The two selection criteria for microbial parameters included here were (1) to be detected using genetic methods and (2) to have an indicator role; pathogens were only included if the indicator role was explicitly stated (e.g., 'viral indicator').

a. **Class.** Six genetic faecal parameter 'classes' were distinguished, where parameter 'class' is defined as a group of similar parameters. General faecal markers, indicating faecal pollution in general (covering human and other animal sources), are represented by two classes: *'traditional general faecal markers'* that target microorganisms or bacteriophages for which the culture-based analysis is standardised and widely used (e.g., *E. coli*, enterococci), and *'new general faecal markers'* that have been more recently developed and target highly abundant *anaerobes of the gut, such as Bacteroides spp.. MST methods are divided into two classes: the various host-associated viral, bacterial or mitochondrial DNA-based markers are in the 'MST markers' class, while the 'MST other' class includes* HTS-based as well as classical library-based, genotyping MST approaches. The class '*community analysis'* covers genotyping- or HTS-based approaches to describe the microbial community. Finally, all other genetic methods for faecal pollution analysis, such as non-library-based genotyping (e.g., *E. coli* population structure using strains or *E. coli* phylogroups) or treatment indicators that are not typical faecal indicators (e.g., pathogens) are included in the *'other'* class.

b. **Target organism.** This study element describes taxonomical groupings covering the major target types in genetic faecal pollution analysis, such as *'prokaryotes', 'viruses'* and the mitochondrial DNA of the host animal itself *('mtDNA').* Other target types, such as eukaryotes using 18S rRNA gene sequencing or if the target organism was not defined, are included in the category *'other'*.

c. **Host.** Target organisms, and therefore nucleic acid targets, may be host-associated, i.e., associated with a particular host species or narrow range of host species or may be general, i.e., associated with a wide range of host species. Four host categories are distinguished, '*general'*, *'human'* (human- or sewage-associated), *'non-human'* (associated with other animals) and *'multiple hosts'* (more than one host was targeted). The category '*not applicable'* was assigned to community analyses (fingerprinting, sequencing, etc.).

d. **Method**. The great diversity of genetic methods for the detection of faecal pollution targets were grouped into four categories. Qualitative PCR methods (and cases where it was unclear whether qualitative or quantitative PCR was performed), are included in the category *'PCR'*. Quantitative PCR and digital PCR are pooled because of their quantitative aspect in the category *'qPCR/dPCR'. 'Sequencing'* covers amplicon sequencing and whole metagenome analysis (shotgun sequencing). Finally, genetic fingerprinting techniques e.g., DGGE or BOX-PCR, hybridisation, isothermal amplification, other methods or in case the method was not defined, are pooled in the category '*other'.*

(ii) Other types of parameters:

Class. All other parameters that the analysed articles reported were assessed on the level of parameter 'class', allowing an overview of the study design. Table 2.1 lists the eleven parameter classes that were identified. The class *'other'* covers diverse parameters with low occurrence, e.g. biological oxygen demand, heterotrophic plate count, observational data on WASH practices.

(iii) **Sample type and use.** Fourteen categories of 'sample type', including various water types, faecal matter and other materials, were identified. In case the authors stated the intended use of the water resource, this was also logged. For a list of 'sample type' and 'use type' categories, please refer to Table 2.1.

(iv) **Data analysis approach**. This study element describes how the dataset, characterised by the three study elements explained above, was analysed by the authors. In contrast to the three study elements, where several items could be logged, depending on the study design of the article, here each article was assigned to one of the six categories listed in Table 2.1. In case where only summary statistics were reported, we have differentiated between qualitative data (occurrences) and

quantitative data (min., max., median, etc.). Correlation analyses, hypothesis testing and simple bioinformatics such as sequence annotation, community analysis (e.g. Bray-Curtis dissimilarities) were grouped together into the category *'correlations, hypothesis tests or simple bioinformatics'*. The category *'multivariate statistics or advanced bioinformatics'* includes multivariate statistics, classification algorithms in case of classical library-based MST, MST algorithms with HTS data as well as HTS-based community analyses involving statistical analysis with metadata. Studies performing Quantitative Microbial Risk Assessment (QMRA) or microbial fate and transport models were grouped together in the category *'QMRA, fate & transport modelling'*. Other data analysis approaches, such as GIS-based data analysis, or, in case of classical library-based MST, genotyping fingerprints without reporting a statistical classification method were assigned to the category *'other'*.

(v) **Application area.** Each article was assigned to one of the seven scientific application areas identified during the study design analysis. The application assignment is based on the predominant research question. For a list of the application areas, please refer to Table 2.1.

The assessment was performed in MS Excel. The resulting study design database was analysed and visualised in R, using *tidyverse*. Co-occurrence networks were computed and visualised using *igraph*, following Ognyanova (2021). Alluvial diagrams that group and visualise categorical data, were done with *ggalluvial*.

2.4 Outcomes of the systematic study design analysis

2.4.1 Broad study design trends across all articles

A systematic scientific literature database search followed by manual screening identified 1,091 scientific articles (Figure 2.1, 'all genetic studies'). Research with genetic methods in this field started in the 1990s with a few articles per year, increasing to up to 100 articles in 2021 (Figure 2.2). The broad categorisation of study design types revealed three distinct phases: (i) the emergence of genetic methods in the 1990s with just a handful of articles published yearly; (ii) between approximately 2003 and 2010, the field started growing with the main focus of research being on the development and validation (establishment) of new methods, namely new general and host-associated faecal markers; (iii) since 2011, the field continues to grow, but there is a clear shift from method establisment activities to implementation across a broad range of applications (Figure 2.2).

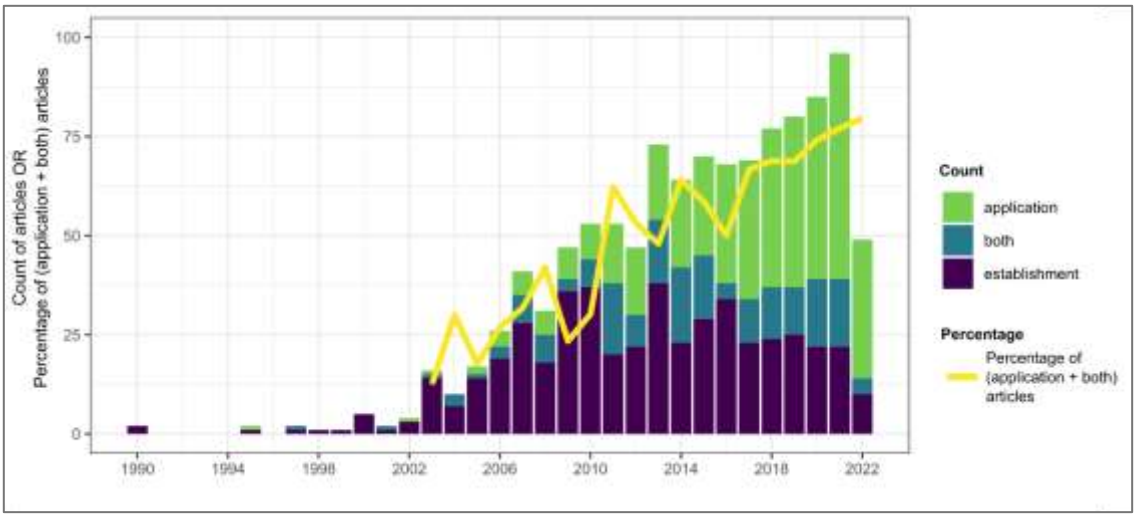


Figure 2.2 Number of publications in the broad study design types over the years in the 'all genetic studies' pool (n=1091, Figure 2.1). The stacked bars show the broad categorisation. The yellow line represents the percentage of pooled 'application' and

'both' categories (i.e. the 'application studies', Figure 2.1) in the 'all genetic studies' pool. The year 2022 is incomplete (last date of retrieval: 06/07/2022).

Since the aspects of establishing methods have been duly reviewed elsewhere (see references in section *'Background information on genetic targets and methods: a historical overview'*), articles focused on these aspects were excluded from further analyses (Figure 2.1).

2.4.2 'Application Studies' trend analyses

'Application studies' (n = 611; Figure 2.1) were reviewed to extract defined study elements ranging from parameters measured to 'application area' (Table 2.1, section *'Methods of the systematic study design analysis'*). The following sections describe study element assignments and occurrence trends.

2.4.2.1 Parameter 'class' assignment and trends

Parameter 'class' assignments were designed to provide a coarse overview of general experimental study design where parameter 'class' was defined as a group of similar parameters. A total of 17 parameter 'class' types, including six genetic and eleven other parameter classes, were identified during the systematic review ranging from 'MST markers' (measured by n = 408 articles) and 'culture-based FIOs' (n = 393) to 'epidemiology' (n = 11). 444 articles (73% of 'application studies') included three or fewer parameter classes. Four parameter classes were reported by 111 articles, while complex study designs with five or more parameter classes were rare with only 56 articles. A co-occurrence network analysis indicated that 'MST markers' and traditional 'culture-based FIO' was the most common combination (n = 266 articles). In fact, not only 'MST markers' were paired often with 'culture-based FIO', but this was the most common combination for each of the genetic parameter classes. Additionally, 'MST markers' were often combined with 'pathogens' (n = 117 articles) and 'physicochemistry and nutrients' (n = 80 articles, Figure 2.3).

2.4.2.2 Genetic parameters: 'target organism', 'host', and 'method' assignments

All 'application studies' were mined for detailed information on the genetic parameters. For each parameter reported, target organism, host organism and analytical method was recorded, resulting in altogether 897 parameter occurrences from across the 611 application studies. The most widely reported target organism was 'prokaryotes' (n = 712 parameter occurrences) followed by 'viruses' (n = 155). In contrast, 'host mitochondrial DNA' and 'other' target organisms collectively accounted for 30 parameter occurrences. Host assignments indicated that 'human' (n = 300) is the most widely researched host animal followed by 'multiple hosts' (n = 200), 'general' faecal (n = 143), and 'non-human' (n = 40). Method assignments suggest that PCR-based methods account for the vast majority of parameter occurrences (n = 688) with 'qPCR/dPCR' methods used 80% of the time. 'Sequencing' was the next most prevalent method assignment group (n = 136). An alluvial plot (Figure 2.4) illustrates linkages or lack thereof between class, target organism, host, and method parameters.

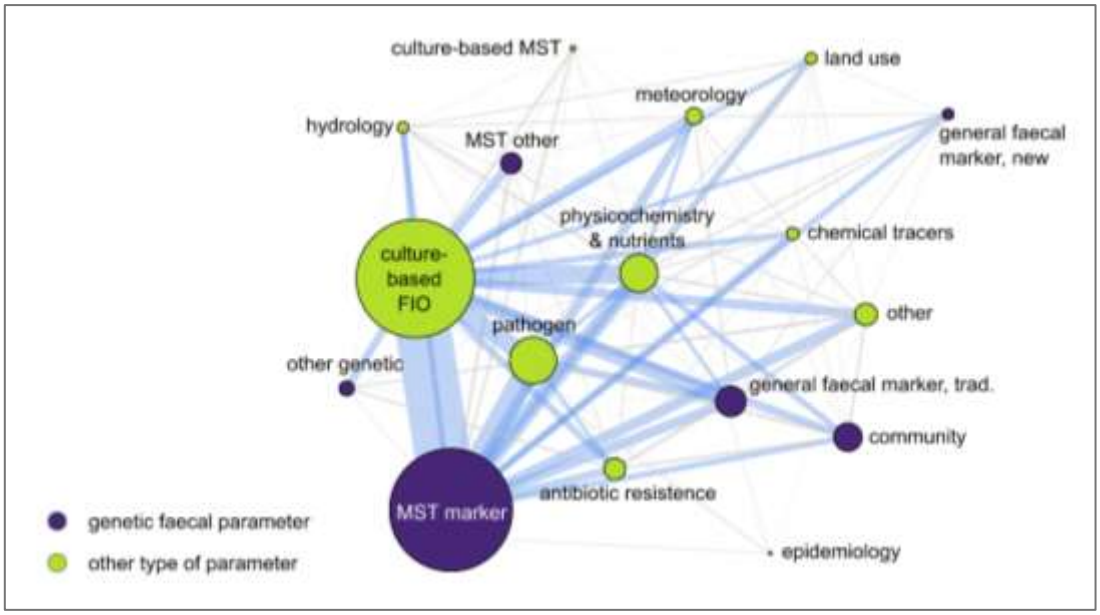


Figure 2.3 Network analysis of the parameter 'class' assignment occurrence in the genetic faecal and other types of parameters (Table 2.1) in the 'application studies' pool (n = 611). The node size is proportional to the number of articles, the line thickness reflects the number of articles for a respective combination. Blue lines mark more than 20 co-occurrences while grey lines show less than 20 co-occurrences.

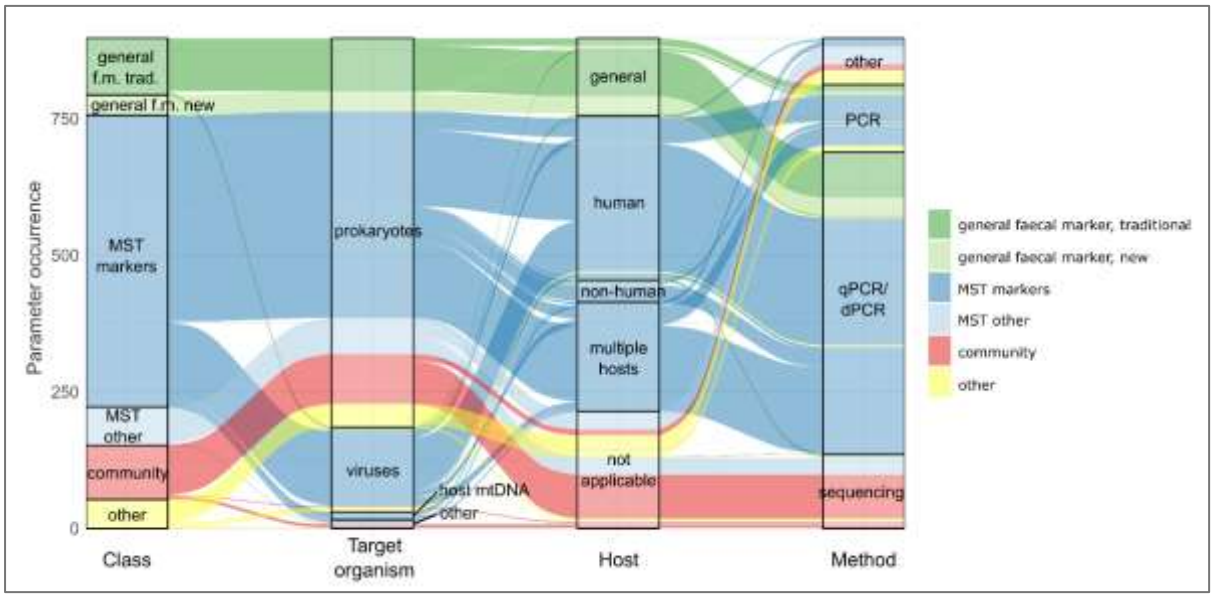


Figure 2.4 Alluvial plot showing the occurrence of genetic parameter types in the 'application studies' pool (n = 611). Each item, i.e., each line corresponds to one parameter measured in one study, so one 'class' – 'target organism' – 'host' – 'method' assignment. The thickness of the stratum (ribbon) corresponds to the number of studies that measured that particular class-organism-host-method combination. However, since a study might have measured several genetic parameters, the y-axis does not correspond to the number of articles in the 'application studies' pool.

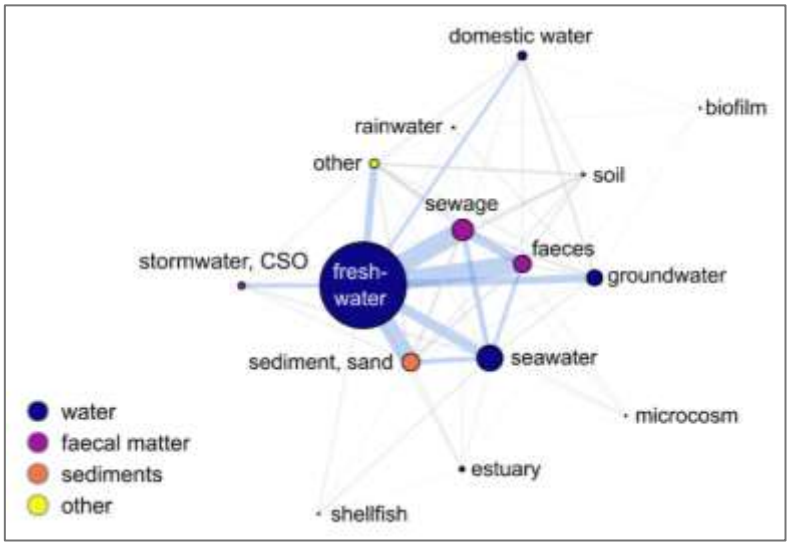


Figure 2.5 Network analysis of the 'sample type' assignment occurrence in the 'application studies' pool (n = 611). The node size is proportional to the number of articles, the line thickness reflects the number of articles for a respective combination. Blue lines mark more than 10 co-occurrences while grey lines show less than 10 co-occurrences. CSO denotes combined sewer overflow.

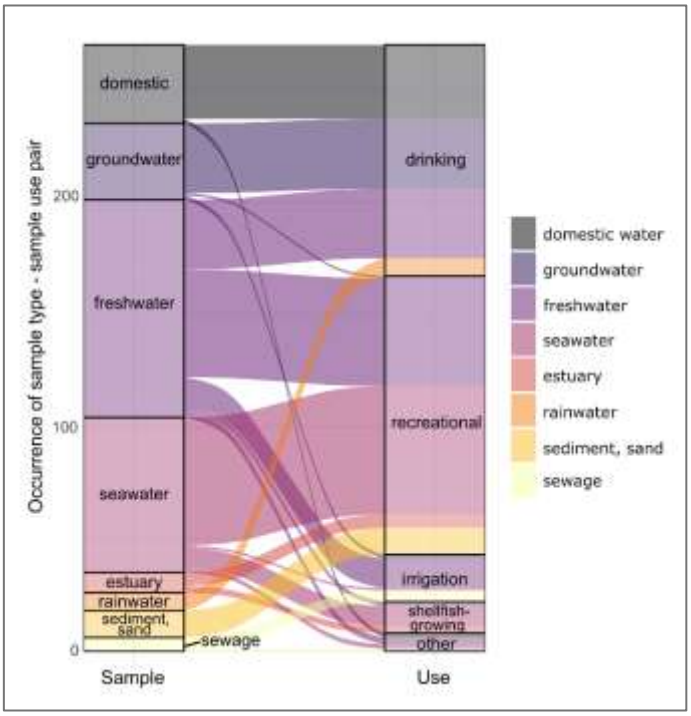


Figure 2.6 Alluvial plot showing the 'sample type' – 'sample use' combinations in the subpopulation of 'application studies' that reported this information (n = 240 articles). Since a study might have analysed several 'sample types', or indicated several 'water uses', the y-axis does not correspond to the total number of articles.

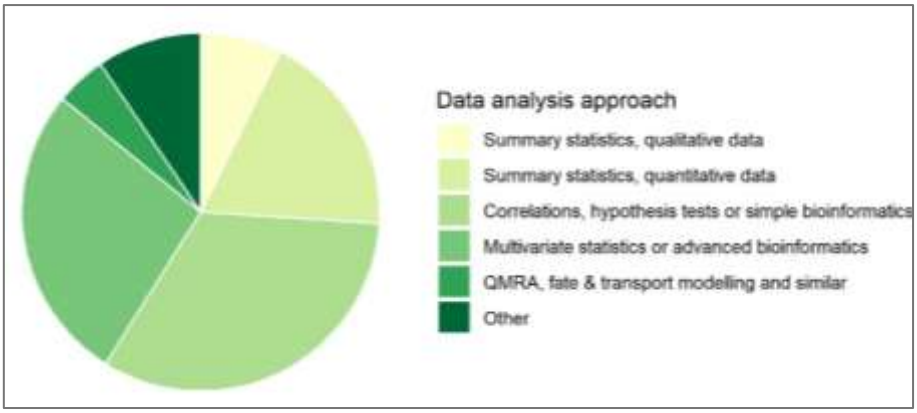


Figure 2.7 Data analysis approach in each article in the 'application studies' pool (n=611).

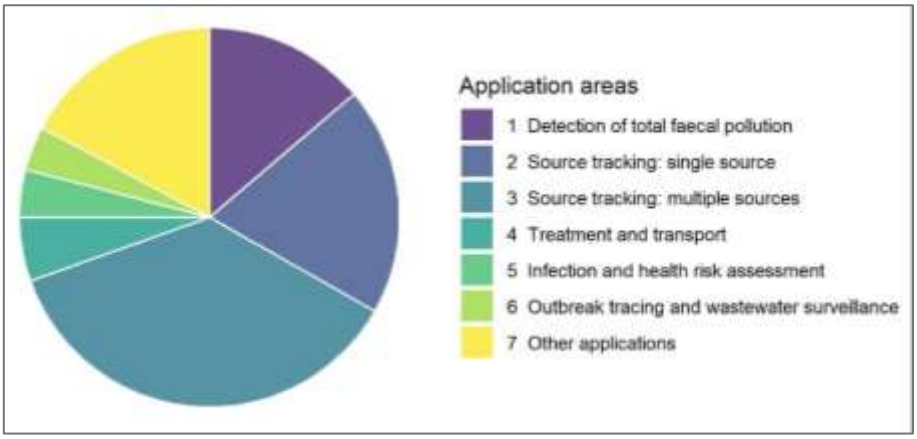


Figure 2.8 Assigned application areas in the 'application studies' pool.

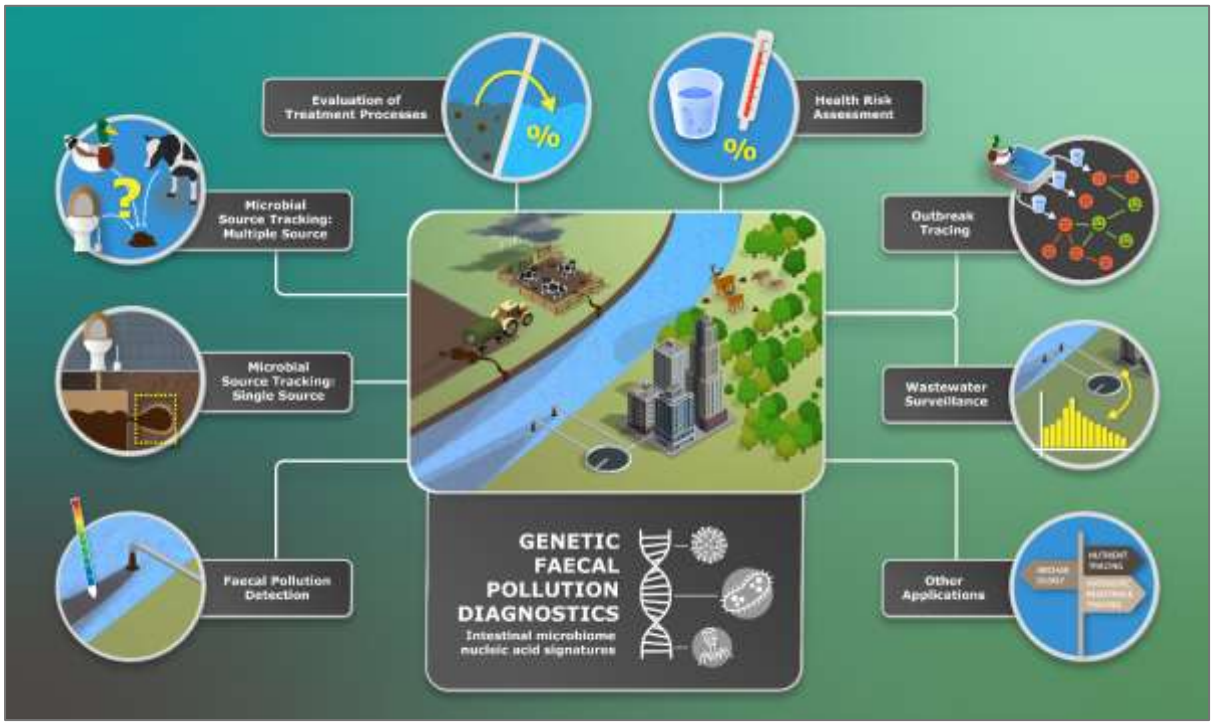


Figure 2.9 Conceptual overview of the assigned application areas of the genetic faecal pollution diagnostics field.

2.4.2.3 Sample 'type' and intended 'use' assignments and trends

A total of fourteen sample types were identified ranging from 'freshwater' (n = 376 articles, 62% of articles) and 'seawater' (n = 112) to 'microcosm', 'shellfish' and 'biofilm' (each n < 10, Figure 2.5). The most common combinations were 'freshwater' with 'sewage (n = 57), with 'faecal matter' (n = 54) and with 'sediments and sand' (n = 51, Figure 2.5), respectively. Of the 793 reported sample types, where an intended use would potentially be relevant (i.e., all water types, 'sewage' and 'sediment and sand'), the intended use was reported for 270 sample types, representing 240 articles. 'Drinking' and 'recreational' water were the most frequently described, accounting for 124 and 103 occurrences, respectively. 'Irrigation' and 'shellfish-growing' were seldom studied (n = 21, n = 14 occurrences, Figure 2.6).

2.4.2.4 'Data analysis approach' assignment and trends

While 159 articles (26%) only report summary statistics (qualitative and quantitative), the majority report more sophisticated data analysis approaches such as correlation analyses, hypothesis tests or simple bioinformatics (n = 201, 33%) or multivariate statistics or advanced bioinformatics (n = 165, 27%). QMRA or microbial fate and transport modelling was found to be done only by a small portion of the articles (n = 28, 5%, Figure 2.7).

2.4.2.5 'Application' type assignment and trends

Seven genetic method application areas were identified in this systematic literature review (Figure 2.8). Besides the detection of total faecal pollution using general faecal indicators ('*Application 1*', 84 articles), MST was the predominant use of genetic faecal markers ('*Application 2*' and '*Application 3*', altogether 341 articles). Most of these studies performed MST in the classical sense, investigating several potential sources ('*Application 3*', 222 articles), while 119 articles target just one source type, mainly human ('*Application 2*'). In a much smaller extent, genetic faecal markers were found in performance assessments of (waste)water treatment and in studies of microorganism fate and transport in groundwater as transport surrogates ('*Application 4*', 33 articles). An equally small, but emerging field is health and infection risk assessment where genetic methods were found to be employed as risk indicators, or as a support in selected steps of quantitative microbial risk assessment (QMRA, '*Application 5*', 25 articles). Host-associated faecal indicators were also used to trace the origin of waterborne outbreaks, to elucidate pathogen transmission routes as well as to support the interpretation of SARS-CoV-2 wastewater surveillance data ('*Application 6*', 23 articles). Apart from these core application areas, genetic faecal pollution diagnostics were also found to support other scientific disciplines, such as the tracking of the source of nutrients or antibiotic resistance genes as well as archaeology. The section '*Application 7*' provides an overview of these additional areas (105 articles).

2.5 In-depth review of the application areas of genetic faecal pollution diagnostic through case studies

The following sections demonstrate the successful implementation of genetic faecal pollution diagnostics in the identified seven application areas of water quality research (Figure 2.9). To do so, trend analyses of select study elements for a given application area are presented at the beginning of each section, followed by an illustration of these findings through a collection of cutting-edge case studies.

2.5.1 Application 1: Detection of total faecal pollution

In general, there are two approaches to detect faecal pollution using genetic methods, and the 84 articles in this application category can be divided along these lines, with just a small overlap: (i) the targeted detection of traditional or new general faecal markers, mostly using qPCR (for definitions, see section *'Systematic analysis of the 'application studies'*, n = 34 articles); (ii) the non-targeted detection of faeces-related taxa using HTS (n = 44);

and (iii) six articles measuring both. 'Traditional general faecal markers' were used more often than 'new general faecal markers' (n = 36 and n = 8 articles, respectively). In most instances, 'traditional general faecal markers' were measured in parallel with the corresponding 'culture-based FIO' parameter (27 of 36 articles). The dominant method for community composition analysis was 16S AmpSeq (42 articles). 'Freshwater', 'seawater' and 'sediments and sand' were the most common sample types while 'recreational' and 'drinking' were the most frequently observed intended use types.

2.5.1.1 Targeted detection of general faecal indicators

Regulatory agencies, such as the U.S. Environmental Protection Agency (USEPA) have begun to capitalize on the potential of qPCR as a rapid monitoring solution for recreational waters, providing same-day results (< 4 hours). In 2012, water quality beach action values for qPCR measurements of enterococci were included in the U.S. Recreational Water Quality Criteria (USEPA 2012a). This addition was based upon epidemiological studies conducted at freshwater and marine beaches that provided evidence that enterococci levels measured by qPCR are predictive for swimmer-related illness ((Wade et al. 2008, Wade et al. 2010), see details in Section *'Application 5'*).

Since then, enterococci qPCR (USEPA Method 1611 and 1609.1) has been applied in several beach monitoring demonstration and implementation programs (Byappanahalli et al. 2018, Dorevitch et al. 2017, Ferretti et al. 2013). In one of the largest studies, nine Chicago beaches were monitored over the course of 894 beach-days in 2015 and 2016, resulting in 1,796 water samples that were analysed by enterococci qPCR while maintaining standard *E. coli* culture testing, which is typically used at the Great Lakes (Dorevitch et al. 2017). Side-by-side comparison of the two approaches showed that enterococci qPCR beach action values were exceeded 3.4 times less frequently than *E. coli* culture beach action values (6.6% vs. 22.6% of beach-days) (Dorevitch et al. 2017). However, generalisations – such as qPCR testing necessarily leads to fewer beach action value exceedances than cultivation-based testing – cannot be made. Several prior studies have found varying levels of agreement between *E. coli* culture and enterococci qPCR beach action value exceedances (Byappanahalli et al. 2018, Haugland et al. 2014). Moreover, data analysis of this large multi-beach, multi-year evaluation study found that prior-day *E. coli* culture results are no better than chance alone at predicting current-day water quality at Chicago beaches (Dorevitch et al. 2017). Based upon these findings, enterococci qPCR testing was expanded by the local authority at up to 20 Lake Michigan beach locations from 2017 onwards and *E. coli* cultivation-based testing was discontinued (Shrestha and Dorevitch 2020).

More recently, the USEPA has developed a draft standard method for qPCR testing of *E. coli* ('Draft Method C', (Sivaganesan et al. 2019)) driven by the need for rapid *E. coli* testing. In a large-scale method comparison effort, data from 101 Michigan (USA) recreational beaches from more than 6,000 samples showed a 91.5% agreement in beach notification outcomes between the cultivation-based standard of 300 MPN or CFU/100 mL and a putative threshold of 1.863 $\log_{10}$ gene copies/reaction, estimated in this study (Haugland et al. 2021). A strong correlation was observed between culture and qPCR results, with a Pearson R-squared value of 0.641 on the pooled data of the 39 sites passing the data eligibility criteria (sample n = 2,092) (Haugland et al. 2021).

The universal *Bacteroidales* marker BacUni, a new general faecal marker, was evaluated together with three cultivation-based FIOs as a predictor of protozoan and bacterial pathogens in samples from rivers and estuaries in California, USA (Schriewer et al. 2010). The universal *Bacteroidales* marker was detected in all water samples in concentrations 2 orders of magnitude higher than cultivation-based FIOs. The results also showed the universal *Bacteroidales* marker to have a comparable or higher mean predictive potential than cultivation-based FIOs (Schriewer et al. 2010). The high abundance of new general faecal markers is certainly an asset, as sensitivity can become a challenging aspect for genetic faecal pollution detection in water resources with low faecal pollution levels (for details, see Section *'Sensitivity of environmental detection of nucleic acid targets'* in the *'Discussion'*).

2.5.1.2 Non-targeted detection of faeces-related taxa using high-throughput sequencing

HTS approaches have emerged in microbial water quality monitoring allowing for new opportunities. From a public health perspective, HTS surveys have shown to identify faecal taxa (e.g. *Bacteroides*) in aquatic microbial communities (Ulrich et al. 2016b, Vadde et al. 2019). For instance, Ulrich et al. (2016b) tracked changes in bacterial community composition in a riverine system during and after Superstorm Sandy (a 100-year storm event in 2012) using HTS and traditional cultivation-based faecal indicator testing. Bioinformatic analyses of 16S AmpSeq data showed a drastic restructuring of the bacterial community, associated with hydrological dynamics. The relative abundances of sequences matching faecal bacteria (*Bacteroides*, *Clostridium*, *Blautia* genera) and potentially pathogenic populations (*Campylobacter*, *Helicobacter*) were observed to increase after the peak of the storm (Ulrich et al. 2016b). Given that HTS applications can provide profiles on microbial communities and information on faeces-associated taxa, such genetic approaches may become useful as a screening tool in the future for identifying potential health risks and for prioritizing sites for follow-up analysis of water samples using targeted quantitative PCR approaches (Jiang et al. 2020, Vadde et al. 2019).

2.5.2 Application 2: MST of faecal pollution from a single source type

Faecal pollution may originate from a multitude of point and non-point sources. The need to identify the source of faecal pollutions rose years ago and since then many different approaches have been developed and validated (Section *'Background information on genetic targets and methods: a historical overview'*). Focussing the investigation on a single type of faecal source often happens (i) if there is some evidence regarding the dominant source of pollution so neglecting other ones is acceptable or (ii) the investigation specifically addresses one source type, because, for example, some faecal sources could represent a higher public health risk than others. In any case, there is the need to validate the hypothesis of the origin of contamination using a reliable analytical tool, since having scientific evidence facilitates posterior effective measures.

Out of the 119 articles in this application area, the single source was human in 107 cases and just a handful of articles focused on non-human sources such as ruminants, gulls, ducks, chicken, or dogs. The majority, 73% of the articles combine 'MST markers' with the measurement of traditional 'culture-based FIOs'. Other parameter classes that often appeared were 'pathogens', 'traditional general faecal markers', 'physicochemistry and nutrients' and 'chemical tracers' (n = 29 to n = 14 articles). Freshwater was most often sampled (n = 75 articles), followed by seawater (n = 23) and sewage (n = 18). Forty-two articles reported 'summary statistics' (qualitative or quantitative), while 44 articles performed 'correlations, hypothesis testing or simple bioinformatics'. A smaller set of articles did more advanced data analyses, such as 'multivariate statistics or advanced bioinformatics' (n = 22) or 'QMRA and fate and transport modelling' (n = 4).

2.5.2.1 Human sources: decentralised wastewater systems

The interpretation of MST results is greatly enhanced by cultivation-based FIO and land-use data or additional parameters that can help to explain the origin and fate and transport of a specific pollution source. For example, in watersheds with more than 1,621 septic systems in Michigan, USA, higher concentrations of *Bacteroides thetaiotaomicron* (human-associated marker) were detected under baseflow conditions suggesting that control measures should include septic system maintenance and construction in the area (Verhougstraete et al. 2015). In this study, analyses were performed using a classification regression tree including riparian buffers, septic tanks, and physicochemical data. Beyond chronic pollution scenarios, rainfall events can impair water quality through combined sewer overflows, septic tanks seepages, agricultural run-offs or other events governed by precipitation. A similar study found that three human-associated *Bacteroides* markers correlated positively with septic tank density during wet weather suggesting them as a significant source (Peed et al. 2011). Since there was no correlation with FIO during baseflow conditions, the authors postulate that other sources might be implicated in the chronic pollution.

2.5.2.2 Human sources: centralised wastewater systems

In some cases, genetic MST markers can be combined with other types of tracers to give strength to the interpretations and to overcome markers' limitations, like low specificity, differing decay rates or different transport. For example, the detection of the human-associated genetic marker HF183 and optical brighteners in private drinking water supplies in rural areas of Virginia, USA, showed sewage as a potential pollution source. However, few samples showed *E. coli* together with the optical brighteners what may be explained for a different fate and transport of these indicators within the aquifer (Smith et al. 2014). In Montreal, Canada, a study applied a multiparameter source tracking toolbox combining chemical source tracking markers for sewage (caffeine, theophylline, and carbamazepine) together with the human-associated genetic markers HF183 and mitochondrial DNA to detect illicit wastewater discharges into storm sewers during dry weather (Hachad et al. 2022). The authors used a composite index of the different markers together with the levels of *E. coli* to identify household cross connections or indirect illicit discharges and verified them successfully with dye tracing.

Hydrological and meteorological data is often indispensable to understand the fate of faecal microorganisms in the environment. For example, hydrological and meteorological data combined with the human-associated marker HMBif, cultivation-based MST parameters and FIO allowed modelling the self-depuration distance of a small Mediterranean river (Pascual-Benito et al. 2020). The obtained models gave information about the recuperation of the river initial conditions after receiving treated sewage discharge. MST tools are also useful after extreme meteorological events. For example, after hurricane Harvey, the detection of HF183 and BacHum and their correlation with FIO indicated a big input of sewage through sewage overflows and stormwater in two catchments in Texas, USA (Kapoor et al. 2018).

HTS applications have also been reported. After the pioneering work of Unno et al. (2010) in South Korea, the study by Newton et al. (2013) was one of the first large-scale studies that also demonstrated the complex challenges in data interpretation. They faced chronic human faecal pollution at an urban site at Lake Michigan, USA, and set out to identify its sources and delivery routes. Through identifying the relative abundance of sewer infrastructure-associated, faecal and human faecal signatures in lake water samples, they identified combined sewer overflows as the dominant pollution source during heavy rainfall events, whereas nonhuman faecal sources exhibited the highest relative abundance during dry weather and non-combined sewer overflow producing rain events. More recently, Zimmer-Faust et al. (2021) tracked the plume of a wastewater treatment plant (WWTP) outfall in the coastal Pacific ocean on the USA/Mexico border and showed its behaviour differs depending on oceanic and meteorological conditions. They used a human-associated MST marker and 16S AmpSeq together with the algorithm SourceTracker, with pristine marine water, WWTP discharge and a nearby river as potential sources to derive the spatial extent and concentration gradient of human pollution.

2.5.2.3 Recreational waters

Coastal waters have an important value for leisure, tourism, and for the coastal ecosystem including shellfish harvesting areas, therefore MST tools have been extensively tested in these areas (González-Fernández et al. 2021, Korajkic et al. 2009). In Thailand, Kongprajug et al. (2021) used two genetic viral MST markers: crAssphage and HPyV at various beaches during dry and wet seasons to verify human waste practices as the main faecal source. Their results reported temporal variability but not spatial, recommending a future monitoring strategy based on a more frequent sampling at a unique sentinel site. Other studies include environmental data like precipitation and solar radiation, oceanographic data like tides and currents, and use correlations or more complex models to be able to predict a potential pattern. For example, at different sites of San Francisco, USA, the human-associated marker HF183 was related with different parameters, mainly 72 h precipitation, but also water temperature, tides or insolation (Jennings et al. 2018). Cao et al. (2018) sought to develop a standardised data analysis approach that incorporates all qPCR measurements from a defined group of samples (i.e., detections, non-detections, and measurements in the range of quantification) to assess average human faecal pollution levels at recreational water sites. The authors proposed a metric, the human faecal score, that combines the HF183/BacR287 qPCR

results with a defined sampling strategy (sampling intensity and number of replicates) and a Bayesian weighted average approach. The score can be used to prioritize sites for remediation and has more recently been used to compare source-associated impacts under wet and dry conditions (Shrestha et al. 2020) and identify trends with cultured FIO paired measurements (Li et al. 2021b). Besides human sources, wild animals can also contribute to faecal indicator bacterial loads in coastal areas with big gull colonies. The presence of the gull-associated bacterium *Catellicoccus marimammalium* in 58% of the water samples and at all sampling sites as well as their correlation with faecal indicators suggested a chronic contribution of gull faeces in the water quality in southern Ontario, USA (Lu et al. 2011). The same marker showed a decrease together with faecal indicators and bacterial pathogens after gull removal in Lake Michigan, USA (Converse et al. 2012).

2.5.2.4 Rural areas, domestic animals

Single source characterization is also relevant in rural areas with a high agricultural pressure where tracking animals such as swine, ruminant or poultry can be of interest (Heaney et al. 2015, Weidhaas et al. 2011, Wiesner-Friedman et al. 2021). These studies include, additionally to the relevant genetic faecal marker, data on land uses, land-applied manure and/or animal feeding operations. For example, after testing for a ruminant-associated marker, BoBac, and including data of animal feeding operations, authors found out that applying manure in the fields implied an increase of faecal indictors in riverbed sediments (Wiesner-Friedman et al. 2021).

2.5.3 Application 3: MST of faecal pollution from multiple sources

Many impaired water bodies are polluted by more than one source. Thus, it is important to characterize key sources because the corresponding health risk as well as the mitigation steps may be different by source. Nevertheless, study design and choice of methods are highly dependent on the water resource type, the intended water use and other factors.

Of the 222 articles with a focus on multi-source MST, MST was achieved predominantly using MST markers (n = 175, 79% of articles) followed by classical library-based MST (n = 32, mostly published before 2015) or HTS (n = 21, mostly published after 2015). In multi-source MST articles, FIO are measured predominantly with culture-based methods (n = 161 articles). In contrast, traditional and new genetic faecal markers played a minor role (n = 16 and n = 18, respectively). The most common parameter combination was MST markers with culture-based FIO (n = 130, 59% of articles). Other common parameter classes were 'physicochemistry and nutrients', pathogens, meteorology and land use (n = 48 to n = 24 articles). The proportion of articles with four or more parameter classes was higher than in single-source MST (31% in multi-source MST and 26% in single-source MST, *'Application 2'*). This higher study design complexity was reflected in the data analysis approach: 35% of articles performed multivariate statistics or advanced bioinformatics (18% in single-source MST, *'Application 2')*.

2.5.3.1 Elevated pollution levels on a watershed scale

The starting point in watershed studies usually is elevated levels of cultivation-based FIO levels in rivers, lakes or coastal waters. Often the spatial scale is relatively large and there are multiple potential sources ranging from human faeces (via leaky infrastructure, treated or untreated wastewater or combined sewer overflows) to livestock (grazing or stabled), pets as well as avian and mammalian wildlife. Often there is limited knowledge on hydrology, meteorology and land-use. An illustrative example is given by three studies conducted over a span of 16 years in the Tillamook Bay catchment in Oregon, USA demonstrating how state-of-the-art genetic MST applications have evolved over time. Bernhard et al. (2003) and Shanks et al. (2006) compared PCR-based ruminant and human marker frequencies with faecal pollution levels considering rainfall patterns and seasonal pollution dynamics to identify pollution sources. Much more recently, Li et al. (2019) used quality controlled and in several cases standardized qPCR assays for five faecal sources, high resolution GIS for land-use and meteorological data to not only identify but also quantify and locate pollution sources and patterns in order to guide remediation efforts and risk assessment. In a similar approach Bushon et al. (2017) ranked tributaries to the

Little Blue River catchment in Missouri, USA, based on estimated contributions to water quality impairment. The studies by Nguyen et al. (2018) and Yamahara et al. (2020) demonstrate how hypothesis-formulation can support study design for genetic faecal pollution diagnostics. Both studies also try to shed light on the potentially confounding role that soil and sediments might have on MST applications, especially in tropical waters. In order to elucidate the relative roles of human and other animal sources polluting the Danube River and its tributaries, Kirschner et al. (2017) used a combination of longitudinal survey along more than 2,500 km of river and a temporal survey over the course of a year at three sites successfully identifying human waste as the dominant source. Bambic et al. (2015) encountered difficulties segregating pollution sources due to the confounding influence of disinfected municipal wastewater. Separating wet from dry weather based on meteorological data allowed data interpretation, with municipal wastewater (human) being the dominant dry-weather pollution source, while during wet weather, agricultural runoff, and stormwater (ruminant and dog) dominate. Using bacterial and viral markers allowed the authors to demonstrate the difficulty to detect the presence of viral pathogens when only using bacterial indicators. The authors used cutting edge data handling methods, including statistical methods to account for the large proportion of non-detects, and an estimation of spatial and temporal variations of same-host contribution using ratios between given *Bacteroidales* MST markers and a general *Bacteroidales* marker (Bambic et al. 2015). Separating the sample set into dry and wet periods allowed Liang et al. (2021) to reveal differing pollution pathways. Results of MST markers agreed with those from 16S AmpSeq and the FEAST algorithm: human as main pollution source in the dry season and ruminant and swine in the wet season at this river site near Beijing, China. MST methods have also been used to more generally identify factors and features that promote or reduce watershed faecal pollution rather than just identifying pollution sources. As an example, Green et al. (2021) used MST and cultivation-based FIO in an investigation of 68 streams in New York State, USA, to identify stream features, land use practices and meteorological patterns that drive faecal pollution levels from multiple sources.

2.5.3.2 Recreational waters

In contrast to general watershed pollution scenarios, bathing water studies are usually triggered by persistently elevated FIO levels at public beaches directly threatening public health of visitors, necessitating beach closures and inflicting considerable economic damage. Study areas are often smaller and the potential sources less diverse (e.g. sewage discharges, birds and pets) (Staley et al. 2018). Prudently, studies often make efforts to consider the influence of hydrology (flows, tides, etc.) and the effect of precipitation and solar radiation on water quality changes and to resolve faecal source contributions (Williams et al. 2022). In a proof of concept study in Xiamen, China, An et al. (2020) used high-throughput qPCR for a large number of assays targeting multiple faecal sources and pathogens to investigate bathing waters.

2.5.3.3 Drinking water

Impairment of drinking water quality is one of the most pressing issues worldwide. The specific challenge in this application field is that low levels of pollution already pose relevant health risks. For example, elevated FIO levels observed in karst and fractured aquifers after precipitation were the starting point for several MST studies. The problem of highly variable pollution dynamics in the course of very short time periods can be tackled by linking sampling to hydrological dynamics (Reischer et al. 2008) and nested sampling with higher sampling frequencies during periods of hydrological fluctuations and during/after rainfall events (Reischer et al. 2011). The very short residence times of faecal pollution in the studied springs also allowed direct source apportionment based on MST marker concentrations in spring water because differential persistence can be disregarded when measuring very recent pollution. In order to determine the source and risk factors for nitrate and microbial pollution in private dolomite karst wells, Borchardt et al. (2021) used multivariate regression models with potential drivers such as land use, precipitation, hydrogeology and well construction.

2.5.3.4 Aquaculture and irrigation water

Shellfish harvesting areas in coastal waters and aquaculture in general also are under a large amount of anthropogenic pressure often resulting in contamination of products with FIOs and pathogens. The applicability of MST approaches to identify and prioritize pollution sources has been demonstrated in shellfish harvesting waters and products such as oysters (Mieszkin et al. 2013). Klase et al. (2019) integrated MST markers, antibiotic resistance gene (ARG) assays and pathogen detection with bacterial community-based analysis to broadly investigate the potential public health risks associated with pollution of fishponds. Similarly, faecal pollution levels, ARG genes and pathogen occurrence was investigated in irrigation waters used for fresh produce to determine sources of pollution and risk factors (Weller et al. 2020).

2.5.4 Application 4: Characterisation of microorganism attenuation during treatment processes

Pathogen removal is one of the primary functions of wastewater and drinking water treatment. However, relying on direct pathogen determination only is not practicable due to the low and varying concentrations in raw water as well as the high number of different pathogens potentially occurring. Thus, treatment performance assessment often relies on treatment indicators used as representative surrogates for pathogen removal (see Glossary, (Momba et al. 2019). While cultivation-based microbial parameters are the most commonly employed treatment indicators (Jofre et al. 2016, Momba et al. 2019), the systematic literature review revealed 33 articles that used genetic markers as treatment indicators. In this article pool, 'MST markers' and 'traditional general faecal markers' were the most often measured genetic parameter 'class' types (15 and 13 articles), whereas 'cultivation-based FIO' and 'pathogens' were the most common other parameter classes (18 and 10 articles). As for the treatment type, 26 articles dealt with *engineered treatment processes,* with the majority, 16 studies, focussing on wastewater treatment. The various steps of drinking water treatment, as well as stormwater and greywater treatment were the topics of the other 10 articles. The *attenuation of microorganisms during groundwater transport* was the focus of seven studies. Five of these involved natural tracers, and two, injected tracers. Riverbank filtration, managed aquifer recharge and the drinking water treatment step slow sand filtration were found to be the main processes studied. Investigations of microorganism attenuation express changes of treatment indicator concentration during a treatment step as percentage reduction or as $\log_{10}$ reduction values (LRV, the difference of $\log_{10}$-transformed concentrations before and after the treatment step) (Momba et al. 2019).

In summary, the identified studies using genetic faecal pollution diagnostics, as representatively shown below, focus on nucleic acid target concentration changes, as an indication for cell- and particle number decrease during biological wastewater treatment or aquifer transport. Important to highlight, investigating water treatment processes also needs to determine disinfection efficacies by characterising the microbicidal and virucidal effect on FIO and pathogens. The challenges and possibilities to determine such effects by genetic investigation methods, and their few application studies so far, will be considered in the Discussion (Section *'Direct detection of nucleic acids: characteristics & challenges'*).

2.5.4.1 Evaluating microorganism removal during engineered treatment processes

For the characterisation of the removal of pathogenic viruses through wastewater treatment, viral qPCR MST markers offer some advantages over traditional indicator viruses such as phages. The most important aspect of qPCR MST markers is that their concentrations in untreated wastewater are expected to be far greater than viral pathogens (Hughes et al. 2017, Kitajima et al. 2018). This is particularly important because an indicator whose concentration is high can be detected consistently and more easily in different stages of treatment processes. The concentrations of coliphages in wastewater were found to be 7-$\log_{10}$ PFU/L, while the concentrations of enteric viruses such as human adenovirus and human polyomaviruses are variable and reported to be on the scale of 6 to 9-$\log_{10}$ copies/L (reviewed in (Ahmed et al. 2020)). Several studies have reported high numbers of PMMoV, crAssphage, *Bacteroides* (HF183) and *Lachnospiraceae* (Lachno3) and other qPCR MST markers in untreated

wastewater (Ahmed et al. 2019, Ahmed et al. 2018, Hughes et al. 2017, Rosario et al. 2009). Furthermore, qPCR MST markers show little variations in untreated wastewater and the concentrations range between 8-10 $\log_{10}$ copies/L (Ahmed et al. 2019, Hughes et al. 2017).

Several studies determined the log reduction values of MST qPCR markers such as crAssphage and PMMoV in full-scale wastewater treatment plants (reviewed in (Ahmed et al. 2020, Sabar et al. 2022)). For example, Hamza et al. (2011) reported approximately 3-$\log_{10}$ reduction of PMMoV in a conventional activated sludge treatment plant in Germany, which were similar to the reduction of polyomavirus and torquetenovirus. Hughes et al. (2017) reported approximately 1.1 -$\log_{10}$ reduction of PMMoV in an activated sludge WWTP which was less than those of HAdV and HPyV but similar to norovirus and enterovirus. Similar log reduction value of PMMoV was reported by Kuroda and colleagues (2015) in a WWTP in Vietnam. Schmitz et al. (2016) reported < 1-$\log_{10}$ reduction of PMMoV during activated sludge and biological trickling filter and the reduction rate was similar to aichivirus, norovirus, sapovirus, adenovirus, and polyomavirus. Based on the log reduction values reported in the literature PMMoV appears to be a conservative viral indicator for the reduction of pathogenic viruses in wastewater treatment plants. Several studies reported the reduction of crAssphage "the most abundant [known] virus" in the human gut in WWTPs with activated sludge. Tandukar et al. (2020) reported a log reduction of 3.3 $\log_{10}$, while Farkas et al. (2018) reported 1.0 to 2.0 $\log_{10}$ reduction.

Asami and colleagues (2016) determined the $\log_{10}$ reduction of PMMoV and JC polyomavirus for coagulation-sedimentation and rapid sand filtration processes in a drinking water treatment plant (DWTP) in Bangkok, Thailand using qPCR. The observed removal efficiencies varied depending on treatment step, season, and raw water quality, with LRVs ranging between 0.4 and 1.6 for PMMoV and between 0.5 and 1.9 for JC polyomavirus.

2.5.4.2 Evaluating microorganism attenuation in groundwater

Pathogen removal during subsurface passage may be studied by investigating infiltrated faecal pollution (e.g., managed aquifer recharge), and monitoring the removal of pathogenic or indicator microorganisms. One way to investigate pathogen removal is to analyse water samples for naturally present microorganisms along a transect. Another way is with tracer tests using an injected target microorganism or surrogates. This can be done either as a laboratory experiment, using columns packed with aquifer material, or in the field.

The vast majority of such transport studies quantify microbial targets with microscopy or cultivation-based methods. Using genetic tools to quantify surrogate or pathogenic organisms (i.e., bacteriophages and enteric viruses) for groundwater transport studies is a relatively novel application of this technology, and therefore, limited literature exists. They allow innovative analyses such as the quantification of multiple microorganism co-transport using multiplex qPCR and differentiating between infectious and inactivated viruses, when qPCR is used together with culture techniques (Bellou et al. 2015, Betancourt et al. 2014, Wang et al. 2022). In addition, genetic methods are a reliable way to enumerate microorganisms attached to particles, such as sediment and microplastics (Hassard et al. 2016). Genetic tools can also be used to confirm possible false negatives derived from microscopy or cultivation-based methods. This is especially useful as field tests are often expensive, labour intensive, and practical (small) sampling volumes often yield negative results. Low concentrations of target organisms require sampling larger volumes, which often presents additional challenges (Forés et al. 2022, Haramoto et al. 2018).

Natural tracers. Managed aquifer recharge involves natural subsurface processes to treat intentionally infiltrated surface water or wastewater effluent. In a study of the treatment efficiencies of three such systems in the USA, (Betancourt et al. 2014) measured viral pathogens and PMMoV, a human-associated viral marker, by qPCR in the infiltrated water and in a series of wells, providing the log reduction rates over given distances. Near the highly polluted Rocha River in Bolivia, surface water and riverbank filtrate are often used for irrigation, another example of indirect wastewater reuse (Verbyla et al. 2016). The removal (log reduction) during riverbank filtration was

assessed for this study using reference pathogens recommended for wastewater reuse, PMMoV, as well as a human-associated bacterial indicator, and a QMRA of the consumption of the irrigated lettuce was done.

Injected tracers. In the case the aim is to study the transport of a pathogenic microorganisms, in field tests a surrogate is often used as a tracer, that mimics the pathogen in size and surface characteristics, while die-off rates are determined separately using batch tests. The transport of the surrogate can be compared to the pathogenic microorganism in small column tests in the laboratory, using aquifer material, while the surrogate is injected or applied at a field site. In this way, it is possible to upscale the transport of dangerous substances using transport models. With this goal in mind, Stevenson et al. (2015) used qPCR to quantify the transport and removal of HAdV and its surrogate, PRD1 phages, in small column tests.. In regards to water treatment, the removal of *Cryptosporidium parvum*, and its surrogate *Clostridium perfringens*, by slow sand filtration was evaluated by Hijnen et al. (2007), as the last step in drinking water treatment using water taken from the River Rhine and spiked with the microorganisms. *C. perfringens* was enumerated using cultivation, and the colonies identified with PCR. Bauer et al. (2011) used qPCR to analyse enteric adenoviruses to evaluate the efficiency of slow sand filtration (SSF) and RBF as drinking water treatment steps. Wang et al. (2022) investigated the transport of MS2 phages, a surrogate for enteric viruses, from a surface water pond to groundwater via riverbank filtration. The authors differentiated between infectious phages by plaque assay versus the total number of phages detected by qPCR.

Synthetic tracers. A unique application of genetic tools is using synthetic DNA as a tracer which can be used as multipoint tracers thanks to the practically unlimited sequence options and their specific quantification using qPCR (Dahlke et al. 2015, Pang et al. 2022). Another innovative idea is the use of DNA-labelled microspheres as surrogates for pathogenic microorganisms (Pang et al. 2014). This enables the enumeration of the pathogen and its surrogate by the same analytical procedure, qPCR, allowing a more direct comparability.

2.5.5 *Application 5: Estimation of infection and health risk*

Genetic faecal pollution diagnostics is increasingly applied to support infection- and health risk estimation regarding human usage of water and water resources. The range of applications is very broad, and includes guidance in hazard identification (e.g., selection of reference pathogen selection), calibration of fate, transport and QMRA models targeted to specific sources, and the genetic detection of risk indicators and markers, as alternative to cultivation-based enumeration techniques.

The study design analysis found 25 articles that estimate health risk by the support of genetic faecal pollution diagnostics: seven epidemiology studies at recreational water sites and 18 QMRA studies, most of which were conducted at recreational waters, four focussed on drinking water and one on irrigation water. The epidemiology studies compare traditional general faecal markers with illness rate, while the QMRA studies apply MST markers to QMRA, using one of the above approaches. The most prominent genetic faecal pollution diagnostics application is qPCR quantification of traditional general faecal markers and MST markers. The relevance of obtaining information on the viability- or infectious status for such types of applications by genetic faecal pollution diagnostics is further addressed in the discussion section (Section *'Direct detection of nucleic acids: characteristics and challenges'*).

2.5.5.1 *Guidance in hazard identification for QMRA*

Host-associated faecal marker quantification in water resources can guide reference pathogen selection for QMRA. This concept has been included into the framework of integrated faecal pollution analysis and management ("3-step approach") of karstic drinking water resources (Farnleitner et al. 2018, Savio et al. 2018). The three steps involve (1) catchment pollution source profiling, (2) monitoring of general faecal pollution, and finally, (3) hypothesis-guided qPCR MST marker enumeration in spring water. At a large, complex and hardly accessible alpine karstic spring water catchment with importance for public water supply in Austria, the results pointed at zoonotic pathogens from ruminants, including cattle as the QMRA reference targets to prioritize in

(Reischer et al. 2011, Savio et al. 2018). The approach introduced by Farnleitner et al. (2018) was later extended to urban river catchments using probabilistic modelling to simulate the occurrence and extent of faecal pollution sources in parallel with zoonotic pathogens from direct human as well as indirect livestock and wildlife faecal pollution sources (Derx et al. 2023). The probabilistic estimates from the catchments and the direct measurements in the river indicated that combined sewer overflows and communal wastewater treatment plants were the largest contributors to faecal pollution at the studied site. The developed approach indicated to be a robust basis for further microbial fate and transport models and the realisation of QMRA (Derx et al. 2023).

MST qPCR maker analysis was also used to associate cases of human illness predicted by QMRA with bovine, human or unknown sources in contaminated private wells in Wisconsin, USA. Although some of the cases of illness were indicated to be of human pollution origin, the results suggested that most of the cases were caused by bovine faecal pollution. This outcome had important implications for land use and water safety and health risk management of the fractured aquifers (Burch et al. 2021). In a study in the Netherlands, MST qPCR marker analysis was applied to trace back the origin of infection risks from *Campylobacter sp.* at a stormwater collection site (water plaza). The presence of human MST markers indicated a cross-connection with the combined sewer system (Sales-Ortells and Medema 2015).

2.5.5.2 Calibration of catchment models to estimate pathogen concentrations for QMRA

Genetic faecal marker quantification also proved valuable for catchment-based QMRA modelling of faecal pollution sources. One of the principles of the "QMRAcatch" philosophy is the catchment-specific calibration of microbial transport (i.e., dilution, advection, dispersion) and fate (i.e., decay/persistence) models for specific faecal pollution sources by the use of MST markers. The calibrated and verified models can be used to derive pollution and management scenarios for given points of interest (e.g., drinking water abstraction sites) based on pathogen transport/fate simulations. Reference pathogens are quantified in the pollution sources or derived from epidemiological data and literature (Derx et al. 2016, Schijven et al. 2015).

In a scenario analysis considering river water as raw water source for drinking water production, the authors calibrated QMRAcatch for human faecal pollution pathways, such as from communal wastewater disposal, using human-associated MST qPCR marker data for the Austrian section of the Danube River (Demeter et al. 2021). By use of a conceptual semi-distributed hydrological model and regional climate model outputs, the authors simulated the interplay of future changes (e.g. climate change, population) and wastewater management measures (enhanced WWTP treatment, prevention of combined sewer overflows) on the infection risks for viral and bacterial reference pathogens (Demeter et al. 2021). The study demonstrated that the degree to which future changes affect drinking water safety strongly depends on the type and magnitude of faecal pollution sources and are thus highly site- and scenario-specific.

More recently, the modelling approach was extended towards source-specific calibration to multiple faecal pollution sources, using MST marker quantification for human, ruminants, pigs, and birds. An improved hydrological module (2-D hydrodynamic flow, rainfall-runoff, differential MST decay) allowed comparing external (allochthonous) an internal (autochthonous) faecal pollution sources and their associated infection risks from zoonotic parasites (*Giardia*, *Cryptosporidium*) for the Danube River (human waste water input) and its floodplains (animal sources) downstream of Vienna (Derx et al. 2021). For best management practices, autochthonous and allochthonous faecal sources during flood and rainfall events contributed pathogen loads in similar orders of magnitude.

2.5.5.3 Infection and health risk indicator role trough epidemiological studies

The traditional way of recreational water quality monitoring of surface waters has been based on the application of cultivation-based FIO. For example, the relative risk of illness for swimmers and non-swimmers in recreational

waters was estimated based on cultivation-based enterococci levels (USEPA, 1986). However, a recent revision of these guidelines in 2012 ("NEEAR study") reported that qPCR measurements of general enterococci concentrations are better predictors of the rate of gastrointestinal (GI) illness among swimmers in recreational waters compared to cultivation-based enterococci levels (USEPA 2012a). This study established a combined approach, using cultivation-based *E. coli* enumeration (beach action value of 235 CFU per 100 mL of water) and genetic enterococci qPCR quantification (beach action value of 1,000 calibrator cell equivalents per 100 mL) with a health-based compliance target of 36 cases of GI illnesses per 1,000 swimmers (USEPA 2012a).

MST marker quantification by qPCR has also been incorporated in epidemiological studies. For example, Griffith et al. (2016) applied several bacterial and viral indicators to predict gastrointestinal illness in three Californian beaches (n = 10,785 swimmers) by comparing qPCR and cultivation-based methods. At one beach, human-associated genetic MST marker levels revealed highest associations with GI. The authors concluded that performance of a selected parameter is likely site-specific. Napier et al. (2017) conducted a prospective cohort GI study also using human-associated genetic MST markers in water (self-reported illness among 12,060 swimmers; six beaches across USA). Inconsistent associations were noted between results, however, the authors concluded that qPCR MST marker data may be useful in assessing human health risks in recreational water bodies.

2.5.5.4 Infection and health risk indicator role through indicator to pathogen ratio and QMRA

An increasing number of studies attempt to establish a link between genetic MST marker concentrations and infection risks in recreational waters using a QMRA modelling framework. One of the first studies of this type were conducted to estimate the risk of GI illness for adults swimming in waters contaminated with untreated sewage (Staley et al. 2012). In this study, norovirus was selected as the reference pathogen. The HF183 marker was detected in sewage dilutions indicating GI risks greater than or equal to the benchmark value of 10/1000 primary contact recreators in several sampling sites based on the 1986 Ambient Water Quality Criteria (USEPA, 1986). Boehm et al. (2015) established a relationship between concentrations of human-associated HF183 and HumM2 qPCR markers and GI risk of swimmers in recreational waters using a QMRA approach. The authors noted that the benchmark GI illness rate of 30/1000 primary contact recreators occurred when the median concentrations of HF183 and HumM2 marker genes were 4,200 and 2,800 GC/100 mL of water, respectively. In a subsequent study, Boehm et al. (2018) incorporated the decay of both human faecal-associated markers and norovirus in the dose-response model to determine risk associated with scenarios when the age of contamination is unknown or water is contaminated by fresh untreated sewage. When an untreated sewage contamination scenario was considered, the risk-based threshold was ~9,700 GC/100 mL. The analysis suggested that a risk-based threshold of 4,100 GC/100 mL is warranted for the HF183 marker gene when the age of contamination is unknown. Schoen et al. (2020) modelled risk-based threshold across different mixture and sewage-age scenario for crAssphage, HF183 and polyomavirus using QMRA. The authors concluded that genetic markers may not be effective when aged sewage contribute the most pathogens relative to fresh contamination. Similar risk-based MST marker thresholds have also been estimated for gull *Catellicoccus*, human *Bacteroides*, and human *Lachnospiraceae* (Boehm et al. 2018, Brown et al. 2017, McLellan et al. 2018).

Such information can be extremely valuable to regulators in interpreting quantitative MST marker data concerning potential human health risk and developing plans for faecal pollution mitigation and to assess human health risks more accurately.

2.5.6 Application 6: Outbreak tracing and wastewater surveillance

The genetic faecal pollution diagnostics toolbox has also proved useful in fields that traditionally focus on the detection and characterisation of pathogens, such as waterborne disease outbreaks or pathogen transmission route characterization. Twenty outbreak and pathogen transmission tracing articles were observed, predominantly employing MST markers with paired measurements of pathogens and cultivation-based FIO.

Additionally, three of the retrieved articles applied MST markers in wastewater surveillance for SARS-CoV-2. Given the importance of this topic, additional literature searches were performed and revealed three different roles where MST markers may be implemented for wastewater surveillance.

2.5.6.1 Outbreak tracing, disease transmission routes and sanitation trials

Waterborne disease outbreaks occur worldwide and may be caused by several factors, for example, in the case of drinking water, these may include raw water contamination, treatment deficiencies, and drinking water distribution network failures. Tracing an outbreak is done predominantly by tracking the pathogen strain from patients through the transmission routes back to the exposure source by genetic typing and sequencing (molecular epidemiology, e.g. (Popa et al. 2021)). Alternatively, host-associated genetic faecal indicators can help identify the source for contamination and support elucidating disease or pathogen transmission routes. While they provide less specific outbreak-related information compared to pathogen typing, these markers are much more abundant than the pathogen in question, making them easier to detect in the environment. For example, host-associated markers were used in outbreak studies in Finland with approximately 450 illness cases to identify the source of pollution and to ensure the success of contaminant removal from the drinking water distribution system (Kauppinen et al. 2019). A novel approach used the human-associated genetic marker HF183 in a norovirus outbreak involving 179 cases in Pennsylvania, USA. It was applied as a microbial tracer to demonstrate the hydrogeological connection between a malfunctioning septic system, drinking water well, and recreational water area and therefore helped inform outbreak prevention strategies in the area (Mattioli et al. 2021). The coastal Biobío Region of Chile had been affected by repeated hepatitis A outbreaks. Human mitochondrial DNA, faecal coliforms and live microbial biomass correlation was investigated and the concordance between human faecal pollution in the coastal waters and a seasonal hepatitis A outbreak strongly suggests that the investigated parameters can be used as a proxy to evaluate the risk of outbreaks of thalassogenic diseases (González-Saldía et al. 2019). During a large *Campylobacter* outbreak in Norway with over 2000 cases and 76 hospitalisations, an old cave as a drinking water pool was identified to be faecally contaminated as indicated by the presence of *E. coli*. Host-associated genetic markers for human, ruminants, horses, pigs and other animals were applied to generate a faecal source distribution profile. This revealed that the faecal contamination was likely zoogenic in origin (horses) (Paruch et al. 2020).

In settings with poor sanitation facilities and practices, pathogen transmission routes can be multiple and therefore planning WASH interventions to reduce pathogen exposure is challenging. A study in an urban slum in Nairobi, Kenya, set out to separate two types human faecal waste: from small children compared to adults, because mitigation steps to reduce could differ (Bauza et al. 2019). Using 16S AmpSeq analysis of faeces from both cohorts and various surfaces and waters, as well as the algorithm SourceTracker, the authors identified child faeces as the dominant pollution source inside households, whereas faecal pollution from adults was more prevalent outside households.

Genetic faecal pollution diagnostic tools can also be used to evaluate WASH interventions. A controlled, before-and-after trial was performed in neighbourhoods of Maputo, Mozambique to estimate the potential health impacts of a sanitation intervention (installation of improved pit latrines). The authors first assessed the transmission routes through a comprehensive sanitary, environmental and socioeconomic survey, including the measurement of a set of general and host-associated faecal indicators. They found widespread faecal contamination in soil, water and food preparation surfaces, including from human sources. However, faecal contamination levels were largely disconnected from these analysed factors (Holcomb et al. 2020). In the before-and-after trial, the authors used a Bayesian hierarchical modelling approach to account for MST marker performance. Bootstrap estimates found no effect of the sanitation intervention on the prevalence of general and human-associated indicators, which highlights the complexity of the system and the need for multisectorial, “transformative” WASH interventions (Holcomb et al. 2021).

2.5.6.2 Wastewater surveillance

Wastewater surveillance, also called wastewater-based epidemiology, seeks to relate the occurrence of a public health target of interest measured in wastewater to the public health of a respective population (e.g. (Choi et al. 2018, Lorenzo and Picó 2019). COVID-19 gave a strong boost to the field, where SARS-CoV-2 RNA occurrence in wastewater is used as a proxy for the prevalence and dynamics of the infection in the population (Ahmed et al. 2022). In contrast to HRWM that focuses on the user of the water (e.g., drinking water, recreation, irrigation), wastewater surveillance is an "upstream approach", looking back at the population´s health. Samples for wastewater surveillance are taken from raw wastewater collected by centralised sewer systems. Surface waters heavily contaminated by sewage may also exhibit an epidemiological indicator function in terms of wastewater surveillance (e.g. (Kolarević et al. 2022, Maidana-Kulesza et al. 2022).

Successful wastewater surveillance application requires the accurate measurement of public health targets in wastewater. However, this can be challenging because the proportion of human waste in a wastewater sample can be highly variable in time and space (i.e., between/within sampling site variability). In addition, the sample matrix may be challenging from the analytical point of view. In response, many scientists have suggested to use faecal markers (e.g., PMMoV, crAssphage, HF183) to support sample characterisation and provide quality control in wastewater surveillance.

One application category is the characterisation of surveillance samples, which mainly aims to quantify the human faecal levels in (waste)water but could also be used to characterize other animal sources. One study looking into the epidemiological indicator function of SARS-CoV-2 in surface waters for countries with poor wastewater treatment, for example, applied an advanced sampling site characterisation approach including measurement of human- (BacHum), ruminant- (BacR) and pig- (Pig-2-Bac) associated genetic faecal markers. By using this approach, they could successfully trace and identify sites with significant raw sewage influence from human populations, which could serve as reasonable sampling locations for wastewater surveillance when no obvious sewage outlets occur (Kolarević et al. 2022).

Besides, MST methods have also been used as internal process controls within wastewater surveillance investigations, either as a proxy for the public health target of interest to ensure adequate recovery and/or as performance metrics of sampling/sample processing protocols. In a monitoring study of SARS-CoV-2 in the wastewater and rivers of Tapachula (Southern Mexico), for example, PMMoV was not only used as a faecal pollution marker but also considered as an analytical control to confirm RNA extraction and amplification (Zarza et al. 2022). In another study investigating the intraday variability in 1-h and 24-h composite wastewater samples, the concentration of the indicator viruses crAssphage and PMMoV were monitored in addition to the less prevalent human pathogen adenovirus (HAdV) in order to inform the design of appropriate wastewater sampling strategies for wastewater surveillance (Ahmed et al. 2021).

The most widely observed use of faecal markers for wastewater surveillance was the normalization of pathogen occurrence data. In this context, different MST markers were used to either describe spatial and temporal trends of the public health target of interest or to support the prediction of community infection trends. For example, Wolfe et al. (2021) describe how normalizing SARS-CoV-2 concentrations from multiple wastewater treatment plants with PMMoV can be used to compare the incidence of laboratory confirmed new COVID-19 cases by accounting for variability in recovery and differences in human faecal loads within or between wastewater treatment plants. Another study investigated the suitability and performance of various normalization parameters and how well they correlated with local clinical cases. They found that normalization by the surrogate viruses crAssphage and PMMoV (amongst others) showed varying performance for different sampling sites (Mitranescu et al. 2022). Similar findings were described for PMMoV in a study by (Nagarkar et al. 2022) suggesting that the most suitable faecal marker for normalization may vary by site and wastewater management practices.

Wastewater surveillance represents an exciting new application for genetic faecal pollution diagnostics. However, additional research is warranted, especially in areas highly relevant for wastewater surveillance, such as the

behaviour of MST targets in sewer systems, distribution between hosts, or protocol performance assessments with wastewater sample processing methods. Although genetic faecal markers have already proven to be valuable, it remains unclear which of the many available methods are most suitable. Optimal method selection will likely vary by use scenario, surveillance target, and geographic location. In addition, the application will likely not be restricted to MST markers, but will use the whole methodological capacity of genetic faecal pollution diagnostics.

2.5.7 Application 7: Other applications

Assessing water resources for the possible presence of faecal pathogens is the foundation of genetic faecal pollution diagnostics. However, these tools have also proven useful in other arenas. For example, 45 out of the 105 articles in this category had antibiotic resistance as primary research focus, complemented with a genetic faecal diagnostic method, mostly MST markers. Twelve articles used MST markers to trace nutrient inputs into ambient waters. Interestingly, three articles were observed from the archaeology field, employing genetic methods for faecal bacteria. These three disciplines are further discussed below.

2.5.7.1 Identification of the sources of antibiotic resistance genes

Antimicrobial resistance (AR) is one of the top 10 global public health threats (World Health Organisation 2021). The spread of antibiotic resistant bacteria (ARB) and their antibiotic resistance genes (ARG) from hotspots such as wastewater treatment plants or agricultural run-off into freshwater and coastal ecosystems is of growing concern (Gao et al. 2018). Identifying such hotspots is therefore a pressing issue. Beyond the monitoring of a large panel of ARB and ARG targets of concern and the genotyping of ARGs (similarly to pathogen typing), two additional approaches have been established that allow tracking their source.

The first relies on the differing AR patterns of the gut microbiota of various host species, reflecting the differing antibiotic usage in human and veterinary medicine. This differing pattern is exploited for MST, where the pattern of the environmental samples of unknown pollution profile is compared to a library of known faecal sources. In the early 2000s, this 'antibiotic resistance analysis' relied on the phenotypic AR characterisation of *E. coli* or enterococci isolates (see also Section *'The early days of genetic methods for faecal pollution diagnostics'* (Mott and Smith 2011). More recently, (Li et al. 2018) adapted the Bayesian source tracking tool SourceTracker, originally relying on 16S AmpSeq data, to ARG data from whole metagenome sequencing. At two rivers in China with dense human and livestock populations and with excess nutrient levels, this tool (Hu et al. 2020) identified WWTPs as the major source of ARG at the majority of sites. At one site, non-human animal faeces proved to be the major pollutant. Correlations with host-associated faecal indicator genera, identified based on 16S AmpSeq data, helped identify swine manure as the main non-human faecal input.

The second approach relies on the co-occurrence of host-associated faecal microorganisms and ARG and/or ARB, because of a common source. Williams et al. (2022) faced persistent faecal pollution in an urban coastal bay in Sydney, Australia. qPCR MST and 16S AmpSeq together with SourceTracker were employed to pinpoint which stormwater drains drive dry-weather or wet-weather faecal pollution. Significant correlations between ARG and the human-associated MST marker HF183 showed that the same stormwater drains were the main sources of ARG and of human faecal pollution. The Bolivian Andes is an intense mining area, and heavy metals exert selective pressure for the co-selection of ARGs. Through a multiple linear regression between the first principal component of a PCA of ARG data as dependent variable and metals, the human-associated viral marker crAssphage and physicochemical parameters as independent variables, Agramont et al. (2020) demonstrated that it is likely human wastewater inputs, rather than heavy metals, that drive ARG concentrations at the three rivers studied.

2.5.7.2 Identification of the sources of nutrient inputs

Nutrients, such as nitrite (NO_2^-), nitrate (NO_3^-) and phosphate (PO_4^{3-}), are essential for plant life. However, excess concentrations can lead to eutrophication and harmful algal blooms (Fenech et al. 2012, Kendall et al. 2007). In

addition, ingestion of high amounts of nitrate, for example through drinking water, may have serious health consequences such as methemoglobinemia of infants (blue baby syndrome), colorectal cancer and thyroid disease (Ward et al. 2018). The World Health Organisation Drinking Water Guidelines recommend setting thresholds of nitrate and nitrite concentrations in drinking water (WHO 2017b) and some countries also regulate surface water and groundwater (European Union: 91/676/EEC and 2006/118/EC, within the frame of 2000/60/EC). Mitigation of excessive nutrient inputs is therefore a key water quality management task. Tracing nutrient inputs relies on the fact that ratios of rare to abundant isotopes of certain elements differ among environmental and biological compartments, due to isotopic fractionation during physiochemical and biochemical reactions. As a typical example, nitrate sources can be tracked using $\delta^{15}N$ and $\delta^{18}O$ isotopes (Fenech et al. 2012, Kendall et al. 2007). Since nitrate has numerous biotic and abiotic sources and isotope tracing cannot separate all source types, a toolbox approach is often useful, that can include MST markers (Fenech et al. 2012).

One of the early studies combining $\delta^{15}N$ and $\delta^{18}O$ isotope tracing with MST was conducted along the Sava River, a tributary of the Danube River, that crosses Slovenia, Croatia, Bosnia and Herzegovina and Serbia. The combined results indicated that soil nitrification and human wastewater were the primary nitrate sources in the Sava River, and the latter was also the main faecal pollution source (Vrzel et al. 2016). Carrey et al. (2021) assessed the main sources of nitrate pollution in surface water and groundwater across Catalonia, Spain in a government-led effort to review vulnerable zones as defined by the European Union Nitrates Directive (91/676/EEC). Nearly 200 samples were analysed for multiple isotopes ($\delta^{15}N$, $\delta^{18}O$, $\delta^{2}H$ and $\delta^{11}B$ from various molecules), viral and bacterial FIO, human-, ruminant- and swine-associated MST markers and complemented by land use data. Each sampling location was interpreted individually. The conclusions from multi-isotopic and MST data agreed or partially agreed in 79% of the samples. The authors gave detailed discussion on the complementary nature of the two approaches and the possible sources of disagreement (Carrey et al. 2021). In the coastal areas of Southwest Florida, harmful algal blooms caused by elevated nutrient levels are a reoccurring problem. Malfunctioning septic tanks were suspected to be the source of nutrients. Brewton et al. (2022) applied $\delta^{15}N$ and $\delta^{13}C$ isotope tracing, elemental composition of particulate matter (C:N:P), a panel of nutrients, chemical tracers, cultivation-based FIO as well as human-, bird- and gull-associated MST markers to tackle the complex challenge. These multiple lines of evidence pointed at a link between septic systems, groundwater and surface water, ultimately resulting in harmful algal blooms. Additionally, chemical tracers and bird- and gull-associated MST markers indicated rainfall runoff to be a contributing factor (Brewton et al. 2022). The Changle River catchment in China has high human population, intensive livestock farming (swine) and agricultural activities, all of them potentially contributing to the high nutrient levels of the river. A Bayesian isotopic mixing model using data from the nitrate dual stable isotope technique ($\delta^{15}N$- NO_3^- and $\delta^{18}O$- NO_3^-) suggested manure and sewage to be the dominant pollution sources (Cao et al. 2022). Since nitrate isotopes cannot differentiate between manure and sewage, Cao et al. (2022) applied MST using 16S AmpSeq together with the algorithm SourceTracker, which suggested untreated and treated domestic wastewater as main sources. Redundancy analysis brought all lines of evidence (isotopes, MST, land use and various ions) together to reveal domestic wastewater as probable cause of nutrient pollution (Cao et al. 2022).

2.5.7.3 Archaeology

Genetic markers can remain detectable much longer in sediments than in the overlaying water column (Korajkic et al. 2019). Sediments may therefore offer time-integrated information on faecal pollution. In a tidal freshwater marsh in South Carolina, USA, the ruminant-associated MST marker BoBac was found in all sections of a soil core, the deepest section of which dated to 1961 (Drexler et al. 2014). While in this hydrogeological system the bacterial community of fresh pollution might migrate through the layers, the findings provide evidence of at least recent, but potentially long-term faecal pollution likely from deer and/or cow manure. On much larger timescales, lake sediments may act as biological archives of sedimentary ancient DNA from autochthonous (in-lake) and allochthonous (from the catchment and beyond) sources (Capo et al. 2021). Among other tools, paleoenvironmental enquiries into ancient human presence and pastoral activities may also use MST markers or

DNA sequencing techniques (Capo et al. 2021). In a study in Northern France, the authors documented a shift from agro-pastoral practices to forested landscape during the Roman Period. Testing for ovine and bovine mtDNA markers revealed sheep as the dominant livestock before the transition (Etienne et al. 2015).

2.6 Discussion

2.6.1 Emergence of a new field in health-related water quality analysis

2.6.1.1 The advent of genetic faecal pollution diagnostics (GFPD)

Our search on peer-reviewed science regarding the analysis of faecal pollution-associated nucleic acid targets in water demonstrates the rapid development of genetic diagnostics in the field of health-related water microbiology (HRWM) since the start of the new millennium. The meta-analysis of the currently existing application types also highlights that this novel scientific discipline goes far beyond the enumeration of genetic MST markers. Many traditional HRWM aspects, such as treatment and microbial transport indications, infection risk assessment and QMRA, as well as the integration into modelling and simulations were found to be supplemented by GFPD (Sections *'Application 1'* through *'Application 7'*). In addition, several novel aspects such as the support of epidemiological outbreak tracing, wastewater surveillance or supplementing ABR research have also been developed.

The emerging scientific field of GFPD still grows; no plateau phase is in view (Figure 2.2). In the past decade, the focus of research has shifted from method establishment to the implementation of these methods in scientific field research. An emphasis on field implementation is also indicated by the frequent use of certain genetic faecal markers, with some of them already standardized at the national level (Section *'Application 1'*). However, the method development has not halted, and it is very likely that expected future technological developments in molecular biological analytics, sequencing and bioinformatics (e.g. (Callaway 2022) will further promote its diversification within the field of HRWM research.

It thus seems justified to define this emerging part of science as a new discipline: *genetic faecal pollution diagnostics* in health-related microbial water quality analysis (see *Glossary*). The ultimate aim of GFPD is to better characterize or to open up the "black box" of microbial faecal pollution of water resources in order to support problem-oriented water safety management, covering aspects such as catchment protection and management, water quality monitoring, health risk management and treatment requirement evaluation. GFPD will find its applications also outside the water sector, as exemplarily indicated by the use in archaeology (Section *'Application 7'*).

2.6.1.2 GFPD analyses distinct nucleic acid-based faecal pollution signatures

Vertebrate gut microbial communities fundamentally differ from environmental "non-digestive" microbial communities (e.g. water, sediment, soil, plant, non-vertebrate), as first demonstrated by the meta-analysis of 16S AmpSeq data by Ley et al. (2008). A very long co-evolution between host vertebrate animals (including humans) and their intestinal microbiomes, driven by many selective forces (e.g. adaptive immune system, host selection pressure, unique biochemical environment), is made responsible for this clear distinction (Ley et al. 2008). Although cosmopolitan populations do occur, strong vertebrate gut-associations also exists on the individual taxa level of microorganisms (McLellan and Eren 2014, Youngblut et al. 2020, Youngblut et al. 2021, Youngblut et al. 2019). This clear intestinal versus non-intestinal microbial community dichotomy forms the essential basis of specific detection of faecal pollution in water, targeting nucleic acid-based signatures from gut-associated bacteria, archaea and viruses.

GFPD of today primarily focuses on the cultivation-independent detection of nucleic-acid based targets in the environment. The literature analysis highlighted that GFPD so far predominately relies on targeted analysis, where faecal pollution-associated sequences are directly detected by amplification methods (e.g. PCR, qPCR, dPCR),

using specific primers and probes. Thanks to the enormous technological developments in HTS, non-targeted approaches, using broader taxonomic sequencing and subsequent specific *in-silico* sequence alignment to faecal-associated signatures, have been on a strong increase during the last decade (Section *'Outcomes of the systematic study design analysis'*, Figure 2.10).

Advances in intestinal microbiomics will certainly further benefit GFPD, expanding our understanding of eco-phylogenetics and providing access to representative sequence databases to support, i) *in-silico* design and evaluation of molecular assays, and ii) bioinformatic analysis of big data from HTS. Human and other animal intestinal microbiome research, with greatest relevance in life sciences and medicine, is a very young discipline, and much is to be expected to come in the future.

2.6.2 Identified revolutionizing aspects for HRWM research

2.6.2.1 Genetic faecal pollution detection and MST: a methodological quantum leap

The use of GFPD has fundamentally changed the way scientific questions on faecal pollution problems in the environment can be addressed and answered (Malakoff 2002). MST using genetic methods has opened the way to identify and quantify many different pollution sources, that cultivation-based methods did not and do not allow. About half of the identified GFPD studies (341 out of 611 articles) dealt with MST, i.e. the characterization and origin determination of faecal pollution. Many cutting edge GFPD studies, covering single and multiple sources in differing types of water resources, including elevated faecal pollution levels in watersheds, recreational waters, groundwater resources, aquaculture and others, could be successfully realized (Sections *'Application 2'* and *'Application 3'*). Very remarkable, almost two-thirds of these studies simultaneously applied MST-marker qPCR quantification and traditional cultivation-based enumeration of FIOs, determined by standardized parameters such as *E.coli* (ISO 1998b, 2012, 2014, 2018) or intestinal enterococci (ISO 1998a, 2000a). The need to determine the causes responsible for faecal pollution in water obviously promotes this most popular "hybrid application" (Sections *'Application 2'* and *Application 3'*).

2.6.2.2 Biobanking: a new key element in HRWM research

Traditional cultivation-based FIO analysis requires sample transport, processing and subsequent cultivation within a short time period (usually < 1 working day). This often significantly constrains the possibilities and extent of research. In contrast, GFPD enables long-term nucleic acid preservation (> 1 year) before performing the diagnostic analysis (Cary and Fierer 2014, De Paoli 2005, Jackson et al. 2011).

The possibility to store nucleic acids for posterior analysis has several essential implications for HRWM research. Assuming that there is enough capacity to establish a representative sample bank over time and space, the researchers can i) focus on selected samples of interest (e.g., pollution event-based analysis), ii) focus on the parameters appearing most appropriate at the time of analysis, and iii) extend the investigation to other samples and/or genetic parameters at any time, as long as sufficient analyte is available. In hydrological sciences, this type of sample archiving for posterior analysis (e.g., isotopes) has already been a standard practice for decades.

In addition, nucleic acid sample conservation during field work also opens the way to international network structures, performing centralized analysis in specialized laboratories (Layton et al. 2013, Mayer et al. 2018b, Reischer et al. 2013). This point is especially interesting for developing regions that lack the infrastructure for advanced GFPD. During the COVID-19 pandemic, infrastructures for molecular biological analysis were established in many urban centres throughout the globe that will likely contribute to centralized GFPD activities in the future.

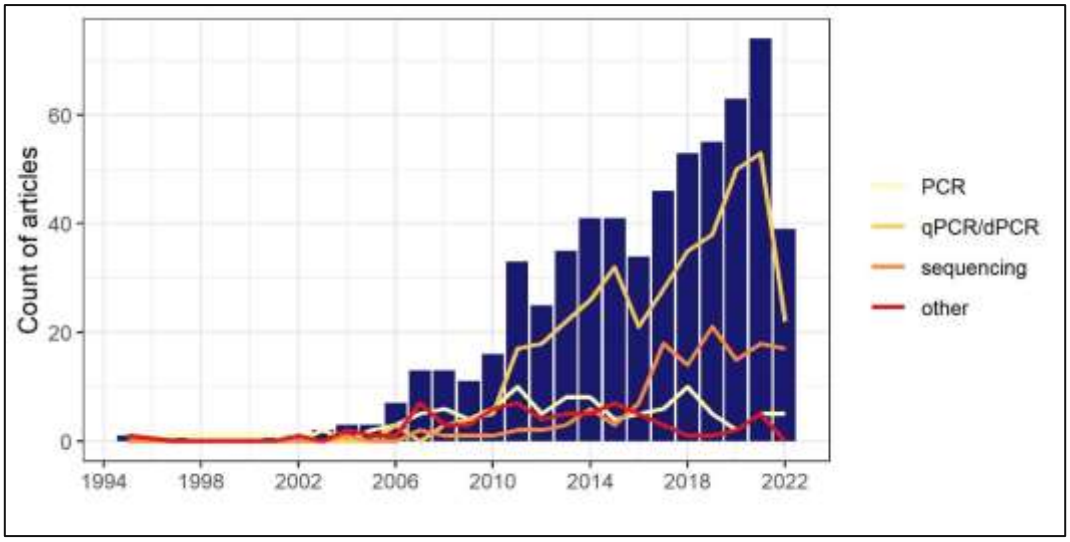


Figure 2.10 Analytical methods over the years in the 'application studies' pool, with the bar chart of all papers per year in the background (n=611 articles). The year 2022 is incomplete (last date of retrieval: 06/07/2022).

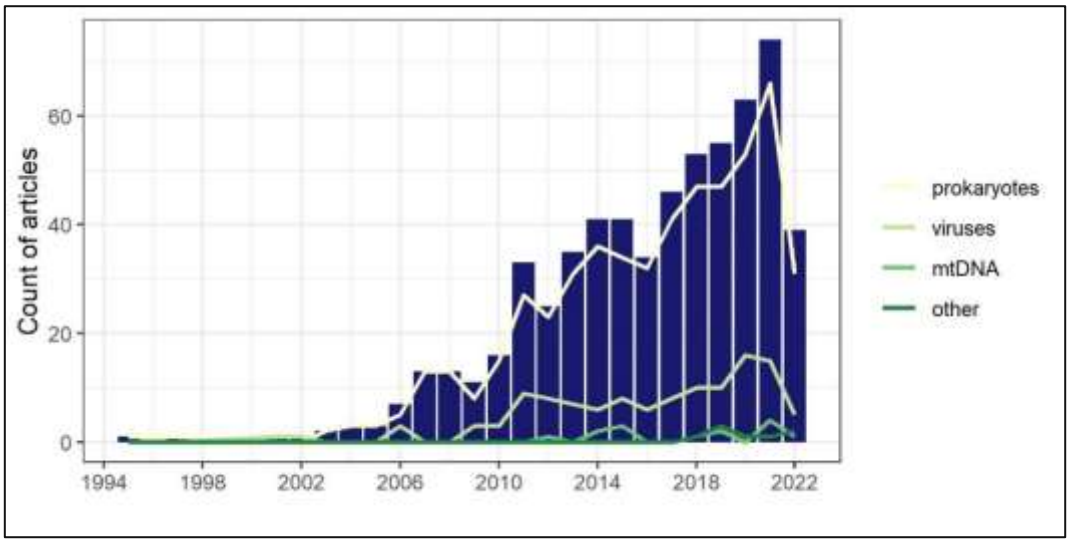


Figure 2.11 Timeseries of microorganisms targeted by genetic methods, with the bar chart of all papers per year in the background (n=611 articles). The year 2022 is incomplete (last date of retrieval: 06/07/2022). mtDNA indicates host mitochondrial DNA.

2.6.2.3 Cutting edge tools (still) require in-depth expert knowledge

Without any doubt, the ability of GFPD methods to detect (what is the pollution problem?), quantify (what is the extent of pollution?) and allocate (what are the sources of pollution?) faecal pollution in water and water resources has revolutionized this area of HRWM research during the last two decades (Malakoff 2002). However, the application of genetic MST markers to generate accurate information on the responsible faecal pollution sources is not trivial. Available genetic MST marker targets (and other FIO) as well as their quantification systems are not harmonized, therefore, targets must be selected so that all relevant biological-diagnostic attributes (Table 2.2) suit the given scientific question with respect to the characteristics of the local water system. Box 1 shows a hypothetical situation to illustrate the confusing effect that differential abundance and persistence of MST markers have for correct indication. A clearly defined MST research goal can often only be addressed for a limited "diagnostic space" using the selected markers (Box 1). In a similar way, appropriate faecal sensitivity and specificity of the applied genetic MST markers is essential (Table 2.2). The minimum acceptable faecal sensitivity

and specificity levels depend on the faecal pollution scenario investigated (e.g. relative abundance of the diagnosed faecal pollution sources) and can be determined by statistical considerations or catchment-based scenario simulations (Derx et al. 2021, Kildare et al. 2007, Reischer et al. 2011). A tailor-made investigation design, based on sound expert knowledge, is thus an essential pre-requisite for cutting-edge GFPD applications.

2.6.3 Direct detection of nucleic acids: characteristics and challenges

2.6.3.1 Characteristics of DNA/RNA-based target analysis

The literature analysis also highlighted that GFPD targeting prokaryotic microbiota (bacteria and archaea) has been almost exclusively relying on DNA analysis, with the 16S rRNA gene as the dominating diagnostic region. The primary aim of tracing intestinal DNA signatures in the environment is the sensitive detection and characterization of faecal pollution. Such DNA analysis does not give any information about the physiological status of the targeted microbiota in the analysed water and water resources. Active, inactive, starving, viable but not culturable, or dead microbial populations are often detected equally. Depending on the applied extraction procedure, DNA attached to cells, organic debris, biofilms, or sediments, and even freely-suspended DNA, is also detectable (Carini et al. 2016). The same is true for viral targets. Detecting viral DNA or RNA does not provide information on the infectious or non-infectious status of the targeted populations.

Notably, it was reported, that the application of ribosomal RNA via RT-qPCR for bacterial general faecal markers and MST markers increases the sensitivity and frequency of faecal pollution detection for several water resources types (Pitkänen et al. 2013). In addition, rRNA analysis may also be interesting for viability considerations (see section *'Generating viability- and infectious status information by molecular tools'*).

2.6.3.2 Relevance of viability- or infectious status information

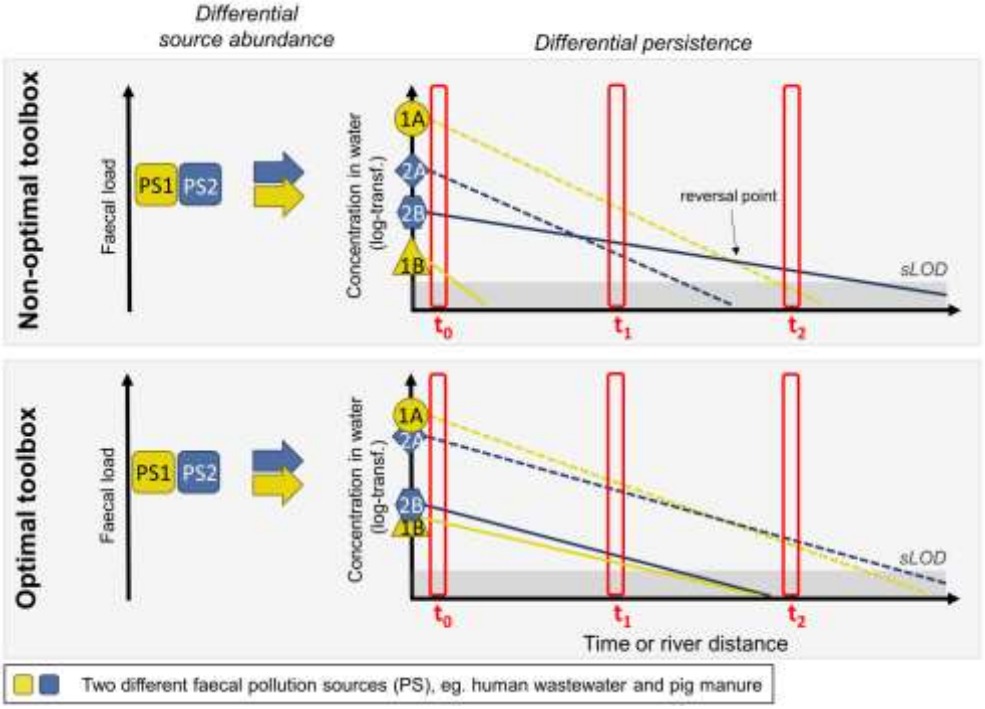
While the majority of genetic detection methods available do not account for information on the viability or infectivity status of the microorganisms or viruses where the nucleic acids originate from, it is important to note, that this is not the main purpose for many GFPD applications. For example, this is clearly the case for most of the identified faecal pollution detection and MST studies throughout the literature analysis (Sections *'Application 1'* to *'Application 3'*). Nevertheless, as outlined above, robust information on the persistence and resistance properties of the genetic targets is essential for the correct selection and application of genetic MST markers and for the appropriate data interpretation (Section *'Identified revolutionizing aspects for HRWM research'*). Other identified GFPD application areas, such as the support of outbreak tracing or wastewater surveillance in raw sewage, are not reliant on the viability status of the microbial targets either (Section *'Application 6'*).

Even the use in recreational water quality monitoring seems to be a realistic exercise, without the need for a viability-end-point (Section *'Application 5'*). For example, a recent investigation on swimming-associated health risks, including 80,000 beachgoers at 13 beaches (pooled data), revealed strongest associations between gastrointestinal symptoms and qPCR quantified enterococci - but not with cultivation-based enumeration (Wade et al. 2022). It was previously hypothesized, that enterococci DNA, as quantifiable by qPCR, better reflects the survival of resistant pathogens during wastewater treatment (e.g. resistant enteric viruses) than cultivable enterococci concentrations (Srinivasan et al. 2011, Wade et al. 2006). Obviously, it is desirable for pathogen die-off kinetics to match decay kinetics of the analysed indicator signals, irrespectively whether viability- or non-viability based parameters are considered. Without any doubt, more research is needed to better understand the principles behind these important relationships in GFPD and health risk assessment. However, the extent of identified innovative research by nucleic acid-based qPCR analysis for infection- and health risk indication holds great promise for the future (Section *'Application 5'*).

Information on viability or infectious status becomes an essential criterion when microbicidal and virucidal treatment is to be characterized. In particular, the efficacy assessment of disinfection, including all technologies (e.g. by heat, chlorine, ozone, UV, etc.), requires the application of representative and reliable indicators for

viability and especially infectivity, often supplemented by selected reference pathogens. The assessment is historically based on cultivation methods, the considered *lege artis* gold standard, especially when disinfection processes and log-reduction targets are to be monitored, validated or verified. For example, a recent EU regulation requires the cultivation-based validation monitoring of reclaimed water for agricultural irrigation (class A) using *E. coli*, somatic coliphages and *Clostridium perfringens* spores, with defined performance targets of ≥5, ≥6 and ≥4 $\log_{10}$ reductions within the treatment chain, respectively (European Union, 2020) .

BOX 1 Microbial source tracking markers: diagnostic scenarios



Simple hypothetical MST situation with two different point sources of pollution (e.g. human wastewater and animal (pig) manure) of equivalent discharge and contamination load for a small river. For reasons of simplicity, only dilution at the time of contamination (t0) and decay of the MST markers is considered (i.e., batch-reactor system with complete mixing and no sedimentation). Three time slots (t0, t1, t2) are chosen to illustrate the different "diagnostic windows" of MST indications at the given detection limit (sample limit of detection, sLOD).

Non-optimal toolbox. All four applied MST markers show different abundance in their respective faecal excreta and persistence in the water body. At t0, all four MST markers allow correct qualitative detection of both sources (differential persistence insignificant). Due to the differential abundances of MST markers, no direct estimation on the relative importance of PS1/PS2 is possible. However, mathematical corrections of concentration differences in excreta would make this possible. At t1, MST marker 1B leads to false negative detection of PS1, due to differential persistence. Even in the case of accounting for differential abundance, only MST markers 1A and 2A can be used to estimate the relative importance of pollution of PS1/PS2 thanks to their similar persistence. At t2, only PS2 is detectable by MST marker 2B, thus the diagnosis would miss PS1 (false negative detection at the given sLOD).

Optimal toolbox. Both selected pairs of MST markers show comparable pollution source abundance in faecal excreta and persistence in the water body. The MST markers pair 1A-2A allow the estimation of the relative contribution of PS1 and PS2 at all times (t0-t2). Due to lower source abundance, the MST marker pair 1B-2B only allow detection and comparison at t0 and t1, but not at t2.

Table 2.2 Overview of essential biological-diagnostic attributes of faecal indicators and associated genetic targets. The overview shows the "big five": sensitivity, specificity, persistence, resistance and mobility. Most of the shown attributes can be divided into sub-characteristics. Various methods and specification metrics have been suggested.

Characteristic	Basic Definition	Remarks	Methods / Metrics
Faecal sensitivity	Occurrence of faecal indicator or genetic target in faecal pollution source(s) to be indicated.	Should be ubiquitous and abundant in targeted pollution source(s). Indicator/genetic target of **total faecal pollution** in human and vertebrate animal faecal pollution sources (primary enteric habitats). **MST** indicator/ genetic marker only in faecal pollution-source-groups to be indicated (human, ruminant, pig, etc.).	**Incidence** or (**binary**) **faecal sensitivity** (% presence in targeted source, e.g. (Ahmed *et al.*, 2009, Farnleitner *et al.*, 2010, Shanks *et al.*, 2010, Shanks *et al.*, 2010)); **Abundance** in target excreta or waste water (conc. per volume/mass, (Farnleitner *et al.*, 2010, Ervin *et al.*, 2013, Mayer *et al.*, 2018)
Faecal specificity	Non-occurrence of faecal indicator or genetic target in the pristine environment or non-targeted compartment	Indicators/targets of **total faecal pollution** should be absent in pristine environments not polluted with faeces. **MST** indicator/marker also absent in non-targeted faecal pollution sources (e.g. human faecal marker not in ruminant excreta).	**False positive occurrence** in pristine habitats (conc. per volume/mass, (Vierheilig et al. 2012)) (**Binary**) **faecal specificity** (% presence in non-targeted source groups, e.g. (Linke et al. 2021, Shanks et al. 2010a, Shanks et al. 2010b)) **25th/75th percentile discrimination metric** (25th percentile target minus 75th percentile non-target,(Reischer et al. 2013)
Persistence	Extent of survival (i.e. viability) of indicator or molecular detectability (i.e. non-degraded amenable nucleic acids) of genetic target in the (aquatic) environment.	Persistence varies widely among microorganisms and genetic targets and is influenced by many potential abiotic and biotic ecological factors, such as sunlight, temperature, salinity, grazing, etc.	**T90, T99** (time in days needed for a 1 $\log_{10}$ (T90) or 2 $\log_{10}$ (T99) reduction in indicator or genetic target concentration, (Mitchell & Akram, 2017)) **decay rate coefficient *k*** of, e.g,, a first-order decay model (Balleste and Blanch 2010, Chick 1908)
Resistance	Extent of survival (i.e. viability, proliferation, infectivity) or molecular detectability (i.e. non-degraded amenable nucleic acids) of indicator or genetic target, respectively, towards chemical substances (e.g. metals, antibiotics) and during technical treatment and disinfection processes.	Resistance varies widely among microorganisms and genetic targets and is influenced by many chemical and physical factors, such as type of chemicals (chlorine, ozone,), concentration and contact time (ct-value), temperature and time in thermal processes, fluence in UV irradiation.	**Inactivation rate and kinetics** obtained under carefully controlled conditions (Hoff and Akin 1986) inactivation rate constants **Log reduction** of the concentration of microorganisms/pathogens; a measure for the effect of a substance or for the efficacy of the process (Guerrero-Latorre et al, 2016). The log reduction to be achieved for a target is determined by risk assessment.
Mobility	Transport characteristics of the indicator or genetic target in the (aquatic) environment	Mobility is influenced by many factors, such as mass and size of the microorganism / phage, its attachment and aggregation behaviour (electrostatic and hydrophobic forces), its detachment behaviour, as well as the motility of certain microorganisms. Mobility characteristics may change as the microorganism decays.	**Sedimentation** onto the river bed applies to larger-sized microorganisms (protozoa) or microorganisms attached to sediment (Jiang et al. 2015, Wu et al. 2019) **Resuspension** of microorganisms attached to the riverbed sediments (Jamieson et al. 2005, Kim et al. 2010, Park et al. 2017) **Straining** due to microorganism size and aquifer material grain size distribution e.g. (Bradford et al. 2003, Tufenkji et al. 2004) **Attachment/Detachment** (Schijven and Hassanizadeh 2000) **Motility** (Becker et al. 2004)

2.6.3.3 Generating viability- and infectious status information by molecular tools

In addition to cultivation-based enumeration by using FIO standards (see above), cultivation-independent, molecular strategies for viability- and infectious status analysis are also increasingly applied in HRWM research. For prokaryotes a vast array of different techniques, including RNA-based methods (rRNA, messenger RNA), membrane integrity (e.g. viability stains, viability PCR), cellular metabolism (e.g. ATP, respiration, isotope labelling), protein-based methods (e.g. BONCAT) or microcalorimetry, have been suggested within the broad field of microbial ecology (Emerson et al. 2017). However, delineation of dead versus viable microbial cells is complex and still under debate (Davey 2011, Kirschner et al. 2021). There is consensus that living microbial cells should have, i) intact functional cell membranes, ii) intact cellular- and energy metabolism, and iii) capability reproduce (i.e. intact transcription/translation mechanisms). Straightforward determination strategies frequently address only one of these aspects of microbial viability (e.g. "live/dead" protocols), leaving room for uncertainty (Emerson et al., 2017). Thus, (more time consuming) multiple criteria are to be applied simultaneously, if precise viability characterisation of the target microbiota is required (Kirschner et al. 2021). Detection of infectious viruses is equally challenging, and no single method is available to detect all of the infectious viruses in water (Gerba et al. 2018). At least three criteria must be fulfilled for infectious viruses, i) sufficient genomic integrity to produce the required proteins for replication and providing an accurate genetic template for subsequent generations, ii) protect the genome from degradation, and, iii) ability to recognise and infect the host cell (Gerba et al. 2018, Pecson et al. 2009).

Viability PCR and a similar approach, enzymatic treatment PCR (ET-qPCR), were introduced to the field of GFPD for more than a decade ago (Bae and Wuertz 2009, Pecson et al. 2009); as exemplarily demonstrated below, these methods have been increasingly applied in HRWM research during in recent years. The original idea of applying viability PCR to bacterial MST markers was to gain information on recent faecal pollution events in water resources (Bae and Wuertz 2012, 2015). More recently, Jager et al. (2018) used qPCR with and without propidium monoazide (PMA) pre-treatment as well as culture-based methods for *E. coli*, enterococci and *P. aeruginosa* to evaluate the removal efficiency of wastewater ozonation, a tertiary treatment step. PMA is an intercalating DNA dye that penetrates cells with impaired membrane and prevents PCR amplification (Nocker et al. 2006). It thus allows selectively detecting viable cells; PMA-qPCR is therefore also called viability-qPCR. Jäger and colleagues' results underline the dependence of disinfection efficiency estimate on the detection method. FIO removal rate estimates were in the following order: culture-based < viability qPCR < qPCR (Jager et al. 2018), emphasizing the difference among the culturable population, the viable but not culturable population and the total bacterial DNA content. Viability qPCR in comparison with qPCR was also applied to FIO (*E.coli*) and viral and bacterial MST markers (crAssphage, JC and BK polyomavirus, human adenovirus, human-associated Bacteroides HF183) in sewage sludge flocs, to reveal their removal and inactivation during potassium ferrate treatment (Wang et al. 2023). Spatial distribution and movement due to the potassium ferrate treatment of the FIO and MST markers could be analysed in different compartments of the sludge flocs, covering different extracellular polymeric substance fractions (EPS). The overall reduction of the MST marker by qPCR was up to 2 orders of magnitudes lower as determined by viability qPCR (Wang et al. 2023).

In analogy, ET-qPCR, applying enzymatic treatment by proteinase K and RNase, was used to estimate infectivity of bacteriophage MS2 particles in water (Pecson et al. 2009). By using multiple-PCR-amplicons (whole genome coverage) and partial treatment with different virucidal agents (heat, UV-B, singlet oxygen) the authors demonstrated that genome damage only partially accounts for viral inactivation, and thus would never generate results equivalent with infectivity assays. Nevertheless, results indicated that ET-qPCR can be used to monitor MS2 infectivity, provided that the statistical ratio to total inactivation by cell-culture under specific treatment conditions applied can be determined (Pecson et al. 2009). A follow-up study investigating UV-C treatment demonstrated that this approach may also be used to estimate viral inactivation in conjunction with mathematical models for JC polyomavirus and human adenovirus (Calgua et al. 2014).

Summing up, molecular tools to generate information on viability and infectious state constitute a very novel and innovative area of research in GFPD. Relatively little experience exists in comparison to traditional PCR and qPCR analysis (section *'Application 4'*). Many problems are still associated with their application, such as problems with methodical reproducibility, cross reaction with background- or free nucleic acids, selection of optimal reagents an experimental conditions and protocols (Codony et al. 2020, Gerba et al. 2018). Furthermore, the success of these methods often depends on the particular mechanism of inactivation (e.g. chemical vs physical agents). Nonetheless, further development activities in future will likely open new windows of opportunities in HRWM as well as in complementing cultivation based-standards. In addition, many potentials within the range of these available molecular tools have not been exploited yet (Emerson et al., 2017). For example, and in contrast to viability PCR applications, RNA-based methods have only very rarely been applied and evaluated in GFPD (Pitkänen et al. 2013). As successfully demonstrated in other fields of environmental microbiology, RNA analysis may significantly contribute to information on the activity status of microbial populations (Amann and Ludwig 2000, Deutscher 2006, Gourse et al. 1996).

2.6.3.4 Sensitivity of environmental detection of nucleic acid targets

A common narrative is that molecular DNA/RNA diagnostics is highly specific and sensitive. This may be true for theoretical considerations. For "real world" applications, this dictum, especially in relation to sensitivity, has to be put in the context of the overall analytical measurement challenge (V. Wintzingerode et al. 1997). For example, an optimally designed qPCR test should be able to detect, in theory, one target molecule of DNA/RNA, if present in a single reaction unit. However, as target molecules follow a stochastic distribution during analyte dilution for parallel analysis, the assay limit of detection (aLOD) cannot be less than three target molecules for a 95% detection probability per qPCR analysis, even if PCR kinetics performs perfectly (Bustin et al. 2009). However, overall considerations require the whole-chain-of-analysis (WCA), including sampling, recovered sampling volume, filtration- and enrichment-, nucleic acid extraction-, and purification efficacies, and finally, the amount of nucleic acid analysed. The resulting overall WCA sensitivity, for instance reported as sample limit of detections (sLOD), can be quite elevated (Domingo et al. 2007). To illustrate, sLOD or alternative estimates on WCA sensitivity for qPCR DNA/RNA target enumeration were reported to be in the range of $\log_{10}$ 1.5 – 3.9 genetic targets per 100 ml sample (Pitkänen et al. 2013).

Selected genetic targets for GFPD often target highly abundant intestinal bacterial and viral populations as occurring in faecal excreta or wastewater, to compensate for the above mentioned WCA sensitivity issues. This fundamental design criterion is achieved by almost all top performing qPCR assays of genetic faecal markers (Green et al. 2014, Layton et al. 2013, Mayer et al. 2018b, Reischer et al. 2013, Sabar et al. 2022). Less abundant intestinal targets, such as traditional *E. coli* or enterococci (Farnleitner et al. 2010) can still be detected using genetic methods, if faecal pollution levels are elevated, as frequently observed for surface waters under communal and agricultural influence. However, in situations with low to very low faecal pollution levels, such as groundwater and drinking water resources, the sensitivity issues of genetic faecal markers can be very limiting. High volume sampling, specific enrichment or alternative amplification systems may bring improved sensitivity to allow to extend the possibilities of GFDP to such situations of applications (Heijnen and Medema 2009, Liu et al. 2012, Min and Baeumner 2002, Rhodes et al. 2011).

HTS applications as identified in our literature analysis (Section *'Outcomes of the systematic study design analysis'*), face challenges in addition to WCA. In fact, the achievable sensitivity of 16S AmpSeq applications, applying general primers for broad taxonomic detection, such as kingdom and phylum level, strongly depends on the relative abundance of faecal pollution-associated intestinal microbiota compared to non-faecal pollution associated microbiota (i.e. environmental "background microbiome"). Water resources, showing low to moderate faecal pollution levels and abundant aquatic microbiomes (e.g., 10^9-10^{11} cells per litre for lakes or rivers, (Kirschner et al. 2004, Velimirov et al. 2011), become problematic, even when applying high amplicon sequencing-depth (Vierheilig et al. 2015). Consequently, identified studies have most frequently focussed on water resources with

significant municipal and agricultural faecal pollution levels (Sections *'Outcomes of the systematic study design analysis'* and *'In-depth review of the application areas of genetic faecal pollution diagnostic through case studies'*).

2.6.4 A toolbox approach

Undoubtedly, GFPD methods and approaches are powerful, however, no method comes without its limitations and no single method can have a universal application. Each genetic faecal parameter has its specific biological-diagnostic attributes (Table 2.2). The scientific investigation should therefore be designed to best suit the given faecal pollution problem including the characteristics of the local water system. A careful selection of microbiological and other parameters, based on their biological-diagnostic attributes (Table 2.2), is essential. Bacterial markers often do not reflect the characteristics of pathogenic viruses ((Harwood et al. 2013) and references therein), which points at the necessity of including both viral and bacterial indicators into the study design. The systematic literature analysis revealed that bacterial faecal markers dominate the GFPD field, but viral faecal markers have seen an increase in the past ten years (Figure 2.11). The importance of biological-diagnostic attributes in GFPD result interpretation is shown here for an MST example. Why concentrations of MST markers in water do not directly imply microbial source apportionment, is illustrated by a hypothetical MST situation in Box 1, and shows the importance of selecting markers with similar abundance and persistence for correct indication. Similarly, appropriate faecal sensitivity and specificity of the applied genetic MST markers is also essential (Table 2.2). Differing biological-diagnostic attributes may be accounted for using statistical considerations or faecal load-based scenario simulations (Ballesté et al. 2020, Derx et al. 2021, Kildare et al. 2007, Reischer et al. 2011). It is also important to collect a (statistically) representative dataset, considering temporal and spatial variations of faecal pollution. For example, sampling frequency may be set to reflect the expected pollution dynamics ('event sampling', (Reischer et al. 2008)). Finally, supporting technologies can give critical information for the correct interpretation of GFPD information. GIS, satellite imagery, and other mapping technologies provide important information on the location of point sources of faecal pollution (e.g. location/amount of WWTPs, septic systems, stormwater discharges), and on land use, indicating potential diffuse sources. The latter may be complemented with field data on land use practices (e.g. agriculture and wildlife population sizes and movements). Hydrological and meteorological data provide essential information on water system dynamics. GFPD can be linked to public health risk outcomes through concomitant epidemiological and clinical data. Thus, a tailor-made investigation design is an essential pre-requisite for cutting-edge GFPD applications.

2.7 Conclusions

→ The tools and approaches developed for **genetic faecal pollution diagnostics** have **revolutionised HRWM research** in the last two decades in terms of faecal pollution detection and microbial source tracking, the current core areas of application. Together with nucleic acid bio-banking, genetic faecal pollution diagnostics represents a new level of methodical possibilities in health-related water quality research in the 21st century, even in remote or less developed regions.

→ Genetic faecal pollution diagnostics is about to **expand to many other application areas** within and outside the field of HRWM. For instance, it will further gain importance in **infection and health risk assessment** (e.g. recreational water quality monitoring) and will increasingly support the evaluation and verification of **water treatment and disinfection** processes, in combination with standardised treatment indicator models and cultivation-based enumeration.

→ The COVID-19 pandemic gave a strong boost to the field of **wastewater surveillance**. Wastewater surveillance for SARS-CoV-2 is currently transforming to a global early warning disease monitoring system. Genetic faecal pollution diagnostics will likely increasingly support wastewater surveillance in data generation,

pollution source characterisation, normalisation and quality assurance. Since both "sister" disciplines use the same molecular biological framework and infrastructure, potential synergies are significant. In general, genetic faecal pollution diagnostics has the potential to support any environmental **global infectious disease surveillance systems**, covering human and other animal populations.

→ As demonstrated by the many identified studies, **internationally accepted, cultivation-based water quality parameters**, such as *E.coli* or intestinal enterococci, can be well **complemented with genetic faecal pollution diagnostics**, thus significantly expanding the methodical possibilities in water quality monitoring and management, when needed (e.g. MST to trace the origin of cultivation-based FIO). Genetic faecal pollution diagnostics constitutes a "toolbox" approach. Tailor-made scientific investigation and monitoring solutions can be rapidly set up by experts.

→ The current century is "the Century of Life Sciences", especially considering how molecular biology and bioinformatics rapidly transform health sciences and medicine. It is also the era of information technology, artificial intelligence and automatization. These driving forces will certainly promote further innovation within genetic faecal pollution detection. **Many technological breakthroughs are expected.**

→ **From science to practice**. The water management sector increasingly needs the tools and approaches offered by of genetic faecal pollution diagnostics to solve future challenges (e.g. challenges related to SDG6). The translation of such tools to practice has to be paralleled by standardization efforts. While some countries have already started such activities (e.g. three assays standardised in the USA), international standards are still lacking. The needs will have to be defined by the water management sector and translated to future genetic faecal pollution detection guidelines and standards by global panels of experts.

→ This meta-analysis provides the **scientific status quo** of the field of genetic faecal pollution diagnostics. It should promote further research to advance the scientific field and serve as condensed information source to the wider audience, including microbiologists, water hygienists, water management professionals, and public health experts.

3 Assessment of the microbiological water quality of the Danube River at Vienna

3.1 Introduction

Water safety planning and management is at the heart of safe drinking water supply. Drinking water supply from rivers with large catchments may pose specific challenges, since a multitude of faecal pollution sources such as urban wastewater, combined sewer overflows, manure, as well as wildlife excreta could affect the microbial quality of river water (Jeong et al. 2019, Kirschner et al. 2017). The scale of the impact may also vary depending on environmental factors, such as precipitation and river discharge, and the dynamics of the pollutions source itself, e.g. the variations in faecal load from a wastewater treatment plant (Burnet et al. 2019b, Flood et al. 2022, Jeong et al. 2019). Assessing such a system is therefore a complex task, requiring careful characterisation involving multiple microbiological and environmental parameters and analysis of the information with adequate statistical and mathematical tools.

The drinking water supply of Vienna partially relies on riverbank filtrate of the Danube River, drawn from a protected floodplain area. The raw water resource may be impacted through infiltration from the Danube River and from backwaters of the floodplains (Frick et al. 2020). During its 2900 km journey from the Black Forest in Germany to the Black Sea, the Danube River drains an area of 800 000 km^2 shared by 19 countries, making it the most international river in the world (ICPDR). Previous research, as well as data from bathing water monitoring at sites in Vienna indicate low to moderate faecal pollution levels with substantial variations (range of *E. coli* measurements between 2004-2014 spanning almost three orders of magnitude, (Frick et al. 2017)) and a dominance of human faecal pollution in the Danube River at Vienna during base flow conditions (Kirschner et al. 2017). The floodplain area is an urban riverine wetland that is only connected to the main channel during floods. The faecal pollution level of the backwaters is generally low, with some exceptions and originates from allochthonous (i.e., main river during flooding, human sources) as well as autochthonous sources (i.e., wildlife on the floodplains, (Frick et al. 2018b, Frick et al. 2020)).

The overall aim of the Groundwater Resource Systems project (2010-2018) was to perform a comprehensive microbiological and related health risk assessment with regard to safe drinking water supply, of the surface waters that may affect the porous groundwater of the Danube floodplains at Vienna (Farnleitner et al. 2015). This involved (i) a detailed microbiological characterisation of the Danube River and its backwaters at Vienna and (ii) microbial fate and transport modelling combined with QMRA for scenario modelling.

The microbiological characterisation of the Danube River and its backwaters at Vienna relies on a long-term (4-7 years) monitoring dataset with a set of traditional faecal indicator organisms, MST markers and viral pathogens (n = 272 water samples). The long-term character is particularly important, as it allows covering seasonal and other variations in the faecal pollution pattern. The microbiological characterisation is described in this chapter.

The robust microbiological dataset guided the setup of the subsequent modelling, including the choice of reference pathogens in QMRA and was directly employed in model calibration. Given the different pollution profile of the Danube River and its backwaters, the two systems were modelled separately, with the research questions tailored to the specificities of the given system. The two modelling studies are described in Chapter 4 (Danube River study) and Annex III (floodplain study).

Figure 3.1 Study area showing the surface water sampling points DSW3 and DSW5 on the Danube River as well as LSW1 and LSW3 on the floodplains of the Danube River. © Federal Office of Metrology and Surveying of Austria (BEV), CC BY 4.0.

3.2 Materials and Methods

3.2.1 Study site and sample collection

The study site is located at the Danube River, in Vienna, Austria (Figure 3.1). The Danube River starts in Germany, approx. 850 km upstream, and shows dynamic variations in river discharge, ranging from 900 to 5300 m^3/s for the characteristic low and high discharges, with a mean discharge of 1900 m^3/s at the study site. The region has a temperate climate where floods are driven by snow melts and heavy rainfall events in the headwater catchment. The Danube River is moderately polluted with faecal matter that originates predominantly from human wastewater (Frick et al. 2017, Kirschner et al. 2017). The Lobau floodplain area is flooded by the main stream through a levee opening at the lowermost end of the floodplain if the river discharge exceeds 2200 m^3/s. The floodwater can only enter the floodplain at this single point. The backwater network is otherwise separated from the main stream by a flood protection levee.

Surface water samples were collected monthly from two sites at the Danube River (DSW3, during 2013-2017 and DSW5, during 2010-2017, DSW stands for Danube Surface Water), and from two sites along the backwater channel (LSW1 and LSW3, during 2010-2015, LSW stands for Lobau Surface Water; Figure 3.1). The sampling location LSW1 is situated at a lateral branch on the river side of the flood protection levee and has therefore a high connectivity to the Danube River ("dynamic backwater"). The site LSW3 is at the main backwater channel and is only connected to the Danube River during floods ("disconnected backwater").

3.2.2 Microbiological analysis

Water samples were analysed for faecal indicators according to international standards: *E. coli* – ISO 16649-1 (ISO 2001), intestinal enterococci – ISO 7899-2 (ISO 2000a), *Clostridium perfringens* spores – ISO 14189 (ISO 2013), and somatic coliphages – ISO 10705-2 (ISO 2000b). Infectious enteroviruses were isolated from 1-L samples according to the inorganic flocculation and ultracentrifugation method of Walter and Rüdiger (1981) and enumerated

following an MPN method (Chang et al. 1958) on Buffalo green monkey kidney cells (Dahling and Wright 1986). The limit of detection was 1 CFU/100 mL for *E. coli*, intestinal enterococci and somatic coliphages and 0.01 CU-MPN/100 mL (1 CU-MPN/10 L) for infectious enteroviruses.

MST markers were quantified in 500-600 mL water samples using quantitative PCR. The human-associated marker HF183/BacR287 (Green et al. 2014), the ruminant-associated marker BacR (Reischer et al. 2006), the pig-associated marker Pig2Bac (Mieszkin et al. 2009), and the duck-associated marker DuckBac (Kobayashi et al. 2013) were selected and applied as described previously (Kirschner et al. 2017). As a robust approximation for the sample limit of detection, 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 mock MST communities), we applied the threshold of detection concept. The threshold of detection assumes one copy detected in the qPCR reaction, neglects loss during filtration and extraction, and reports the value per 100mL water sample, taking into account the filtration volume and dilution of the DNA extract used in qPCR (Reischer et al. 2008, Reischer et al. 2007). The quantitative microbial source tracking results were expressed as marker equivalents per 100 mL (ME/100 mL) (Reischer et al. 2008, Reischer et al. 2007). The unit molecule equivalents (ME) is used to account for neglecting filtration/extraction losses. The threshold of detection of MST markers varied between 12 and 171 ME/100 mL, with a median value of 29 ME/100 mL.

3.2.3 Environmental data

Daily mean discharge of the Danube River was available for the station Wildungsmauer, 30 km downstream of Vienna. Daily average water temperature and water turbidity of 15-min resolution were available for the station Nußdorf in Vienna. The discharge, temperature and turbidity data was provided by the Austrian Danube River waterway management company *viadonau*, Precipitation during the 48 hours prior to the sampling timepoint was summed across eight stations covering the 200-km section upstream of Vienna (Linz, Enns, Amstetten, Melk, Krems, Langlebarn, Vienna, Groß-Enzersdorf, courtesy of GeoSphere Austria, earlier ZAMG, Central Institution for Meteorology and Geodynamics). UVB dose was summed for the 26 hours prior to the sampling timepoint, up to 13h before sampling time from the UV station in Steyregg/Linz, afterwards from the station in Vienna. Data was provided by the Austrian UV monitoring network, courtesy of the Medical University of Innsbruck (http://www.uv-index.at/). The assignment to UV-stations was set based on water travel times that were calculated using mean flow velocity and the distance of the meteorological stations to the sampling site.

3.2.4 Data treatment and analysis

Data was gathered in MS Excel and further processed and visualised in R *tidyverse* (R Core Team 2019, Wickham et al. 2019). For the statistical analysis, non-detects were replaced by the half of the limit of detection (FIOs and enterovirus) or half of the threshold of detection (MST markers). For visualisation purposes, non-detects were assigned a constant value below the limit/threshold of detection.

The conditional probability of correct detection of MST markers (i.e., that the detected marker originates from the target species and not from cross-reaction with non-target species) was calculated based on Bayes' Theorem following Kildare et al. (2007). Eq. (1) estimates $P(C^+|T^+)$, the probability that the targeted source of contamination (C) is present in the analysed water sample given that the test (T) signals positive with the given assay:

$$P\,(C^+|T^+) = \frac{P\left(T^+|C^+\right) P\,(C^+)}{P\left(T^+|C^+\right) P\,(C^+) + P\left(T^+|C^-\right) P\,(C^-)} \quad (1)$$

where $P(T^+|C^+)$ is the probability of a positive signal with the given assay in a faecal sample from the targeted species (assay sensitivity); $P(T^+|C^-)$ is the probability of positive signal with the given assay in a faecal sample from non-targeted species (1-assay specificity); and $P(C^+)$ is the background probability of detecting the given marker

in a specific catchment. It is assumed that the background probability can be approximated by the occurrence of the given assay in water samples from the studied catchment (Kildare et al. 2007). In the current study, $P(C^+)$ is set equal to the occurrence of the given marker in water samples from the given site (reported in Figure 3.3). Binary faecal specificity and sensitivity were available from earlier investigations in the study area: i) HF183II assay, 78 and 100%, ii) BacR assay, 86 and 100% and iii) Pig2Bac assay, 91 and 100%, respectively (Mayer et al. 2016, Steinbacher et al. 2021). The faecal specificity and sensitivity of the DuckBac assay are 75% and 91%, respectively (unpublished data).

To investigate correlation among microbial and environmental parameters, Spearman rank correlation tests were performed using the *psych* package (Revelle 2022) and visualised using the *corrplot* package (Wei and Simko 2021) in R. The p-values were corrected for multiple testing using the method of Holm (Holm 1979), embedded in *psych*. The threshold of significance was set at p = 0.05. The Analysis of Similarities (ANOSIM) was computed using the anosim function of the *vegan* R package (Oksanen et al. 2022).

3.3 Results

3.3.1 Faecal indicator and pathogen monitoring results

The multi-year faecal indicator dataset showed that the faecal pollution level of at the two Danube River sites was low to moderate, occasionally critical, while at the floodplain sites it was low, according to the *E. coli* and intestinal enterococci classification system set up by Kavka et al. (2006) (Figure 3.2). This observation is further supported by the concentration values of persistent *Clostridium perfringens* spores and somatic coliphages and is in line with the findings of Frick et al. (2017) who reported a decade-long monitoring data of faecal indicators at the study site. The Danube River samples were also tested for infectious human enteroviruses that showed a low environmental occurrence (< 50%) and concentrations (max. 104 MPN-CU/10L). While no apparent seasonal pattern was observed with the faecal indicator organisms, the infectious enteroviruses showed a clear absence in the summer months (Figure 3.2).

3.3.2 Microbial source tracking results

Based on previous research into the faecal pollution sources in the study area, including the assessment of all major animal groups as potential sources of FIOs at the floodplain area (Frick et al. 2018b), a human-, a ruminant-, a pig- and a duck-associated marker was selected for this study. Since the results paint a different picture about the Danube River sites than the floodplain sites, the two waterbody types are described separately below.

Danube River. The human-associated marker was detected in almost all Danube River samples, with median concentrations of 3.1 and 2.9 log10 ME/100 mL at DSW3 and DSW5, respectively (Figure 3.3). The conditional probability of a correct detection of the human marker (i.e., that the detected marker originates from humans and not from other animals, (Kildare et al. 2007)) indicated almost 100% probability of correct detection. The ruminant- and pig-associated markers were found with an occurrence of approx. 50% and 20%, respectively, with very low concentrations, often close to the detection limit. Despite the low environmental occurrence, the conditional probability of correct detection was between 0.7 and 0.89, due to high marker sensitivity and specificity. The duck-associated marker was found to have a median concentration of 3.1 log10 ME/100 mL and an occurrence of 70% (only DSW5 tested). Positive detections of the duck-associated marker had a conditional probability of correct detection of 0.89. For subsequent analyses, the data of the two Danube River sites was pooled, as no statistically significant difference was found between the two sites in terms of their mean rank of Bray-Curtis dissimilarities (ANOSIM statistic R=0.012, p=0.148). The dissimilarity matrix was calculated based on the combined microbial dataset of FIOs and the human-, ruminant- and pig-associated markers (restricted to the overlapping years: 2013-2017).

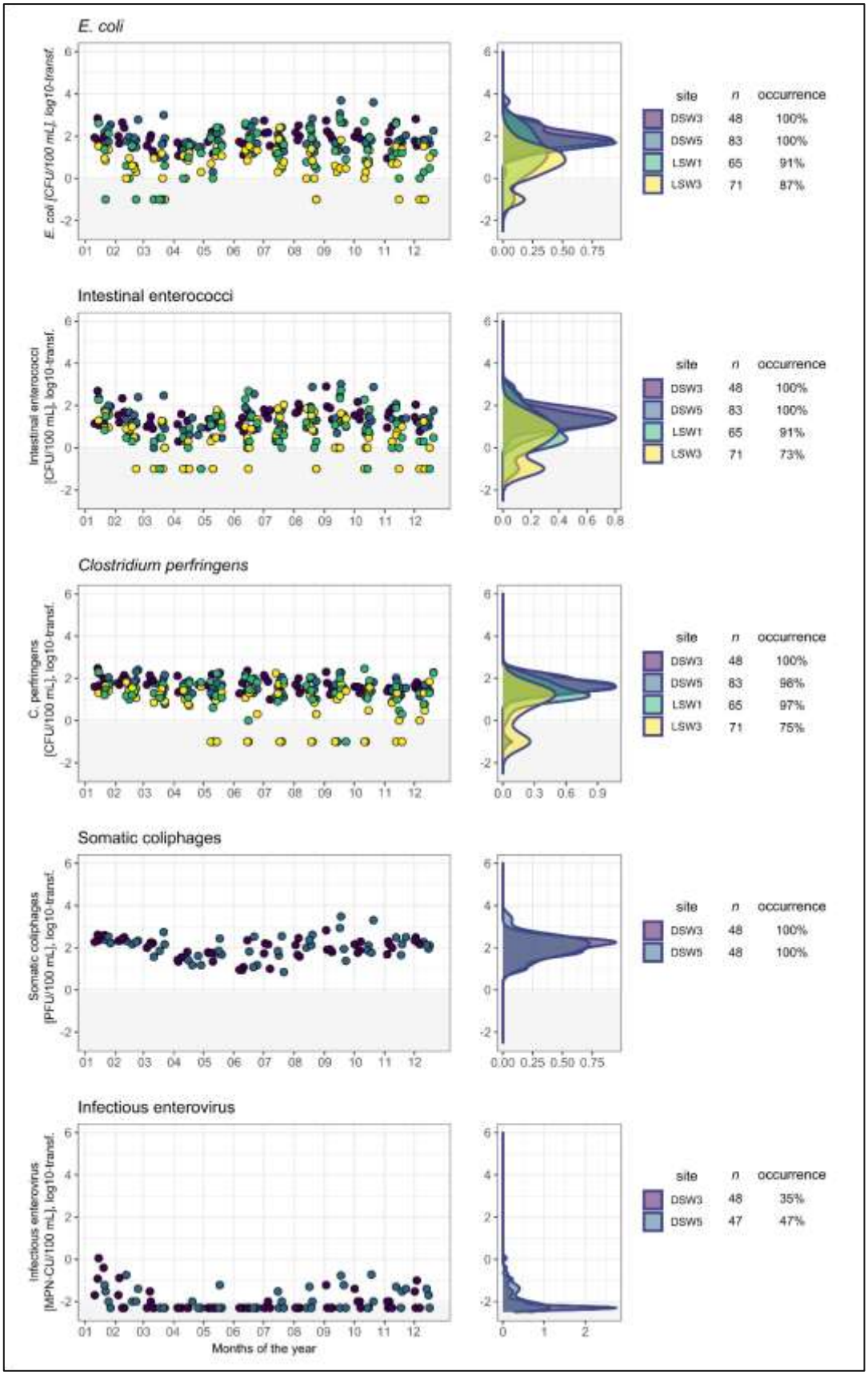


Figure 3.2 Annual pattern and density plot of the monitoring dataset (2010-2017) of faecal indicator organisms *E. coli*, intestinal enterococci, *C. perfringens* and somatic coliphages, as well as infectious enteroviruses. The grey area marks the range below the limit of detection. CFU: colony forming unit; PFU: plaque forming unit; MPN-CU: most probable number of cytopathic unit. For visualisation purposes, non-detects were assigned a constant value below the limit of detection.

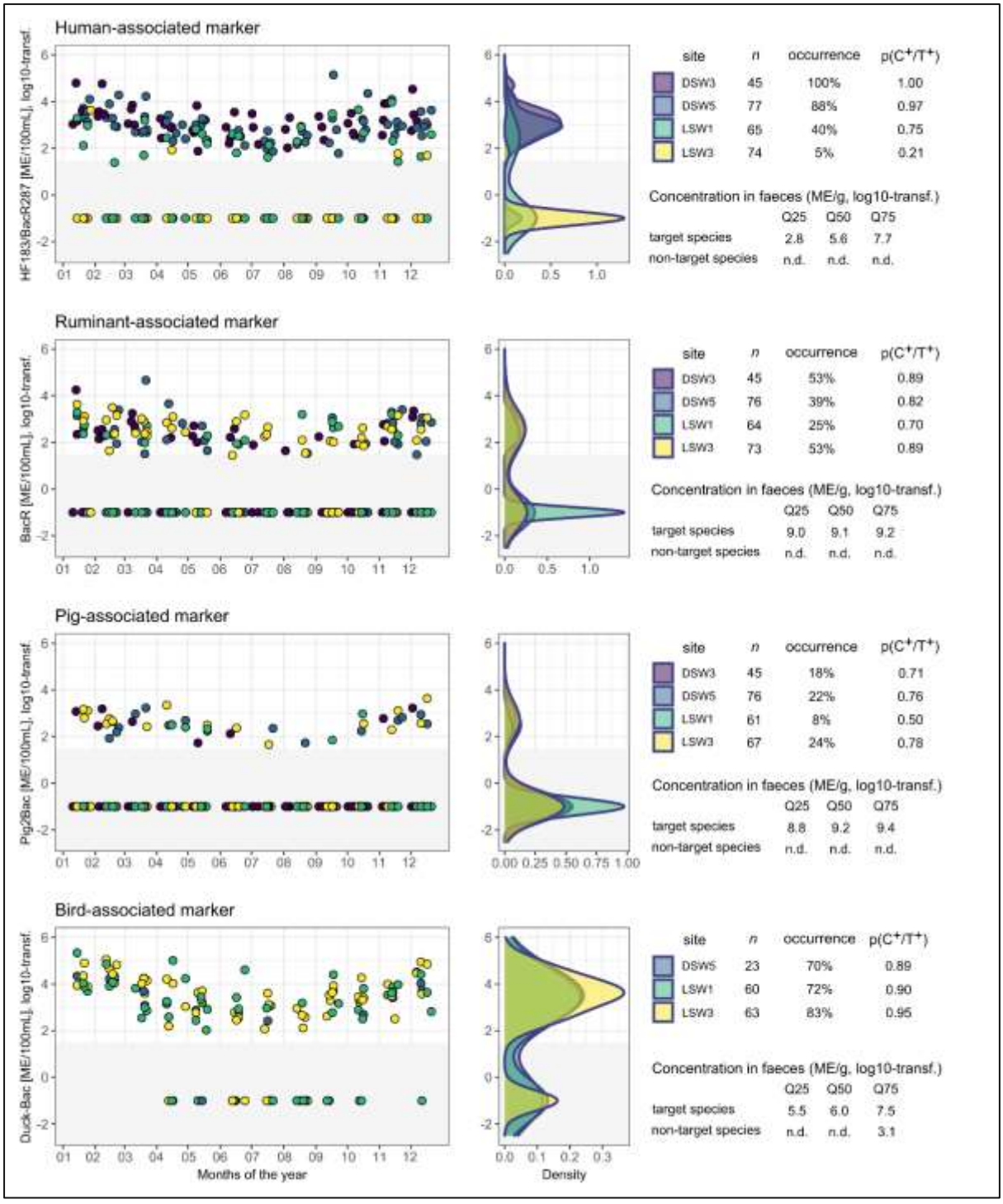


Figure 3.3 Annual pattern and density plot of the monitoring dataset (2010-2017) of human-, ruminant-, pig- and duck-associated MST markers in water samples and their key performance statistics. The grey area marks the range below the median threshold of detection. For visualisation purposes, non-detects were assigned a constant value below the threshold of detection. Q25: 25th quantile; Q50: 50th quantile (median); Q75: 75th quantile; ME: marker equivalent; n.d.: not detected; n.a.: not available; occurrence: occurrence of the MST marker in water samples; $p(C^+/T^+)$: conditional probability that the marker detected originates from the target species instead of a non-target species (Kildare et al. 2007); -SD: mean minus standard deviation; +SD: mean plus standard deviation. Data sources: faecal sensitivity and specificity (for the calculation of the conditional probability) as well as concentrations in faeces for the human-, ruminant- and pig-associated MST markers were published by Mayer et al. (2016) and Steinbacher et al. (2021), and for the duck-associated marker, unpublished data.

Floodplains. The occurrence rates of the human-, ruminant- and pig-associated marker were low with values below 25% at both floodplain sites, with the exception of the human-associated marker in the dynamic backwater (40%, LSW1) and the ruminant-associated marker in the disconnected backwater (53%, LSW3). In positive samples, the concentration values were generally low but occasionally elevated with concentrations of 3.0 to 4.0 log10 ME/100 mL. The conditional probability of correct detection was between 0.21 and 0.89. The duck-associated marker showed a more widespread occurrence with rates of 72% and 83% and median concentrations of 3.0 and 3.4 ME/100 mL (LSW1, LSW3, respectively) as well as a conditional probability of correct detection around and above 0.9 (Figure 3.3)

Annual variability. Across all four sites investigated (both Danube River and floodplain sites), the four MST markers showed a seasonal pattern, with summer concentrations being 0.5 to 1 order of magnitude lower than during the rest of the year. Additionally, all four markers showed large variability (ranges between 2 to 4 orders of magnitude). These two observations point at the role of environmental factors and/or varying faecal inputs into the system (Figure 3.3).

3.3.3 Correlation analyses

In order to further explore the microbiological dataset and to reveal relationships among parameters, correlation analyses were performed. Due to the high proportion of non-detects in the dataset of some MST markers, the Spearman rank method was chosen. The correlation matrices were calculated for the three distinct sites: Danube River (both sites pooled), and the dynamic and the disconnected backwater sites. In the Danube River, the bacterial, viral and spore FIOs as well as the human-associated MST marker were highly correlated, suggesting a common source. In the dynamic backwater site, the FIOs showed high correlations but the human-associated marker had a lower correlation with them than in the Danube River. The ruminant-associated marker correlated with the pig-associated marker. In the disconnected backwater site, the highest correlations were observed among the MST markers (Figure 3.4).

Environmental data, such as precipitation, UV radiation, water temperature, water turbidity and river discharge, were available for the Danube River sites, allowing further insights into the driving forces of pollution dynamics. The microbiological dataset was restricted to those parameters that had an occurrence above 80%. The various faecal indicators, especially the two bacterial FIOs showed a positive correlation with river discharge, turbidity and 48-hour precipitation. Negative correlations were seen between faecal indicators and water temperature as well as UV radiation, most prominently with somatic coliphages and the human-associated MST marker (Figure 3.5).

3.4 Discussion and conclusions

The presented annual pattern assessment and correlation analyses of the large microbiological and environmental dataset (nine microbial and four environmental parameters, 272 samples from four sites) allows an insight into the faecal pollution dynamics.

If we put all the evidence together, a different picture forms for the Danube River than for the floodplain sites. At the Danube River, faecal pollution levels were low to moderate, sometimes critical. High concentrations of the human-associated MST marker and its strong correlation with FIOs suggests that human pollution sources are dominant, especially under base flow conditions. The high occurrence rate of *C. perfringens* spores, a microorganism closely associated with municipal wastewater influence (Vierheilig et al. 2013), also point to human faecal pollution. These findings are in line with the results by Kirschner et al. (2017). The upstream catchment is home to approx. 10 million people, 99% of whom is served by wastewater treatment plants discharging secondary treated sewage into the Danube River or its tributaries (European Commission 2019, Schreiber et al. 2005), which make urban wastewater the main human faecal pollution source. The ruminant- and

the pig-associated marker showed a low occurrence and low environmental concentrations. Since their concentrations are very high in faecal matter (Steinbacher et al. 2021), the low detection of these markers suggest that the faecal load from ruminant and pig sources is probably very low. Additionally, the duck-associated marker was often detected in Danube River samples, suggesting input from wildlife.

In contrast, the floodplain area showed low faecal pollution levels, which is in line with the findings of Frick et al. (2020) at the investigated two sites, however, Frick et al. (2020) also observed a few animal faecal pollution hotspots in other backwaters of the floodplains. The two backwater sites were found to be impacted by all four faecal sources tested to a moderate extent. The dynamic backwater site showed a somewhat elevated occurrence (40%) of the human-associated marker that had significant positive correlations with the three bacterial FIOs.

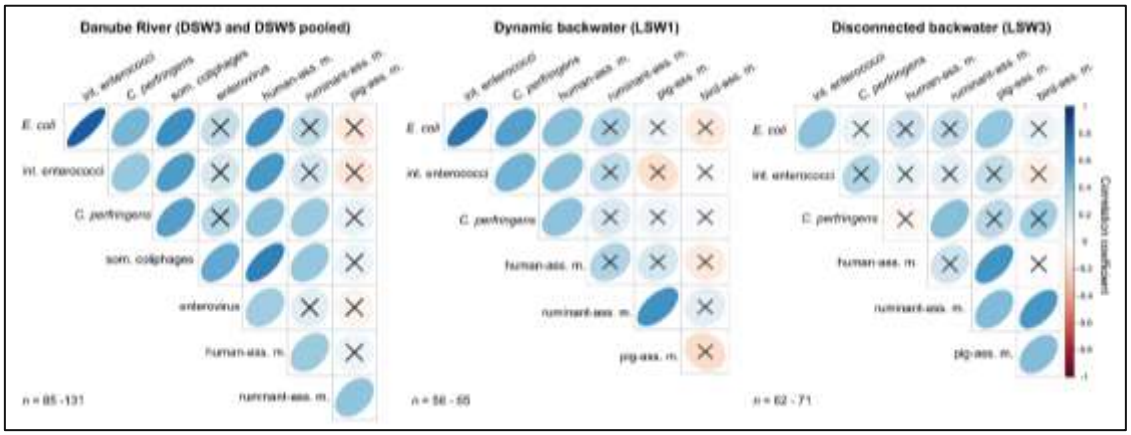


Figure 3.4 Spearman rank correlation matrices among microbial parameters in water samples from the Danube River (left, DSW3 and DSW5 pooled), from the dynamic backwater (middle, LSW1) and the disconnected backwater (right, LSW3) of the floodplain area. The colour gradient indicates the value of the correlation coefficient. The p-values were corrected for multiple testing using the method of Holm (Holm 1979). Non-significant ($p > 0.05$) results are crossed. n indicates the number of data pairs tested.

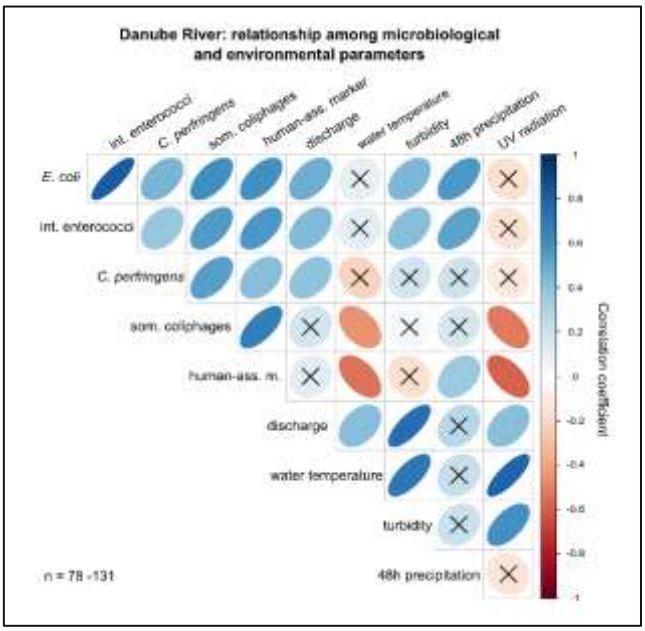


Figure 3.5 Spearman rank correlation matrix among microbial and environmental parameters in water samples from the Danube River (DSW3 and DSW5 pooled, during the period 2013-2017).The colour gradient indicates the value of the correlation coefficient. The p-values were corrected for multiple testing using the method of Holm (Holm 1979). Non-significant ($p > 0.05$) results are crossed. n indicates the number of data pairs tested.

These lines of evidence suggest that the dynamic backwater site is occasionally under the influence human faecal pollution carried by the Danube River. The floodplain area is part of a national park, with no livestock, only wild animals present (Frick et al. 2018b). The pig-associated marker showed very low occurrences and concentrations, while the ruminant-associated marker was detected in over 50% of the samples at the disconnected site, showing that the site is probably occasionally impacted by ruminant wildlife, such as deer. The duck-associated marker was detected with high occurrence and concentrations, in line with the fact that the area is a partially wooded marshland, with significant waterfowl populations. A pollution source profiling study of the area indicated birds as potentially important source of FIO (Frick et al. 2018b).

In consequence, for modelling, (i) at the Danube River, human faecal pollution sources, more precisely, municipal wastewater sources are relevant, and (ii) for the floodplain area, both allochthonous human and autochthonous wildlife sources are important. This information not only guides the factors and compartments to consider in the model, but also indicates which reference pathogen(s) to select for the QMRA (i.e., human pathogens for the Danube River model and zoonotic pathogens for the floodplain model).

4 Modelling the interplay of future changes and wastewater management measures on the microbiological river water quality considering safe drinking water production

4.1 Introduction

Rivers are important for drinking water supply worldwide, yet, they are often under pressure from multiple pollution sources. The most widespread health risk associated with drinking water is contamination with pathogens that originate from faecal matter (WHO 2017b). In densely populated large river catchments, discharges of municipal wastewater treatment plants (WWTP) and combined sewer overflows (CSO) are major contributors to the faecal pollution load (Rickert et al. 2016, WHO 2017b). Additional faecal pollution sources include wildlife and domestic animal waste. Understanding this plethora of pressures and their future changes, along with their impact on the drinking water source, poses a great challenge for water safety planning (Rickert et al. 2016).

The future climatic and population changes may affect faecal pollution sources, the microbiological quality of surface water, and ultimately drinking water safety. Population growth and the associated increase in (treated) wastewater discharges may result in the deterioration of river water quality, and the concerns may be further aggravated by climatic changes (WHO 2017a). According to climate projections, the frequency and intensity of extreme rainfall events will increase in many areas (Myhre et al. 2019). This will result in runoff flushes and, in places with a combined sewer system, more frequent and intense CSO events (Bi et al. 2015, Nie et al. 2009, Willems et al. 2012). In addition, the hydrological regimes and temperatures of rivers are likely to change (e.g., Blöschl et al. 2019), affecting their buffering capacities in terms of dilution and inactivation of pathogens. Droughts and the resulting low river discharges would concentrate contaminants in river and groundwater resources (WHO 2017a), while floods are often accompanied by short-term deteriorations of the water quality due to agricultural runoff and CSOs (e.g., Derx et al., 2013) . In contrast, higher water temperatures expected due to climate change may facilitate the inactivation of enteric pathogens (Boehm et al. 2019). If the pollution sources are mainly urban wastewater discharges, possible strategies to reduce faecal contamination include enhanced wastewater treatment and CSO prevention. As a final step in wastewater treatment, ozonation and advanced oxidation processes as well as UV-treatment and chlorination allow a considerably reduced pathogen load in the final effluent. Measures to prevent CSOs include reservoirs or any form of green infrastructure affecting runoff water quantity and quality at different spatial scales (Golden and Hoghooghi 2018).

The sum of climatic and demographic changes were previously found to deteriorate the microbiological water quality to a limited degree, with less than 0.5 $\log_{10}$ increase in the mean concentration of faecal indicator bacteria or index pathogens until 2040 – 2070, as shown for large rivers in Canada (Jalliffier-Verne et al. 2017, Jalliffier-Verne et al. 2015), Bangladesh (Islam et al. 2018a), Pakistan (Iqbal et al. 2019), and a fictive river in the Netherlands (Sterk et al. 2016). The impact of CSOs and WWTPs on the microbiological water quality of rivers has been analysed from various perspectives. Upstream short-term pollution events, such as via CSO discharges (Taghipour et al. 2019) or WWTP bypass and pumping station overflow events (Sokolova et al. 2015) were shown to be less important for drinking water safety if relying on surface water than the optimal treatment performance of the drinking water treatment plant itself. The simultaneous reduction of multiple faecal inputs in two catchments with high human population and livestock numbers was found beneficial under a sustainable future scenario, in comparison with the uncontrolled future scenario (Iqbal et al. 2019, Islam et al. 2018a). Medema and Schijven (2001) calculated that the majority of *Cryptosporidium* oocysts in Dutch rivers originates from treated sewage, while *Giardia* was rather

linked to untreated discharges, pointing at the different strategies needed to reduce their concentrations in river water. Sterk et al. (2016) found an elevated infection risk through bathing downstream of the discharge point of a WWTP all-year round, while even higher risks, although intermittent, downstream of a CSO. The various measures that can be taken to reduce the input of pathogens into the river have not yet been analysed systematically. Questions also remain as to how climate and demographic changes would alter the effect of these measures.

Assessing faecal pollution dynamics and their possible future developments at the catchment scale is a complex problem as it involves large uncertainties of the source and transport variables (Cho et al. 2016). Most of these microbial fate and transport models focus on faecal indicator organisms (Islam et al. 2018b, Kim et al. 2017), while some also include microbial source tracking markers (MST) enabling source-specific model calibration (Sokolova et al., 2012) or pathogens allowing the assessment of health risks directly (Dorner et al. 2006, Fauvel et al. 2017). Depending on the purpose, the developments range from deterministic, e.g., hydrodynamic, models that simulate water currents requiring much computational effort, to process based pathogen fate and transport models requiring a high number of input parameters (e.g., SWAT, Kim et al. 2017). Recently, studies have combined fate and transport models with quantitative microbial risk assessment (QMRA) to estimate the health risk associated with drinking (Sokolova et al. 2015) or bathing (Eregno et al. 2016, Sterk et al. 2016). QMRAcatch was one of the first models of this kind. Its microbial fate and transport module follows a mass balance approach to simulate microbial concentrations in river water and accounts for the uncertainty of model input variables (e.g., concentration of microorganism in raw wastewater) by using a probabilistic approach. The QMRA module of QMRAcatch simulates the infection risks associated with the ingestion of pathogens contained in drinking water as well as the required treatment to produce safe drinking water (Derx et al. 2016, Schijven et al. 2015).

This study aimed to test a new integrative approach for deciphering the interplay between the effects of climate and demographic changes and wastewater management measures on the microbiological river water quality with regards to the required treatment to produce safe drinking water. We investigated the effects of future climatic and demographic changes up to 2050, as a 'no management changes' scenario, as well as these effects combined with measures that aim to reduce the pollution from upstream WWTPs, CSOs, or both. Additionally, we investigated the effects of increased CSOs in a systematic sensitivity analysis. The approach was tested at a Danube River study site in Vienna, representative of large rivers where the dominant source of faecal pollution is upstream discharges of human wastewater. The scenarios were analysed for two viral reference pathogens: enterovirus and norovirus, which are mainly associated with human wastewater and are often used as references for infection risk assessment from water resources (WHO 2017b). To meet our aim, we significantly extended QMRAcatch (Schijven et al. 2015) (v1.0 Python) now available as open source, which we calibrated and validated based on concentrations of a human-associated genetic MST marker and infectious enteric viruses, measured at the study site monthly over a period of four years. In the scenario analysis, river discharges were simulated using a conceptual semi-distributed hydrological model and four regional climate model projections covering the range of expected climate change pathways.

4.2 Materials and methods

4.2.1 Study area

The study site is located at the Danube River, in Vienna, Austria (Figure 4.1). The Viennese drinking water supply relies partially on water from the Danube River. The Danube River starts in Germany, approx. 850 km upstream, and shows dynamic variations in river discharge, ranging from 900 to 5300 m^3/s for the characteristic low and high discharges, with a mean discharge of 1900 m^3/s at the study site. The region has a temperate climate where floods are driven by snow melts and heavy rainfall events in the headwater catchment. The Danube River is moderately polluted with faecal matter that originates predominantly from human wastewater (Frick et al. 2016, Kirschner et al. 2017). The catchment upstream of Vienna is home to approx. 11 million inhabitants (Schreiber et al. 2005). Considering that 99% of the human population in the study area is connected to a WWTP (European Commission

2018), urban wastewater is the main source of human pollution. This site is therefore representative of a large river polluted by upstream discharges of urban wastewater.

4.2.2 Modelling approach

Two models are applied in this study (Figure 4.2). A hydrological model is used to simulate the river discharge in the Danube for a reference period (2003-2017) and a future period (2035-2049) based on regional climate model outputs (Parajka et al. 2016). The hydrological model domain encompasses the entire Danube subcatchment drained by the Danube up to the study site (104,000 km^2). A newly adapted version of the microbial fate and transport and infection risk model QMRAcatch is used to simulate the concentrations of viruses in river water at the study site and the required reduction of human viruses in source water to achieve safe drinking water (log reduction value, LRV). Its model domain is the 190-km-long Danube River section directly upstream of the study site and includes all sources of human wastewater, represented by: i) the effluent of five WWTPs situated 20, 24, 43, 77, and 193 km upstream of the study site and ii) the corresponding CSOs.

4.2.3 QMRAcatch

Model overview

The probabilistic-deterministic microbial fate and transport and infection risk model QMRAcatch (Schijven et al. 2015) was extended for this study and newly coded as open source (v1.0 Python). QMRAcatch was used to estimate the microbial concentrations and the required reduction of reference pathogens to produce safe drinking water at the study site. The model comprises the functionality of the original Mathematica version *QMRAcatch06062019.cdf* with the following extensions:

- Simulation time over multiple years in contrast to one year in the previous version.
- All model calculations are repeated for 100 to 1000 Monte Carlo simulations until results remain stable, to account for the natural variability and uncertainty of the model input parameters listed in Tables 4.1 and 4.4. In the previous version random values of all stochastic input variables were generated only once for each day in the simulation period.
- Pathogen loads are calculated from the simulated concentrations in river water at the study site.
- One CSO is located at each WWTP, as previously. The CSO discharge volumes and frequencies are now set independently from the continuous WWTP discharges. The days when CSOs occur are set to the days of observed rainfalls (during model calibration and validation) or randomly over the year based on a uniform probability distribution for each Monte Carlo run (scenario and sensitivity analysis).
- The transverse spreading of a continuous point source in a wide river flow was accounted for according to Jirka et al. (2004).
- In contrast to the previous version, the temperature-dependent microbial inactivation coefficients (for which site-specific values are not available) were set during model calibration within constrained limits based on reported persistence data.

Microbial fate and transport module

Faecal inputs. In QMRAcatch, the microbial concentration entering the WWTP (C_{raw}) is multiplied by the fraction of pathogens passing the WWTP (Log_{10} F_{wwtp}) to determine the concentration discharged to the surface water (C_{wwtp}). It is assumed that microbes in water samples at low concentrations are Poisson distributed. In Bayesian inference, the conjugate prior for the rate parameter of the Poisson distribution is the Gamma distribution. C_{raw} is therefore described by a gamma distribution (Table 4.1). The lognormal distribution was used here to describe treatment efficiency of water (F_{WWTP}). It is convenient in that it describes the skewness of the treatment efficiency well and it is easy to interpret. We generated random values for each Monte Carlo run by drawing from these distributions to

reflect the temporal concentration variability. A constant discharge of treated wastewater (Q_{WWTP}) was attributed to WWTPs 1-5 based on previous annual discharge measurements at WWTPs 1-5 and in the rest of the QMRAcatch model domain (Figure 4.1). The WWTPs provide secondary (conventional biological) treatment without chlorination or other tertiary treatment.

During CSO events, untreated wastewater mixed with rainwater discharges into the river. The microbial concentration in CSO water (C_{CSO}) is therefore a fraction of C_{raw}. The yearly CSO volumes were roughly estimated by Thomas Ertl and Florian Kretschmer (personal communication, Clara et al. 2014) based on the mean annual precipitation, the contributing runoff areas, the proportion of the combined sewer system, and the theoretical fraction of water piped to the WWTPs (ÖWAV 2007).

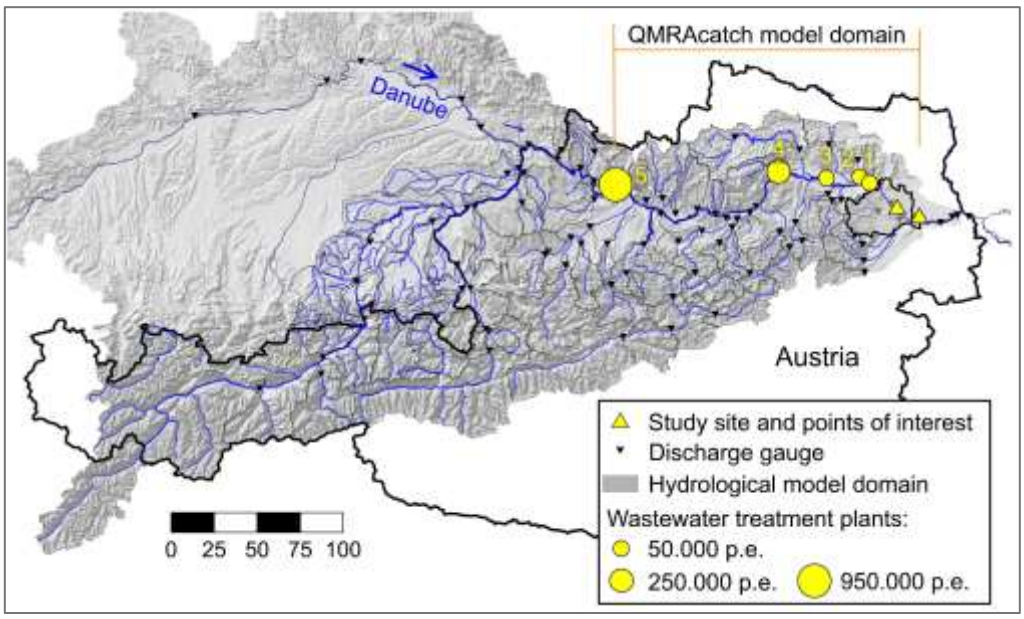


Figure 4.1 Map of the study area

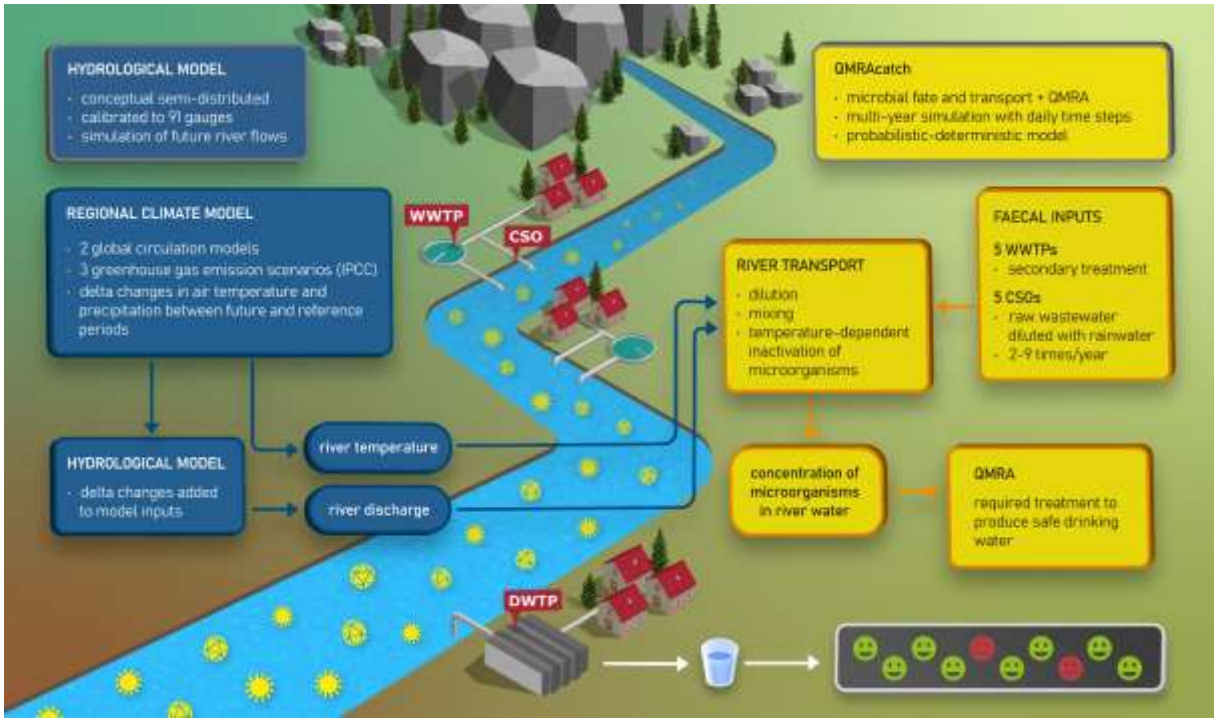


Figure 4.2 Overview of model components.

Table 4.1 Model input parameters for norovirus and enterovirus (see Table 4.5 for the parameters varied in the scenario analysis). Gamma probability distribution function (mean, 95th percentile) of the microbial concentrations in raw wastewater (C_{raw}), normal probability distribution function (mean, 95th percentile) of microbial removal by wastewater treatment (F_{WWTP}), WWTP effluent discharge rate (Q_{WWTP}), inactivation rate coefficients (a_0, a_1), and dose-response parameters α and β.

Parameter	Unit	Distribution	Details	Microorganism	Value	Reference
C_{raw} (mean, 95th perc.)	N/L	Gamma	WWTP 1,2,4,5 WWTP 3	Human MST marker	$(1.84, 5.82) \times 10^9$ $(1.04, 3.52) \times 10^9$	Schijven et al. (2015)
				Enterovirus	$(1.0, 2.0) \times 10^3$	(WHO 2017b)
				Norovirus	$(1.0, 2.0) \times 10^5$	Katayama et al. (2008) Lodder and Husman (2005)
F_{WWTP} (mean, 95th perc.)	Log_{10}	Normal	WWTP 1,2,4,5 WWTP 3	Human MST marker	2.63, 2.15 2.25, 1.39	Derx et al. (2016)
				Enterovirus	1.8, 0.2	This study (calibrated)
				Norovirus	1, 0.75	Lodder and Husman (2005)
Q_{WWTP}	m^3/s	n.a.	WWTP1 to 5	-	0.30, 0.34, 0.17, 0.67, 7.24	EPA Austria (2018)
a_0, a_1	-	n.a.	first order decay in function of water temperature	Human MST marker	0.6, -0.035	This study (calibrated)
				Enterovirus	0.68, -0.036	This study (calibrated)
				Norovirus	2.3, -0.035	Bertrand et al. (2012) (mean value)
α, β	-	n.a.	dose-response relationship: hypergeometric with beta-distributed parameters	Enterovirus	0.253, 0.422	Teunis et al. (1996)
				Norovirus	0.04, 0.055	Teunis et al. (2008)

Dilution in river water. The influx of microorganisms to the river water, through either a WWTP or a CSO, is diluted in river water. The mixing happens gradually as the water flows downstream. To calculate the cross-sectional concentration in the surface waters at X meters downstream of the emission, the transverse spreading of a continuous point source in a wide river flow (W >> h) was accounted for according to Jirka et al. (2004). The distance L_{mh} to the location where complete horizontal mixing over the river cross-section takes place was calculated by

$$L_{mh} = 0.4 \frac{UW^2}{E_y} \quad (1)$$

where U is the flow velocity [m/s] calculated according to Manning (1891) and W is the river width [m] (Table 4.2). The horizontal diffusivity E_y is calculated according to Fischer et al. (1979)

$$E_y = \alpha_y u^* h \quad (2),$$

where α_y is the diffusivity constant with a possible range of 0.5 ± 0.25 [-] for rivers without strong meanders and lateral dead zones, u^* is the friction velocity [m/s] and h is the river depth [m] (Table 4.2). Vertical mixing was computed to be complete after a few hundred meters. The dilutions of microbial loads with river water are then calculated as

$$C_{river} = \sum_{i=1}^{n_{WWTP}} [\frac{(C_{WWTP_i} Q_{WWTP_i} + C_{CSO_i} Q_{CSO_i})}{Q_{river}} \frac{L_{mh}}{X_i}] \quad (3),$$

where Q_{WWTPi} [m^3/s] is the discharge of treated wastewater, Q_{CSOi} [m^3/s] is the CSO discharge, Q_{river} [m^3/s] is the river discharge, and X_i is the distance of the point of interest to the pollution source [m].

Inactivation during transport. The degree of removal of pathogens during transport depends on the travel time or flow rate. Inactivation during transport is described as a first order decay reaction, where the decay rate in water (μ_w [d^{-1}]) during transport is a function of the water temperature (T [°C]):

$$\mu_w(t) = \frac{ln10}{10^{a_0 + a_1 T}} \quad (4)$$

where a_0 [$\log_{10}$ day] and a_1 [$\log_{10}$ day °C^{-1}] are microorganism and pathogen-specific inactivation rate parameters (Bertrand et al. 2012). After a travel time of *m* days to the study site, microorganism concentrations ($C_{m,T}$ [m^{-3}]) are calculated as:

$$C_{m,T} = C_0 exp\left(-\sum_{i=1}^{m} \mu_{W_i}\right) \quad (5)$$

where C_0 is the initial concentration [m^{-3}] and T_i is the temperature [°C] on the i^{th} day. The parameters a_0 and a_1 were determined using linear regression between reported times to first $\log_{10}$ reduction (TFL) versus water temperature. Inactivation rates that were reported in the literature were reviewed and summarized in Figure 4.3.

QMRA module

Daily probabilities of infection for enterovirus and norovirus can be estimated using a hypergeometric dose-response relation (Teunis and Havelaar 2000):

$$P_{inf} = 1 - {}_1F_1(\alpha, \alpha + \beta, D) \quad (6)$$

$$D = C_{RW} \times 10^{LRV} \times I \times V \quad (7)$$

where P_{inf} is the daily probability of infection for a person per exposure, α, β are the parameters of dose-response models (Table 4.1), D is the dose of ingested microorganisms, C_{RW} is the microorganisms' concentration in the river water, LRV is the required treatment reduction of viruses, I is the infectious fraction, and V is the consumed unboiled water volume (1 L/person/day (WHO 2017b)). As the daily health-based target (hbt), $1 \cdot 10^{-6}$ infections/person/day was adopted in this study (Signor and Ashbolt 2009). LRV was estimated iteratively until the criterion $P_{inf} \leq$ hbt according to eq. (6) was fulfilled for both the mean (μ) and 95th percentile values of P_{inf}.

$$\text{LRV} = \max[log_{10}(\frac{\mu^{P_{inf}}}{hbt}), log_{10}(\frac{95\%^{P_{inf}}}{hbt})] \quad (8)$$

The dose-response model parameters for enterovirus were based on results from a human challenge study conducted with rotavirus where the minimum infectious dose for enterovirus was 1 focus forming unit (Ward et al. 1986). We used enterovirus data based on a cell culture method, detecting infectious enterovirus. The fraction of infectious to total viral particles is unknown for norovirus, as their enumeration method is based on PCR methods. However, the frequent occurrence of these viruses in outbreaks suggests high infectivity (Teunis et al. 2008). As the same authors pointed out, the resulting risk estimates might still be unbiased if the ratio of total to infectious numbers of viruses is constant because the exposure estimates in our scenarios are based on the same enumeration methods as in the human challenge study.

Table 4.2 River dimensions and intermediate calculations for dilution of microbial concentrations with river water.

River geometry			
W	Width of the river	250	[m]
h	Depth of the river	4	[m]
s	Slope of the river bed	0.0004	[-]
Constants			
n	Manning coefficient	0.024	[m $s^{-0.3}$]
α_y	Diffusivity constant, calibrated	0.35	[-]
g	Gravitation constant	9.81	[m s^{-2}]
Intermediate calculations			
R_h	Hydraulic radius	$=\frac{W \cdot h}{W+2h}$ =3.9	[m]
U	Average flow velocity (Manning 1891)	$=\frac{R_h^{2/3}\sqrt{s}}{n}$=2.1	[m/s]
u*	Friction velocity	$=\sqrt{g \cdot h \cdot s}$=0.1	[m/s]

4.2.4 *Hydrological model*

To simulate the daily river flow rates of the Danube at the study site, we used a conceptual spatially-distributed hydrological model (Blöschl et al. 2008), which we extended for operational river flow forecasting. The structure is similar to that of the HBV model (Bergström 1976) but several modifications were made including an additional groundwater storage, a bypass flow (Blöschl et al. 2008, Komma et al. 2008), and a modified routing routine. For each raster cell (5 km x 5 km), snow processes, soil moisture processes, and hill slope scale routing are simulated at an hourly time step. In the snow routine, snow accumulation and melt are represented by a simple degree-day concept. Runoff generation and changes in soil moisture storage are calculated by a soil moisture accounting scheme as a nonlinear function of rainfall and evaporation. Runoff is generated as a combination of outflows from three reservoirs representing overland flow, interflow, and deep groundwater flow processes. Runoff routing in the stream network is described by a cascade of linear reservoirs (Szolgay 2004). More details and application examples are given, e.g., in Blöschl et al. (2008), Komma et al. (2008), and Reszler et al. (2008).

In the extended version, the model input consists of spatially distributed fields of precipitation, air temperature, and potential evaporation. Meteorological and hydrological data were available for the period from 2003 to 2017 (provided by ZAMG and HZB, the central services for meteorology and hydrology in Austria). The data set includes several extreme flow periods, such as the 200-year flood in 2013 or the drought in 2015. The existence of hydro-meteorological extremes in the data set helps to estimate more robust and appropriate model parameters for predictions and extrapolation to future scenarios. For parameter identification, a hydrologic response unit approach based on spatial information about land use, soils, hydrogeology, and topography combined with a manual calibration procedure (Reszler et al. 2008) has been adopted. In this study, 15 different hydrologic response unit types were used to describe the different hydrological response behaviours. The parameters have been calibrated and validated against hourly discharge data at 91 discharge gauges at the Danube and its tributaries. The simulation period included a calibration (2012 to 2018) and a validation period (2003 to 2011). The Nash and Sutcliffe coefficient of runoff model efficiency (Nash and Sutcliffe 1970) at the 91 discharge gauges ranged between 0.72-0.84 for the calibration and between 0.66-0.85 for the validation period. The model efficiency for the calibration and validation period at the Danube gauge was 0.78 and 0.73, respectively.

4.2.5 Microbial characterization of wastewater and river water

Microbiological analyses of wastewater. Values for C_{raw} and C_{wwtp} of the human-associated MST marker at WWTP2 and 3 were available from single 1-L samples collected from 2010 to 2013 (n=72, Schijven et al., 2015, Table 4.1,). Additionally, values for C_{wwtp} of enterovirus at WWTP2 were isolated from 1-L samples between 2011 and 2017 (n=73) according to the inorganic flocculation and ultracentrifugation method of Walter and Rüdiger (1981) and enumerated following an MPN method (Chang et al. 1958) on Buffalo green monkey kidney cells (Dahling and Wright 1986). For the concentrations of microorganisms in wastewater, QMRAcatch estimates the parameters of a Gamma distribution based on mean and 95th percentile values. The enterovirus concentrations were MPN values, i.e., maximum likelihood estimates, assuming Poisson distributed count observations. Given that these are concentration estimates already, concentration estimates of zero could be included in the calculation of the mean and 95th percentile values. We assumed a mean and 95th percentile of 1×10^3 and 2×10^3 MPN/L for C_{raw} of enterovirus, respectively (WHO 2017b). F_{wwtp} was adjusted until the mean and 95th percentile of generated random values of C_{wwtp} matched the respective observed values (Table 4.1). Mean C_{raw} and F_{wwtp} values of 1×10^5 gene copies (gc)/L and 1-$\log_{10}$ for norovirus were assumed, respectively, according to Katayama et al. (2008) and Lodder and Husman (2005) (Table 4.1).

Microbiological analyses of river water. Surface water samples were collected from the Danube riverbank as grab samples at two sampling points (Figure 4.1) alternately on a monthly basis over four years, resulting in a bi-weekly dataset of independent values (2013-2017, n=87-94). Because of the vicinity of the two points, they were treated as one point of interest for drinking water production, called hereafter 'the study site' (Figure 4.1). The samples were analysed for the human-associated MST marker HF183/BacR287 as well as for infectious enterovirus. The human-associated MST marker was quantified in 500–600-mL water samples using quantitative PCR as described previously (Green et al. 2014, Mayer et al. 2018a). The human-associated MST marker was detected in all samples with a median concentration of $1.1\text{x}10^4$ ME/L (marker equivalent per litre) and a range of $4.0\text{x}10^2$ to $1.4\text{x}10^6$ ME/L (n=87, Figure 4.4). The filtration volume, the use of 2.5-µL of undiluted DNA extract in qPCR, and the minimal theoretically detectable marker concentration per reaction defines the detection threshold ($3.0\text{x}10^2$ ME/L) (Reischer et al. 2008). Infectious enteroviruses were enumerated from 10-L water samples according to the method described above. Enteroviruses were detected in 42% of the samples with a maximum concentration of 11.3 CU-MPN/L (cytopathic unit, most probable number, n=94). The limit of detection was 0.1 CU-MPN/L.

4.2.6 Calibration, validation, and application of QMRAcatch

Due to the limited availability of pathogen data in river water, we took a three-step approach for the calibration, validation and application of QMRAcatch: (1) model calibration and validation using the source-specific and highly abundant human-associated MST marker by adjusting the calibration parameters; (2) model calibration and validation using the reference pathogen enterovirus by adjusting just the microorganism- and virus-specific calibration parameters (the others taken over from step one); and (3) application of the calibrated and validated model to various pathogens (enterovirus and norovirus) by using measured data or values assumed from the literature as model inputs (overview in Table 4.3, data in Table 4.1).

The datasets of observed concentrations of the human-associated MST marker and enterovirus (n=87 and 94, respectively) were split into two time periods. Data for the period from July 2013 to June 2015 was used for calibration, and for the period from July 2015 to June 2017 for validation, for both microorganisms (Table 4.3). Non-detects were set to the limit of detection in the calculations.

The mean absolute error (MAE) was used as a performance metric (Willmott and Matsuura 2005). Log_{10} transformed concentrations were used in the MAE computations because microorganisms typically follow a lognormal distribution, and the use of logarithms minimizes the influence of outliers present in the data (Hong et al. 2018). The Mann-Whitney test was used for the distribution comparisons of the simulated and observed

datasets and the p-value of the Mann-Whitney statistic was a metric of model performance. During model calibration, the calibration parameters were adjusted to minimize the objective function (OF).

$$OF = MAE + (1 - p) \quad (9)$$

The calibration parameters can be grouped into (i) not faecal microorganism and pathogen-specific and (ii) faecal microorganism and pathogen-specific parameters. The parameters of the first group describe the discharge and mixing processes which are assumed to be the same for faecal indicators and pathogens in river water. These are the constant diffusion coefficient α_y (Table 4.2, eq. 1 and 2), the frequency and discharge volumes of CSOs 1-5, and the microbial concentration in CSO water as a fraction of the concentration in raw wastewater. The parameters of the second group are the microorganism- and virus-specific inactivation parameters a_0 and a_1. In the first step (calibration with the human-associated MST marker), calibration parameters of both groups were adjusted, and the best combination used for the validation. In the second step (calibration with enterovirus), the calibrated values of the not microorganism- or virus-specific parameters were taken over from step 1 and kept constant, and only the microorganism- or virus-specific parameters were adjusted. The best combination of the calibration parameter settings was used for the validation. The CSO volumes per year were constrained to $\pm$ 25% of the yearly estimates (Section 4.2.3). The CSO events were constrained to days when the daily amount of rainfall exceeded 13 mm/d. Recorded precipitation data were used at gauges Vienna Hohe Warte (at WWTPs 1 and 2), Langenlebarn (at WWTP 3), Krems (at WWTP 4), and Linz-City (at WWTP 5, ZAMG, 2018). Inactivation rates of the human-associated MST marker, enterovirus, and norovirus that were reported in the literature were collected and summarized in Figure 4.3. Boehm et al. (2019) conducted an extensive literature review on inactivation studies for viruses and conducted a multiple linear regression analysis between environmental variables and first-order decay rates. Enterovirus inactivation rates showed a statistically significant relationship with temperature, method and sunlight, therefore we restricted our selection to studies conducted with cell culture (so that they are comparable to our results, see Section 4.2.3) and in natural or artificial sunlight. The coefficient 'water type' was not significant in the multiple linear regression, therefore we included all water types (marine and estuarine – no study was conducted in freshwater in sunlight). Norovirus only showed a significant relationship with temperature so we included all studies listed by Boehm et al. (2019). An ordinary least square method was used to fit the time-to-first-log (TFL) as a function of water temperature (dashed lines in Figure 4.3). During the adjustment of intercept a_0 and slope a_1 (used in eq. 4, solid lines in Figure 4.3), it was ensured that the inactivation as function of temperature obtained through the calibration lay within the prediction interval of the ordinary least square regressions (Figure 4.3, shaded area, left and centre).

Table 4.3 A tiered approach to the application of the model to various targeted microorganisms and pathogens. n.a.: not applied.

Microorganism/pathogen	QMRAcatch calibration	QMRAcatch validation	Scenario simulations
human-associated MST marker	Yes dataset 2013-2015	Yes dataset 2015-2017	n.a.
enterovirus	Yes dataset 2013-2015	Yes dataset 2015-2017	Yes, input data partially measured, partially from literature
norovirus	n.a.	n.a.	Yes, input data from literature

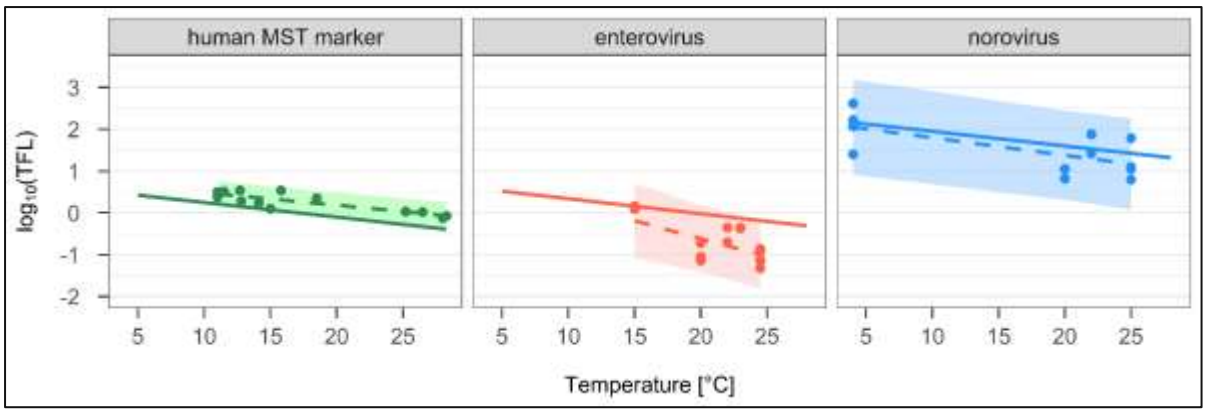


Figure 4.3 Inactivation of the human-associated MST marker, enterovirus, and norovirus as applied in this study (solid lines) and as reported in experimental studies (dots), plotted as time to first $\log_{10}$ reduction (TFL, days) values $\log_{10}$-transformed ($\log_{10}$(TFL)) in function of the temperature. Ordinary-least-square regressions (dashed lines) were fitted to the literature values, shown with their 95% prediction intervals (shaded). The intercept and the slope of the solid lines were the result of the model calibration for the human MST marker and enterovirus, and are the reported values of Bertrand et al. (2012) for norovirus. These values were used as model input parameters a_0 and a_1 in eq. 4 (Table 4.1).

4.2.7 Scenario analysis

We defined a reference scenario and the following future scenarios: i) climate and demographic changes and ii) three scenarios of wastewater management measures that aim to reduce the faecal load from WWTPs and CSOs. Table 4.4 provides an overview of the scenarios. Additionally, we conducted a sensitivity analysis to investigate the impact of CSO changes. For all scenarios and the sensitivity analysis, the concentrations of enterovirus, norovirus, *Cryptosporidium* and *Campylobacter* in the Danube were simulated using the input settings as described in Tables 4.1, 4.5 and S3. Subsequently, a QMRA was conducted for assessing the required LRV to achieve the health-based target.

Reference scenario

We simulated the concentrations of norovirus and enterovirus in river water at the study site, as well as the required LRVs to produce safe drinking water for the reference period from 2003 to 2017. This period included hydrologically extreme years and was therefore deemed a robust basis for the scenario analysis (Tables 4.4 and 4.5).

Table 4.4 Overview of the scenario analysis. +: taken into account, -: not applicable / not applied.

Scenario	Climate change (river discharge and temperature)	Population growth (WWTP discharge)	Enhanced wastewater treatment	CSO prevention
Reference	-	-	-	-
No management changes	+	+	-	-
CSO prevention	+	+	-	+
Enhanced wastewater treatment	+	+	+	-
CSO prevention and enhanced wastewater treatment	+	+	+	+

Future scenarios

Climate and demographic changes: 'No management changes' scenario

In this study, flow projections of future climate scenarios were modelled using a hydrological model forcing from a delta change approach as described in detail by Parajka et al. (2016). In a first step, regional climate model (RCM) outputs were used to estimate monthly differences in air temperature and precipitation between reference (control) and future periods (2035 – 2049). These differences (delta changes) were then added to the observed precipitation and air temperature data and used as model inputs to simulate future hydrologic changes. The daily precipitation was scaled by the relative delta changes for each month, and the frequency of rainy days was kept as in the reference period. The daily air temperature was changed by the mean daily delta changes each month. To obtain future delta changes in water temperature, the delta changes of daily air temperature were multiplied by seasonal conversion factors derived from the observed changes in air and water temperatures of the Danube from 1900 – 2010 (Standhartinger and Godina 2013, p. 46, Fig. 11). The conversion factors for December, January, and February resulted in 1.22; for March, April, and May they resulted in 0.52; for June, July, and August they resulted in 0.76; and for September, October, and November they resulted in 1.5.

The RCM scenarios used in this study are based on the results of the reclip:century project (Loibl et al. 2011, Parajka et al. 2016). The ensemble climate projections are represented by COSMO-CLM RCM runs forced by the ECHAM5 and HADCM3 global circulation models for three different Intergovernmental Panel on Climate Change (IPCC) emission scenarios (A1B, B1, and A2; Nakicenovic et al. 2000). These represent a large spread of different emission pathways based on no change in greenhouse gas emission practices (A2), a scenario with a moderate decline in emissions after 2050 (A1B), and a scenario indicating considerably reduced emissions from the present onwards (B1). For this study, the projections by the ECHAM5 model were selected for the three emission scenarios (A1B, A2, B1), as well as the projections by the HADCM3 model for one emission scenario (A1B).. Although these scenarios are meanwhile replaced by the Representative Concentration Pathways (RCPs, van Vuuren et al. (2011)) these older scenarios are still comparable to the newer RCPs with respect to their climate change signals. Moreover, the model setup for RCM simulations of reclip:century are specifically tailored for the complex terrain of the Alpine Region and therefore provide more robust estimates of the future climate change in the Alps and surrounding areas (Blöschl et al. 2018, Blöschl et al. 2017).

The reclip:century scenarios project, for the study area, changes in air temperature and precipitation between the future period 2035-2049 and the reference period 2003-2017. Precipitation projections show that winters will become 5% (HADCM3 A1B) to 22% (ECHAM5 A2) wetter and extreme precipitation quantities will increase. Predictions of future precipitation changes for summer range from 4% (HADCM3 A1B) to -21% (ECHAM5 A2). These changes are generally in line with the newer generation of RCM simulation from the EURO-CORDEX initiative where an ensemble of simulations for RCP4.5 and 8.5 are downscaled for the Austrian domain. Only the EURO-CORDEX ensemble mean summer precipitation change signal for Austria is +3% for RCP8.5, showing somewhat different results compared to ECHAM5 A2, however, the ensemble spread in EURO-CORDEX is rather large pointing towards higher uncertainties during the summer season.

For the study site of the Danube, all climate scenarios project a general decrease of river flows during the low flow period (summer) and a slight increase or no change of river flow during the high flow period (end of winter/spring). The river flow in the Danube study catchment is expected to decrease on average by 14% (ECHAM5 A1B) to 25% (ECHAM5 A2), with a slight increase of about 10% - 15% in January and February for the ECHAM5 A1B scenario and an almost 50% decrease in August for the ECHAM5 A2 scenario.

Population growth will result in a corresponding increase in urban wastewater discharges into the Danube. An increase in wastewater discharge volumes by 14% until 2050 was considered at WWTPs 1-5, according to the projected population growth of Lower Austria, the state covering the majority of the model domain (Austria 2017, Tables 4.4 and 4.5).

Scenarios of wastewater management measures

'Enhanced wastewater treatment' scenario. The current EU regulations for WWTPs require a reduction of organic carbon, nitrogen, and phosphorous, but there are no microbiological requirements or obligations for disinfection (European Commission 1998). A possible strategy to reduce the load of pathogens from WWTPs is to include ozonation and/or UV irradiation as a tertiary wastewater treatment. According to previous reports, the efficiency of disinfection during wastewater treatment on reducing virus concentrations can remarkably vary, depending on the dose of chemicals or the UV fluence, from 1.5 to 4 $\log_{10}$ particles/L by ozonation (Gerrity et al. 2012, Owens et al. 2000, Paraskeva and Graham 2002) or UV irradiation (Campos et al. 2016, Francy et al. 2012). We considered an additional treatment step at at WWTPs 1–5 that reaches a reduction of entero- and norovirus by 4 $\log_{10}$ in the scenarios (Tables 4.4 and 4.5).

'CSO prevention' scenario. A further possible measure is to prevent CSO events using, for example, stormwater reservoirs, retention basins, rain barrels, green roofs, permeable patios, or grassed swales (Demuzere et al. 2014, Lewellyn et al. 2016, Pazwash 2016). We assumed that the measures are capable of completely preventing CSOs (Tables 4.4 and 4.5).

'CSO prevention and enhanced wastewater treatment' scenario. A combination of the above two wastewater management measures was considered in the fourth future scenario.

4.2.8 Sensitivity analysis to investigate the effects of increased storm events

The extreme storm event frequency is thought to increase with warming at a rate similar to the water vapour holding capacity of the air, the so-called Clausius-Clapeyron rate, at ~ 7% / °C (Molnar et al. 2015). CSOs are therefore likely to happen more frequently, but their reaction to changes in rainfall is highly non-linear (Willems et al. 2012). Considering this high uncertainty, we did not include an increased rate or intensity of CSOs in the future scenarios (they were kept the same as in the reference scenario) but conducted a sensitivity analysis to investigate how changes in CSO discharge volumes and frequencies would modulate the future scenarios. We took the 'no management changes' and 'enhanced wastewater treatment' scenarios as baselines. The above-listed two variables were varied individually, while the settings for all other parameters were kept the same as those for the baselines (Table 4.5).

4.3 Results

4.3.1 Model calibration and validation

The QMRAcatch model was calibrated and validated in two consecutive steps: First, against data on the human-associated MST marker and second, against data on enterovirus measured at the study site (Table 4.3). From the calibration parameters, the model proved to be the most sensitive to the microorganism and virus-specific inactivation rate parameters a_0 and a_1 during the manual calibration. The parameters were constrained so that the resulting time to first log reduction versus water temperature function remained within the 95% prediction interval of the regression line fit to experimental values for both microorganisms (Figure 4.3 and Table 4.1).

The Mann-Whitney tests indicated that the simulated and observed concentrations were not significantly different for the human-associated MST marker and enterovirus ($p>0.05$, Table 4.6). The cumulative distribution plot of the simulated and observed concentrations are shown in Figure 4.4.

The model errors within the 5-95th percentiles ranged from -1.3 to 1.3 $\log_{10}$ N/L for the human-associated MST marker and from -1.1 to 1.5 $\log_{10}$ N/L for enterovirus. The error distributions were very similar for the calibration and validation periods. The OF values were almost the same in the validation period as in the calibration period for the human-associated MST marker. The OF values for enterovirus were the same as for the human-associated MST marker for the calibration period, but slightly higher for the validation period.

Table 4.5 Model input parameters for the reference scenario (2003 – 2017) and future scenarios (2035 – 2049) as well as for the sensitivity analysis, for the study site in the Danube.

Parameter	*Dimension*	*Reference scenario*	*Description of future change*	*Future scenarios / Sensitivity analysis*
Population growth and climate change				
Effluent discharge at WWTPs 1-5	m^3/s	See Table 4.1	Population growth	+14% (Austria 2017)
Daily river discharge at study site	m^3/s	Hydrological modelling for period 2003-2017	Climate scenarios ECHAM5-A1B, A2, B1, HADCM3-A1B (Loibl et al. 2011, Parajka et al. 2016)	Hydrological modelling from 2035-2049
Daily river water temperature	°C	Data records at Danube gauge Greifenstein from 2003-2018 (viadonau, 2019)		Delta changes in air temperature and season-specific conversion factors (Sec. 2.4.2.1)
Changes due to wastewater management measures				
Log_{10} reduction by wastewater treatment (F_{wwtp})	Log_{10}	See Table 4.1	Additional treatment	+ 4 (Campos et al. 2016, Francy et al. 2012, Gerrity et al. 2012, Owens et al. 2000, Paraskeva and Graham 2002)
CSO frequency	N/year	At WWTP 1: 2 At WWTP 2: 2.5 At WWTP 3: 9.5 At WWTP 4: 5.5 At WWTP 5: 4 (calibrated)	Complete prevention of CSOs through, e.g., reservoirs	0
CSO discharge at WWTPs 1-5	m^3/s	At WWTP 1: 0.89 At WWTP 2: 1.01 At WWTP 3: 0.52 At WWTP 4: 2.00 At WWTP 5: 21.73 (calibrated)	Complete prevention of CSOs through, e.g., reservoirs	0
Sensitivity to changes in CSOs				
CSO frequency at WWTPs 1-5	N/year	Calibrated (see above)	More frequent extreme rainfall events	up to 3-fold increase
CSO discharge at WWTPs 1-5	m^3/year	Calibrated (see above)	More frequent extreme rainfall events	up to 3-fold increase
Concentration of pathogens in CSO water	-	0.1 (calibrated)	Fraction of the concentration in raw wastewater	0.1 and 1.0 (de Man et al. 2014, Sterk et al. 2016)

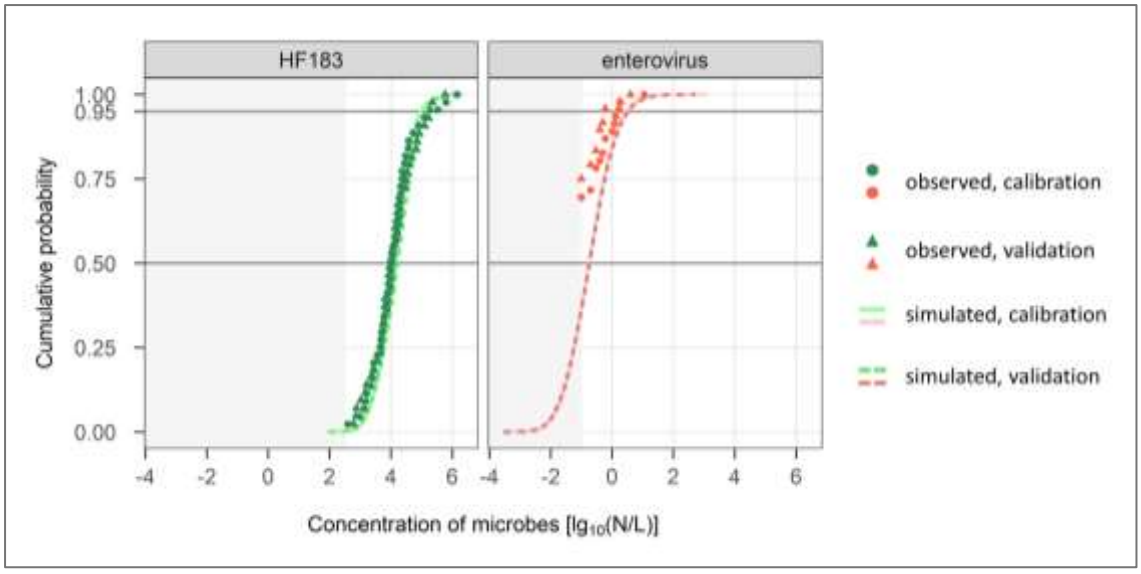


Figure 4.4 Simulated and observed concentrations of the human-associated MST marker and of enterovirus during both calibration and validation (pooled dataset shown). The light grey area marks values under the detection threshold for the human-associated MST marker (qPCR) and under the limit of detection for enterovirus (MPN method).

Table 4.6 Model performance for simulated microbial concentrations at the study site after 1000 Monte Carlo runs.

	Parameter	Time period	n (n of detects)	Mann-Whitney test, p-value	Mean absolute error [log_{10} (N/L)]	Objective function (eq. 9)
Calibration	Human MST marker	2013 - 2015	44 (44)	0.61	0.54	0.9
	Enterovirus	2013 - 2015	46 (17)	0.75	0.62	0.9
Validation	Human MST marker	2015 - 2017	43 (43)	0.59	0.63	1.0
	Enterovirus	2015 - 2017	48 (22)	0.13	0.64	1.5

4.3.2 Scenario analysis: Virus concentration and required LRV

QMRAcatch was applied to simulate the reference and four future scenarios using the calibrated settings but with river flows and temperatures as simulated by the regional climate and hydrological models (Figure 4.2, Tables 4.4 and 4.5). For these scenarios, we simulated pathogen concentrations in river water at the study site and calculated the required treatment reduction (LRV) of pathogens from river water for the production of safe drinking water. The viral reference pathogens norovirus and enterovirus were the focus of the analysis.

Reference. For the reference period, the median and range of concentrations of enterovirus at the study site were -0.55 (-2.84 to 2.54) log_{10} N/L, while they were 3 orders of magnitude higher for norovirus: 2.30 (1.23 to 3.23) log_{10} N/L (Figure 4.5). The required LRV was 6.3 and 8.4 log_{10} for enterovirus and norovirus, respectively (Figure 4.6).

Future climate, population, and no management changes. Four regional climate scenarios were tested, covering the entire range of expected climate pathways. They showed similar results in terms of the simulated concentrations of enterovirus and norovirus in river water as well as the LRVs (Figure 4.7). The climate scenario ECHAM5 A2, based on no efforts taken to reduce greenhouse gas emissions, was chosen as the basis for all future scenarios. Based on this climate scenario, the discharge of the Danube River at the study site will be up to 50% lower during low flows (summer-autumn), while only slightly higher during high flows (late winter-spring). The temperature of river water will be 2°C higher on average (Figure 4.8).

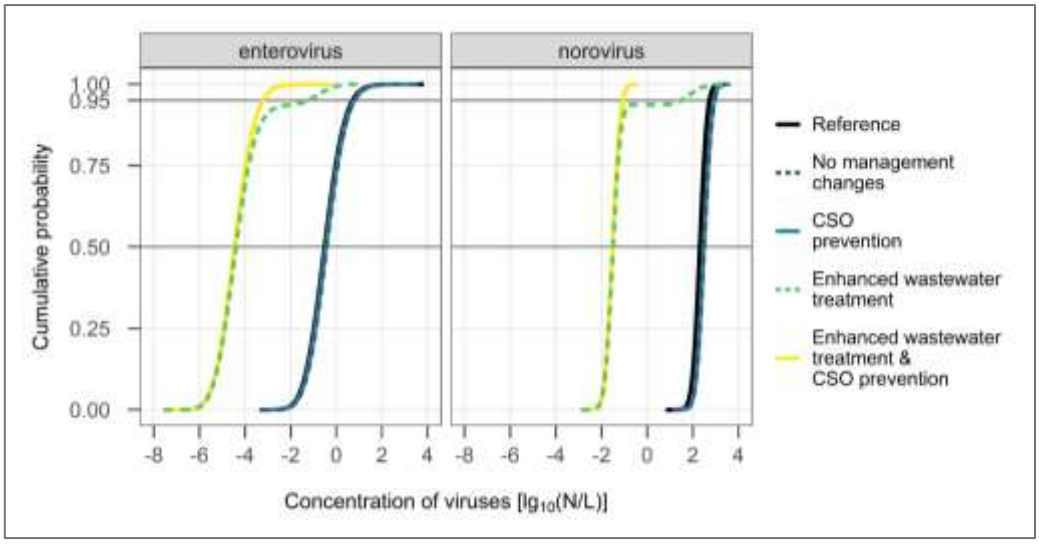


Figure 4.5 Scenario simulations for norovirus and enterovirus, simulated concentrations in river water. The 'CSO prevention' scenario entirely overlaps with the 'no management changes' one.

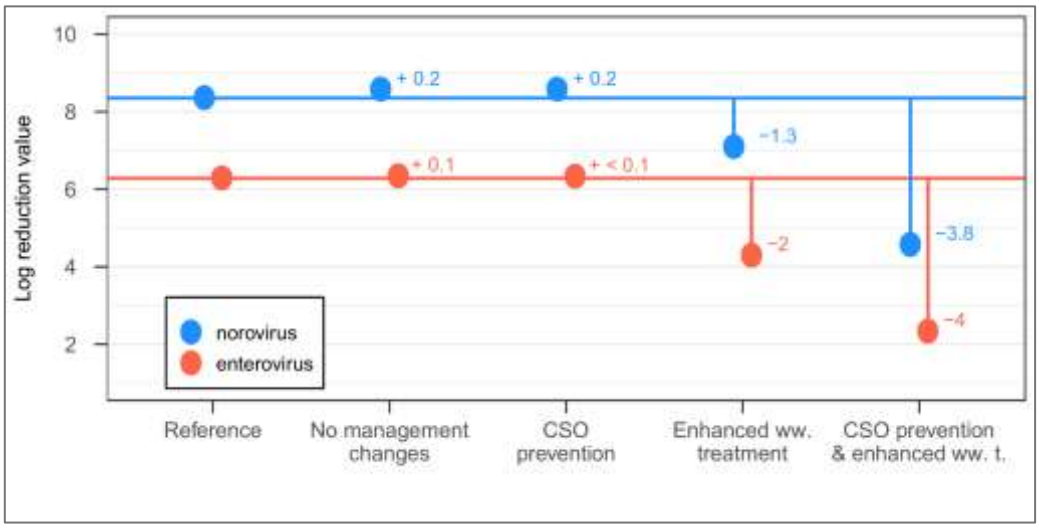


Figure 4.6 Scenario simulations for norovirus and enterovirus, required virus log reduction values for safe drinking water.

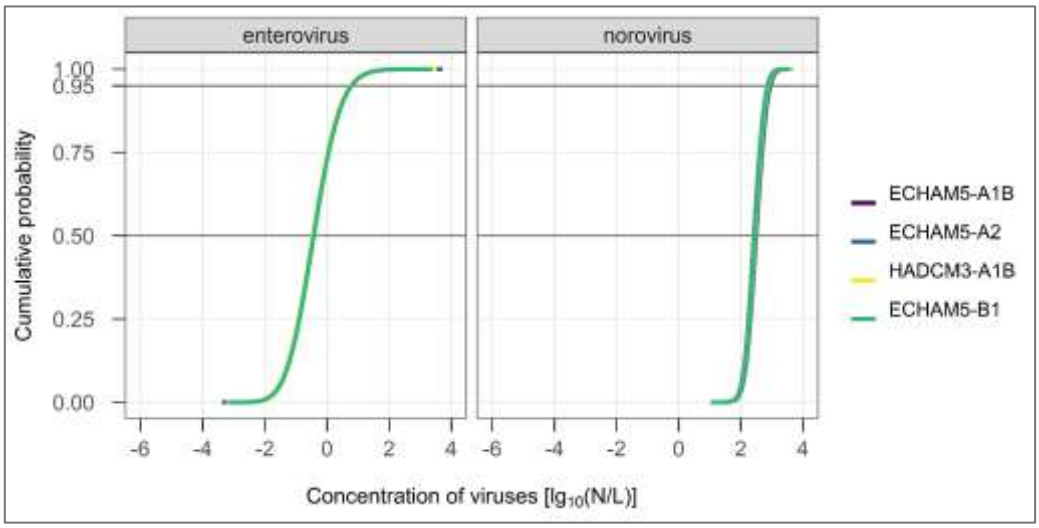


Figure 4.7 Simulated concentrations of norovirus and enterovirus in river water according to the four climate scenarios considered in this study.

In comparison to the reference period, the projected climatic and demographic changes showed a negligible effect on the concentrations of enterovirus and norovirus in river water as well as on the required LRVs (Figures 4.5 and 4.6).

Future climate, population, and prevention of CSOs. While preventing CSO events precluded a few peaks of virus concentration in the river, it did not affect the overall distribution of concentrations, which remained similar to the reference and 'no management changes' scenarios (Figure 4.5). The LRVs were not affected either (Figure 4.6).

Future climate, population, and enhanced wastewater treatment. The installation of a tertiary treatment step at the five WWTPs (assumed effect: 4 $\log_{10}$) reduced the median concentrations of enterovirus and norovirus in river water by 3.9 and 3.8 $\log_{10}$, respectively, compared to the reference scenario. However, the maximum concentrations were only slightly reduced (1.9 and 0.1 $\log_{10}$ lower), and a batch of virus concentration peaks 3 to 5 $\log_{10}$ higher than the median remained (Figure 4.5). The LRVs for enterovirus and norovirus were 2.0 and 1.3 $\log_{10}$ lower than in the reference scenario (Figure 4.6).

Future climate, population, and combination of enhanced wastewater treatment and prevention of CSOs. The measure reduced both the median and maximum virus concentrations at the study site by approximately 4 $\log_{10}$ compared to the reference scenario (Figure 4.5). The LRVs were 3.9 and 3.8 $\log_{10}$ lower than in the reference scenario (Figure 4.6).

4.3.1 Sensitivity of future scenarios to uncertainties in CSO predictions

Considering the lack of information regarding the effect of climate change on CSOs, we conducted a sensitivity analysis to see how changes in CSOs would modulate the two future scenarios with CSO events ('no management changes' and 'enhanced wastewater treatment'). We varied two factors: the frequency of CSO events and the volume of CSO events (up to a 3-fold increase compared to the reference). We calculated the LRVs for enterovirus and norovirus.

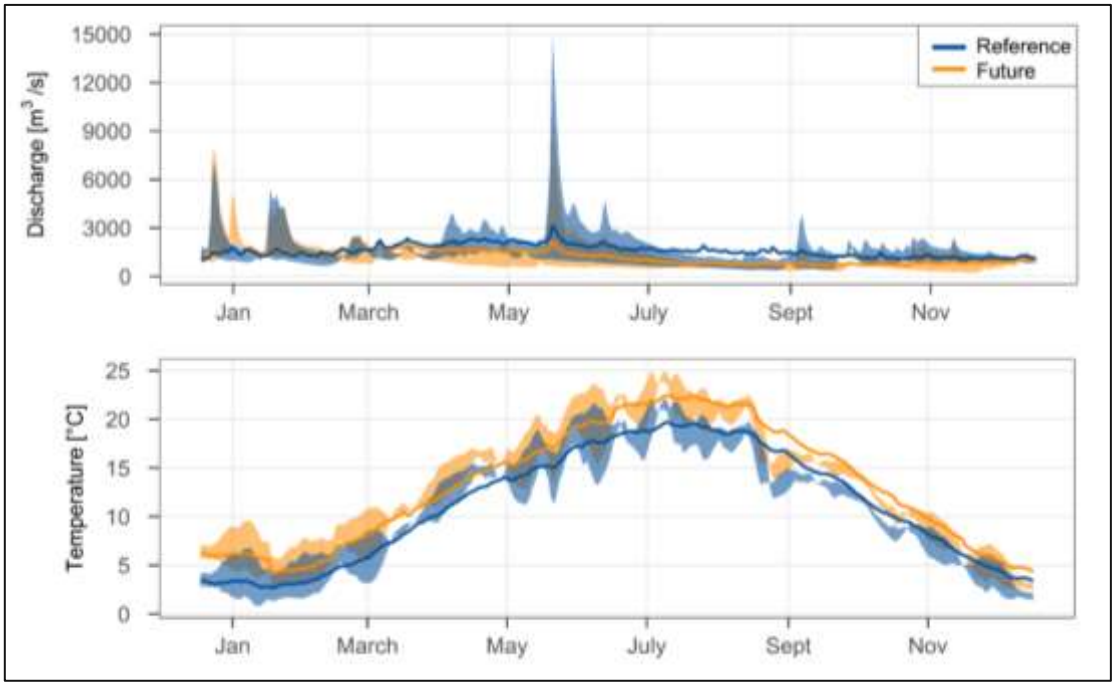
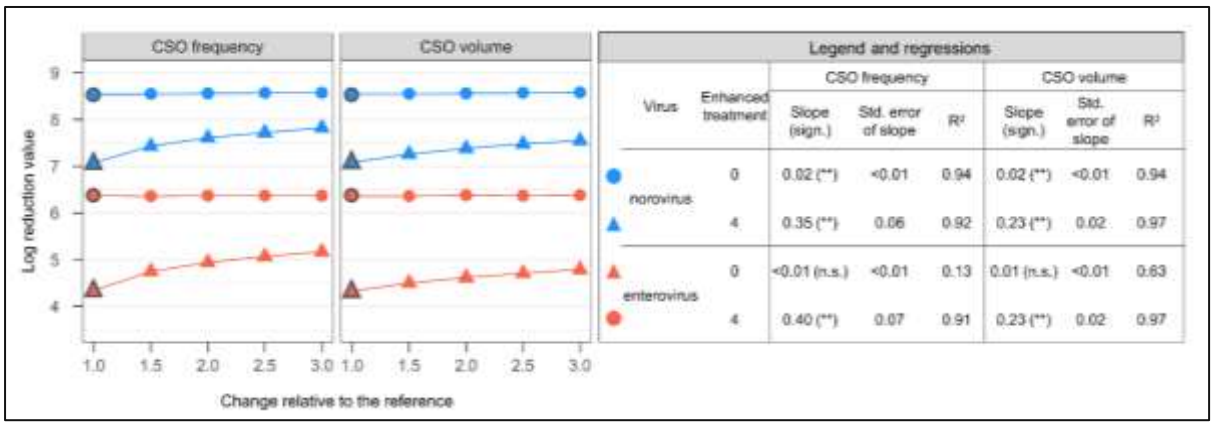


Figure 4.8 Discharge (upper panel) and water temperature (lower panel) of the Danube in the study area for the reference period (2003-2017) and the future period (2035-2049). The projections are based on the ECHAM5 A2 climate scenario. The line shows the mean across the 15 years, while the ribbon shows the range between the year with the lowest yearly mean discharge / temperature and the year with the highest one.

In the 'no management changes' scenario, varying the frequency and the volume of CSO events had no or very little effect on the LRVs. In contrast, in the 'enhanced wastewater treatment' scenario, the same variations in the frequency and volume of CSO events had a considerable effect on the LRVs. An increase in the frequency of CSOs had a more pronounced effect on the LRVs, with 0.35 to 0.40 $\log_{10}$ higher LRVs for a 100% increase in CSO frequency, compared to 0.23 $\log_{10}$ higher LRVs for a 100% increase in CSO volumes (Figure 4.9).

The above results show that the 'no management changes' scenario is not sensitive to an increase in CSOs. However, in the 'enhanced wastewater treatment' scenario, the required LRV for enterovirus and norovirus could be up to 1.03 and 0.74 $\log_{10}$ higher, respectively, depending on how the frequency and volumes of CSOs are affected by climate change (Figure 4.9).

Additionally, we assessed the effect of the virus concentration in CSO water on the LRV. In the two baseline scenarios, the virus concentration in CSO water is assumed to be 10% of that of raw wastewater. Increasing it to equal raw wastewater did not cause changes in the predictions for the 'no management changes' case; however, it increased the LRV values by 1 $\log_{10}$ for the 'enhanced wastewater treatment' case (results not shown).



Virus	Enhanced treatment	CSO frequency: Slope (sign.)	CSO frequency: Std. error of slope	CSO frequency: R^2	CSO volume: Slope (sign.)	CSO volume: Std. error of slope	CSO volume: R^2
norovirus	0	0.02 (**)	<0.01	0.94	0.02 (**)	<0.01	0.94
	4	0.35 (**)	0.06	0.92	0.23 (**)	0.02	0.97
enterovirus	0	<0.01 (n.s.)	<0.01	0.13	0.01 (n.s.)	<0.01	0.63
	4	0.40 (**)	0.07	0.91	0.23 (**)	0.02	0.97

Figure 4.9 Effect of various future CSO changes on the required log reduction value (LRV). The black contour shows the 'no management changes' (circle) and 'enhanced wastewater treatment' (triangle) scenarios. The table shows the results of linear regression analyses for enhanced treatment achieving an additional reduction of viruses of 0 or 4 $\log_{10}$; n.s., not significant; **, $p < 0.01$.

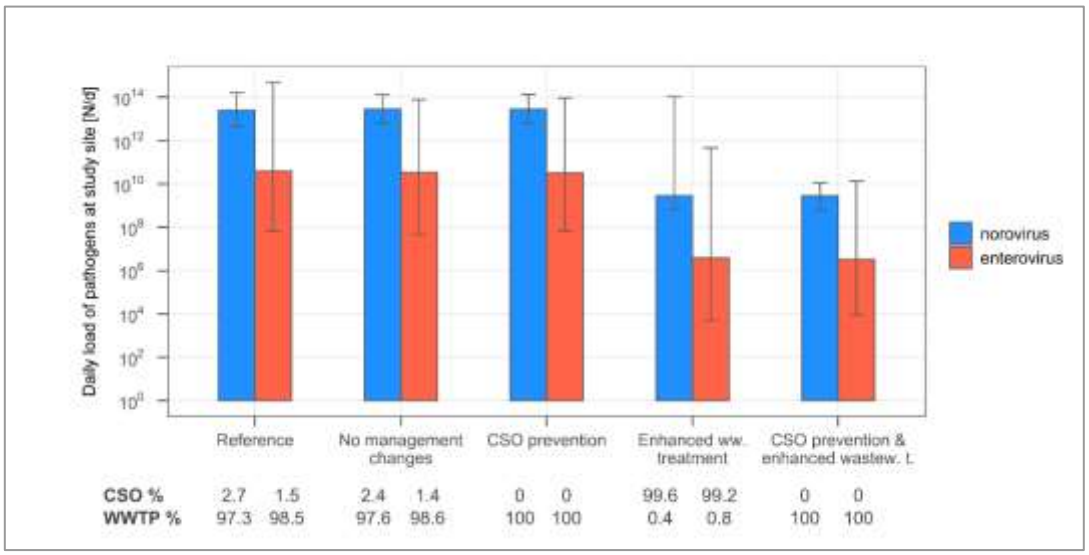


Figure 4.10 Scenarios: Median daily load of norovirus and enterovirus at the study site and relative source apportionment. The whiskers show the range of simulated daily loads.

4.3.2 Scenario analysis: Source apportionment of the load of viruses at the study site

The above results of the scenario analysis raise intriguing questions about the effect of the wastewater management measures targeting WWTPs and/or CSOs (Section 4.3.2.) and their interplay with climate and population changes (Section 4.3.3). To better understand this effect, we conducted a source apportionment of the load of pathogens at the study site originating from WWTPs and from CSOs. We did this by running each scenario twice: once by setting all pathogen inputs from WWTPs to zero and once by setting all inputs from CSOs to zero. We then calculated the daily load of pathogens at the study site by multiplying the simulated daily concentrations at the study site by the daily river flows. Figure 4.10 displays the sum of loads from WWTPs and CSOs, i.e., the entire load of viruses in each scenario together with the percentage contribution of WWTPs and CSOs.

The analysis revealed that under the current situation and in the 'no management changes' scenario, WWTPs discharging secondary treated wastewater were the major contributors to the load of viruses at the study site (10^{10} N/d enterovirus and 10^{13} N/d norovirus, 97 - 99% of the total load). The rest, 1 - 3%, originated from CSOs that discharge raw wastewater diluted with rainwater (Figure 4.10). This explains why changes in CSOs (neither their prevention nor their increase due to climate change) did not affect virus concentration distributions and LRVs (Figures 4.5 and 4.6). It also explains why enhanced wastewater treatment was effective at improving river microbial water quality (LRVs reduced by 1.3 and 2 $\log_{10}$, for norovirus and enterovirus, Figure 4.6): It addressed the main contributor to the pollution load.

This source contribution relationship between WWTPs and CSOs flipped once enhanced wastewater treatment was in place: the main contributors were then the CSOs, with 10^{6} N/d enterovirus and 10^{9} N/d norovirus contributing over 99% to the total load. The median daily load was reduced by 4 $\log_{10}$; however, the maximum daily loads were reduced by only 0.2 and 3.1 $\log_{10}$ for norovirus and enterovirus, respectively (Figure 4.10). This means a highly unequal distribution of the pollution load over time: While on days with no CSO events the daily load is relatively low, showing the beneficial effect of enhanced wastewater treatment, on days with CSOs the load is up to 5 $\log_{10}$ higher. The same pattern is visible in the virus concentration results (Figure 4.5). Also, this predominance of CSOs explains why a climate-change-driven increase in CSOs affects LRVs so remarkably if enhanced wastewater treatment is in place (Figure 4.6), and why the prevention of CSOs ('CSO prevention and enhanced wastewater treatment ' scenario) resulted in such a pronounced additional decrease of LRVs (an additional 2.5 and 1.9 $\log_{10}$ reduction of viruses, Figure 4.6).

4.4 Discussion

In this study, we tested an integrative modelling approach that combines CO_2 emission scenarios of the IPCC, a regional climate model, a conceptual hydrological model of the catchment as well as the significantly extended version of the microbial fate, transport and infection risk model QMRAcatch. The latter was used to estimate the source apportionment and the pathogen concentration in river water for QMRA. The combination of these methodological aspects was the key in gaining insights into the effects of future climatic and demographic changes and their interplay with possible upgrades in wastewater infrastructure on the microbiological water quality of rivers.

4.4.1 Implications of the assumptions and uncertainties of the modelling approach

Here, we discuss the model assumptions in this study, the uncertainty in the choice of input parameters, and their implications on the results. To assess the effects of climate change, monthly differences of air temperature and precipitation between simulations for a reference (2003 – 2017) and a future period (2035 – 2049) were calculated based on regional climate model outputs and used as input to a hydrological model. To investigate the changes

in intense rainfall events and the impact on CSOs, however, methods that account for the spatial and temporal variability of rainfall would be needed (Muller-Thomy et al. 2018). Hydraulic modelling of the sewer system, e.g., by using the urban stormwater model SWMM, would enable the studying of these effects on CSOs. For example, Bi et al. (2015) found a 15-500% increase in the volume discharged by CSOs in 2050 as compared to 2013 in Canada. This highlights that the relationship between changes in precipitation and CSO variables is not linear. How the contaminant concentration in CSO water will change in the future is also very much specific to the urban area and the sewer system drained by the CSO. An in-depth and location-specific analysis of urban sewer systems and their response to climate change was beyond the focus of this study.

As a wastewater upgrade for WWTPs, we assumed that enhanced treatment achieves an additional 4-log reduction of enterovirus and norovirus concentrations. While we added this value to the mean and 95th percentile of the assumed normal probability distribution of secondary treatment, a more realistic approach would be to apply distributions of microorganism- and process-specific values. The concentration of pathogens in WWTP effluent was assumed to be the same in the future as it is now. However, there may be differences in the disease burden in the future (Levy et al. 2016).

The above-listed uncertainties and assumptions affect the absolute LRVs to achieve safe drinking water for all scenarios likewise. We aimed to study the effects of various changes of the system on microbiological drinking water safety requirements, not absolute LRV values.

4.4.2 *Accurateness of the pathogen fate and transport predictions*

In order to accurately predict the reference pathogen concentrations and loads, the model calibration and validation of the fate and transport model based on site- and source-specific data are seen as an essential step. To evaluate QMRAcatch, we used measured MST and enterovirus concentrations collected at the study site over four years.

Our literature survey on reported MST marker and virus inactivation rates revealed a current lack of studies conducted in real-world and natural light conditions, in particular for norovirus, which creates a source of model uncertainty. In order to overcome this limitation, we set the microbial inactivation coefficients during calibration within constrained limits based on reported persistence data for the human-associated MST marker and enterovirus.

The use of human-associated MST data allowed for source-targeted calibration and validation of the model (Mayer et al. 2018a, Zhang et al. 2019). The human-associated MST marker thus provides a better basis for calibration and validation in the context of our research questions (on point sources of human wastewater) than a standard faecal indicator organism, such as *E. coli* would do, since *E.coli* may, for example, also originate from other non-faecal sources (Frick et al. 2018a). Integrating pathogen data in the calibration-validation process is an essential confirmation, and it allows to assess health risks directly (Boehm and Soller 2013, Lodder et al. 2015). However, pathogens can hardly serve as the basis for a comprehensive calibration on their own, since their concentrations in environmental waters are often very low, and the required large sample volumes and processing efforts render the establishment of large data series unfeasible. Still, a smaller pathogen dataset may complement the calibration process. The human-associated MST marker has approx. 4-6 $\log_{10}$ higher concentrations in raw wastewater than most pathogens, and often maintains high concentrations in environmental waters. Therefore, the model calibration followed a nested approach to make optimal use of both the host-associated MST marker and pathogen data. Discharge and mixing processes of faecal pollution associated microorganisms and pathogens in river water could be robustly calibrated using sufficiently abundant source-targeted MST marker data. The pathogen data were then used to adjust the model to the pathogen-specific inactivation rate coefficients. The calibrated and validated model was then able to simulate the low, non-detectable but significant ranges of the enteric pathogens in question or even simulate new pathogens, where

only information on the concentrations in sewage and environmental persistence is available (Table 4.3). Despite the advantages of this systematic approach, only a few microbial fate and transport modelling studies have used it so far (Derx et al. 2016, Schijven et al. 2015).

4.4.3 Deciphering the interplay of future changes and wastewater management measures

Several studies investigated the effects of future changes in climate, population or wastewater infrastructure on the microbiological river water quality so far, but the controlling factors remain yet unclear (Iqbal et al. 2019, Islam et al. 2018a, Jalliffier-Verne et al. 2017, Sterk et al. 2016). This study brought new insights into this question by integrating source apportionment, concentrations of reference pathogens and risk assessment into a modelling analysis. Source apportionment was previously used to identify the dominant sources of faecal indicator bacteria (Soller et al. 2014, Stapleton et al. 2011) or to study the effects of sociodemographic and climate changes on faecal indicator bacteria loads into a river (Iqbal et al. 2019). Our study identified source apportionment together with the other methodological aspects of the integrative modelling approach as the key for understanding for the first time the interplay of future changes and wastewater management measures. We showed how this interplay affects pathogen loads into rivers, and the pathogen concentrations in rivers considering safe drinking water production.

For the scenario with no management changes at our example study site, changes in river flows and water temperatures were shown to have a minor negative effect on the microbiological river water quality, in line with the predictions for other regions (Iqbal et al. 2019, Islam et al. 2018a, Jalliffier-Verne et al. 2017, Sterk et al. 2016). According to the reference scenario, WWTPs discharging secondary treated sewage are the major contributors to the pathogen loads, not CSOs. Therefore, neither potential increases in CSO events due to climate change nor their prevention affected the drinking water safety requirements. In contrast, if enhanced wastewater treatment was in place at the WWTPs, CSOs suddenly became the major contributors to the pathogen loads. Here, climate-change driven increases in CSO events resulted in significantly higher treatment requirements. While this issue was addressed earlier by (Sterk et al. 2016) in the context of bathing water infection risks, our modelling results for the first time identify the conditions and extent to which increased CSOs affect the microbiological river water quality in the context of safe drinking water production.

The greatest improvement in the microbiological water quality of the riverine water intake was achieved with measures targeting both WWTPs and CSOs. The Austrian Waste and Wastewater Association estimates that an additional yearly investment of 150 million Euros are needed to tackle upcoming problems in this sector in the coming years (ÖWAV 2020). Since the current regulatory standard is secondary treatment at the WWTPs, it is very important to evaluate the benefits enhanced wastewater treatment would bring, which is currently discussed in the context of micropollutant abatement. Furthermore, it is important to estimate the impact of urban soil sealing, an important yet often neglected aspect in city development, with respect to the microbiological water quality of the receiving waters.

4.5 Conclusions

The pathogen fate and transport and infection risk model QMRAcatch (v1.0 Python) was significantly extended and is now available as open source.

Climatic and demographic changes had little impact on the microbiological river water quality considering safe drinking water, where 98 % of the pathogen loads stemmed from WWTP discharges. Strong climate change effects are shown in the scenario with enhanced WWTP treatment, where CSOs are the major faecal pollution sources.

The required log reduction value (LRV) to produce safe drinking water was 6.3 and 8.4 $\log_{10}$ for enterovirus and norovirus in the scenario with secondary WWTP treatment. Enhanced wastewater treatment led to a reduction of

LRVs by 0.5 to 2.0 log_{10}. This measure combined with preventing CSOs had the most significant positive effect with a reduction of LRVs by up to 4 log_{10}.

The integrative modelling framework is demonstrated at a large, wastewater-impacted river, and is applicable at other catchments and types of pollution sources for long-term water safety planning.

5 Automated online monitoring of fecal pollution in water by enzymatic methods

5.1 Introduction

The prevention of waterborne diseases requires a systemic framework including water quality monitoring, pollution characterization and health risk assessment (Farnleitner et al. 2018). The monitoring of fecal pollution is a key element in this approach. Fecal pollution patterns in water may vary greatly on short temporal and spatial scales (Boehm 2007, Enns et al. 2012). However, culture-based monitoring standards using fecal indicator bacteria (FIB, such as *E. coli*, intestinal enterococci) only provide a result after 18-24 hours and grab samples are collected at large intervals (often >> 1 day). Pollution peaks might be missed, or if caught, the result is only available retrospectively. Therefore, there is a need for continuous and (near-)real-time monitoring of fecal pollution in water. Such devices may be applied for monitoring and strategic management throughout the water sector, from drinking water supply to recreational waters (Figure 5.1). Wired or wireless data transmission enables remote control and thus the method may become an integral part of an increasingly digitalized water industry.

Methods based on the fluorometric measurement of the enzymatic activity of ß-D-galactosidase (GAL) and ß-D-glucuronidase (GLUC) in water were suggested over two decades ago as rapid surrogates for the culture-based determination of coliforms (GAL) and fecal coliforms or *E.coli* (GLUC) (Berg and Fiksdal 1988, Farnleitner et al. 2001, Fiksdal et al. 1994, Fiksdall and Tryland 2008). Fluorogenic and chromogenic enzymatic substrates had been well known for a long time as diagnostic supplements in bacterial media (e.g. (Edberg and Edberg 1988) included now in ISO 9308-2:2012 for the detection of *E. coli* (ISO 2012)). During the last decade, fluorogenic substrate technologies were incorporated into online instruments enabling the automated and rapid determination of specific enzymatic hydrolysis rates in water (Koschelnik et al. 2015, Ryzinska-Paier et al. 2014, Zibuschka et al. 2010).

Here we provide an update and extension of the milestone review of Fiksdal and Tryland (Fiksdall and Tryland 2008) by focusing on online, automated enzyme measurement platforms intended for fecal pollution monitoring in water resources. The emphasis lies on the direct determination of enzymatic hydrolysis rates in water (not involving a culture step) because the short time to result supports near-real-time monitoring applications. The focus is on GLUC activity rates, because the studies available to date in peer-reviewed literature cover almost exclusively this parameter.

Definitions

Rapid detection: there is no widely accepted definition, Noble and Weisberg suggest „methods that provide results in less than 4 hours“ (Noble and Weisberg 2005).

Online measurement: continuous and automatic monitoring of a parameter. Intranet and/or internet connection allows controlling the results remotely (2020b).

Proxy or surrogate parameter: a parameter which is used as an indicator of the presence of another parameter in the absence of a direct measure (2019, 2020c).

Automated: carried out by machines or computers without needing human control (2020a).

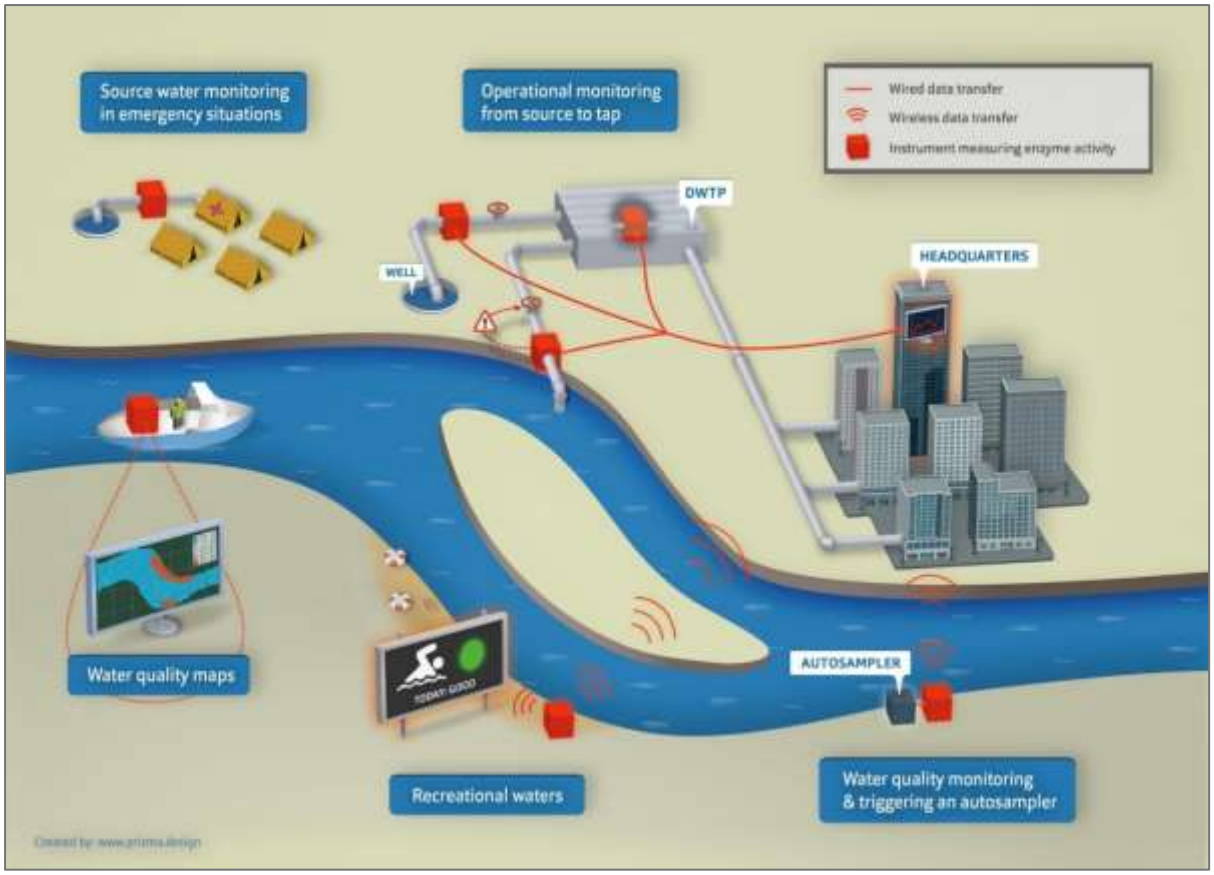


Figure 5.1 Potential applications of rapid online enzymatic methods for the detection of fecal pollution in water. The instruments may be placed at various monitoring points in natural waters or at critical control points along the drinking water supply chain. Connection with existing infrastructure allows the instrument triggering the action of another instrument, such as an autosampler to allow cross-comparison with laboratory-based standard microbiological assays. Connection to the headquarters and/or to cell phones allows data management and central monitoring. DWTP: Drinking Water Treatment Plant.

5.2 Does the automation work? The technical realization of rapid automated GLUC measurement.

Device principles. The technical developments necessary for the enzymatic assay to be operated remotely and fully automated have been achieved and are well documented (Koschelnik et al. 2015, Movig et al. 2017, Ryzinska-Paier et al. 2014, Zibuschka et al. 2010) (Table 5.1, upper panel). The devices typically consist of a sample intake, reagent stocks, a temperature-controlled reaction chamber, a UV emitter and optical sensor as well as a control unit and a user interface (for references, see Table 5.1). Technical applications (casing, power supply, etc.) have been reported for operation in buildings, remotely as a stationary device or as a mobile outdoor device (Ryzinska-Paier et al. 2014, Stadler et al. 2016a, Stadler et al. 2017). Reported sample volumes range from 6 mL to 5,000 mL, with the possibility to concentrate large sample volumes (Koschelnik et al. 2015, Ryzinska-Paier et al. 2014, Zibuschka et al. 2010). Measurement intervals between 15 and 180 minutes have been described (Ryzinska-Paier et al. 2014, Stadler et al. 2016a, Stadler et al. 2017).

Analytical performance. The available evaluations have indicated high analytical precision for the automated GLUC activity measurements with coefficients of variation below 5% (Burnet et al. 2019a). Widely used cultivation based FIB standards achieved a lower analytical precision with coefficients of variation between 16% and 31% (Burnet et al. 2019a). The general performance of GLUC activity measurements was reported to be comparable

with manually performed analysis and the simultaneous determination of the limit of quantification can be integrated into the automated data analysis by the instrument (Ryzinska-Paier et al. 2014). It should be noted that the reported units differ among manufacturers and studies (hydrolysis rate *versus* Fishman units per volume), although conversions can be achieved.

The *robustness* of the automated GLUC activity measurements in freshwater types having a wide range of physicochemical and microbiological characteristics was demonstrated by recent studies in pristine waters (Ryzinska-Paier et al. 2014), surface waters with elevated suspended solid loads (Ender et al. 2017, Stadler et al. 2016a) and waters impacted by treated and/or untreated municipal sewage (Burnet et al. 2019a, Stadler et al. 2016b). However, marine waters were only tested so far using laboratory-based direct GLUC assays (Fiksdal et al. 1994, Lebaron et al. 2005). Reported environmental factors influencing measurement accuracy and error-free running time are ambient temperature and suspended organic matter (Fiksdall and Tryland 2008, Stadler et al. 2016a). Both factors are now managed well by specific adaptations of the devices, including the specific design of the reaction chamber, sample pre-filtration, adapted cleaning procedures and data-correction algorithms (Stadler et al. 2017). Such devices were successfully operated outdoors *in situ* for up to two years (e.g. (Ryzinska-Paier et al. 2014, Stadler et al. 2019a)).

Alternative laboratory-independent methods based on GLUC activity. A portable device has been developed based on the direct measurement of GLUC activity after cell lysis ((Heery et al. 2016), Table 5.1, lower panel). In addition, automated devices based on enrichment in selective growth media prior to the measurement of enzymatic activity have been successfully realized, with several fluorometric and one voltammetric method based on this principle. Some instruments have been designed for online monitoring others as field-deployable devices (Table 5.1, lower panel).

5.3 Where has it been used? Field studies using automated GLUC measurement.

Automated GLUC activity measurement devices have been deployed with the aim to characterize the temporal and spatial patterns of GLUC activity and describe the relationship to cultivation-based standard *E. coli* detection methods in various water resources (Table 5.2).

Vulnerability assessment of water resources. The first demonstration of the technical feasibility of near-real time monitoring of GLUC and GAL activity was provided by Ryzinska-Paier et al. (2014) at an alpine karst spring and an alluvial aquifer in Austria over a period of two years. The seasonal dynamics of GLUC activity at a karstic spring environment were described for the first time (>5000 successful automated measurements). In a freshwater resource for urban drinking water supply in Canada, Burnet et al. used a 1.5 year long GLUC activity time series to identify the dominant fecal pollution source among multiple wastewater discharges and to uncover the hydraulic connection between an upstream wastewater treatment plant and the drinking water treatment plant (Burnet et al. 2019b). Ender et al. (2017) demonstrated the feasibility of automated near-real time monitoring of GLUC activity in a remote karst spring in Northern Vietnam using a portable instrument designed to operate under limited resources settings.

Table 5.1 Published methods for the laboratory-independent measurement of enzymatic activities intended for the monitoring of fecal pollution. GLU: β-D-glucosidase; * including a sample concentration step

Enzyme	Method	Measurement principle	Substrate	Time to result	Automated or manual	Literature reference: method	Literature reference: field applications	Commercial realization
Online, automated devices for direct measurement of enzymatic activity								
GLUC (GLU, GAL)	Fluorometric	Direct enzymatic	4-methylumbelliferyl-β-D-glucuronide	15 min	Automated online	(Koschelnik et al. 2015, Stadler et al. 2016a)	(Burnet et al. 2019a, Burnet et al. 2019b, Cazals et al. 2020, Ender et al. 2017, Stadler et al. 2019a, Stadler et al. 2016a, Stadler et al. 2019b)	ColiMinder (VWMS, Austria)
GLUC (GAL)	Fluorometric	Direct enzymatic	4-methylumbelliferyl-β-D-glucuronide	75 min*	Automated online	(Ryzinska-Paier et al. 2014, Stadler et al. 2017, Zibuschka et al. 2010)	(Ryzinska-Paier et al. 2014, Stadler et al. 2016a)	BACTcontrol (microLan, The Netherlands), previously ColiGuard (mbOnline, Austria)
Alternative methods for laboratory-independent monitoring of fecal pollution based on enzymatic activities								
GLUC	Fluorometric (ColiSense)	Enzymatic after lysis	6-chloro-4-methyl-umbelliferyl-beta-d-glucuronide (6-CMUG)	75 min	Manual, field-portable	(Briciu-Burghina et al. 2015, Briciu-Burghina et al. 2017, Heery et al. 2016)	(Briciu-Burghina et al. 2019)	---
GAL	Fluorometric	Enzymatic after selective culture	GAL: 4-methylumbelliferyl-D-galactoside	15-120 min	Manual, field-portable	(Tryland et al. 2016)	(Tryland et al. 2016)	Colifast Field kit (Colifast AS, Norway)
GLUC, GAL	Fluorometric	Enzymatic after selective culture	GAL: 4-methylumbelliferyl-D-galactoside GLUC: not disclosed	2.5-15 h	Automated online	(Tryland et al. 2016, Tryland et al. 2015)	(Tryland et al. 2016, Tryland et al. 2015)	Colifast ALARM, Colifast CALM (Colifast AS, Norway)
GLUC, GAL	Fluorometric	Enzymatic after selective culture	pyrene-glucuronide and anthracene-galactoside	2-18 h	Automated online	(Brown et al. 2013)	(Burnet et al. 2019a)	Tecta B16 (Endetec, Canada)
GLUC	Fluorometric	Enzymatic after selective culture	4-methylumbelliferyl-β-D-glucuronide	2-12 h	Automated field-deployable and manual field-portable	(Angelescu et al. 2019)	---	ALERT System, ALERT Lab (Fluidion SAS, France)
GLUC	Voltammetric (EcoStat)	Enzymatic after selective culture	methyl-β-D-glucuronide sodium salt	≤ 10 h	Automated	(Zuser et al. 2019)	---	---

Table 5.2 Applications of GLUC online enzymatic activity measurement devices

Enzyme	Intended Application	Duration	Water resource type (mean discharge)	Location	Land use (major fecal pollution sources)	Meteorological conditions	Literature reference
GLUC	Automated near-real time monitoring of source water quality	2 years	Karst aquifer spring (5 m^3/s)	Northern Alps, Austria	Forested and summer pastures (domestic and wildlife ruminants)	Dry weather, rainfall	(Ryzinska-Paier et al. 2014)
			Alluvial aquifer	Danube River, Vienna, Austria	Protected wetland and floodplain forest (wildlife ruminants)	Dry weather, rainfall	
GLUC	Comparison of two automated online technologies for investigation of catchment-based transport of *E. coli*	1 year	Stream (2.7 10^{-3} m^3/s)	Hydrological Open-Air Laboratory (HOAL) catchment, Austria	Agricultural cropland (swine manure)	Dry weather, rainfall	(Stadler et al. 2016a)
GLUC	Automated near-real time monitoring of source water quality in remote and resource-limited settings	10 days	Karst spring (0.6 -0.9 m^3/s)	Seo Ho River, Vietnam	Agricultural (livestock, manure, untreated domestic sewage)	Dry weather, rainfall	(Ender et al. 2017)
GLUC	Ship-borne automated surface water quality mapping at various spatial scales	3h - 1 day	Lake	Yahara lakes, Wisconsin, USA	Predominantly agricultural with urban areas. Sources: diffuse agricultural pollution, leaks from sanitary sewers, urban stormwater outfalls, birds	Dry weather, rainfall	(Stadler et al. 2019b)
		5 days	River (5700 m^3/s)	Lower Columbia River, Oregon/Washington, USA	Agricultural and urban	Dry weather	
		1 day	River (1300 m^3/s)	Upper Mississippi River, Wisconsin, USA	Predominantly agricultural with urban areas. Sources: Diffuse agricultural sources, wastewater treatment plant effluents	Dry weather	
GLUC	Investigation of catchment microbial dynamics at seasonal to hourly time scales	2 years	Stream (2.7 10^{-3} m^3/s)	Hydrological Open-Air Laboratory (HOAL) catchment, Austria	Agricultural cropland (swine manure)	Dry weather, rainfall	(Stadler et al. 2019a)
GLUC	Identification of dominant fecal pollution sources in an urban drinking water supply	1.5 years	River (300 m^3/s)	Greater Montreal Area, QC, Canada	Predominantly urban, small agricultural tributaries (treated and untreated sewage discharges, diffuse agricultural sources)	Dry weather, rainfall, and snowmelt, spring flood	(Burnet et al. 2019b)
GLUC	Automated near-real time monitoring of recreational water quality	2 months	River (7,500 m^3/s)	Greater Montreal Area, QC, Canada	Combined sewer overflows	Dry weather, rainfall	(Cazals et al. 2020)
		4 months	River (300 m^3/s)	Greater Montreal Area, QC, Canada	Predominantly urban, small agricultural tributaries (treated and untreated sewage discharges, diffuse agricultural sources)	Dry weather, rainfall	
		2 months	River (12,600 m^3/s)	Quebec City, QC, Canada	Mixed (Combined sewer overflow discharges, diffuse agriculture runoff, gulls)	Dry weather, rainfall	
		3 months	River (150-450 m^3/s)	Waikato River, Hamilton, New Zealand	Agricultural (diffuse runoff from livestock grazing, effluent spreading and wildlife, stormwater oufalls)	Dry weather, rainfall	

Catchment microbial/biochemical dynamics. The automated near real-time monitoring of GLUC activity as biochemical indicator has a considerable potential. Stadler et al. (2016a) first demonstrated that two different commercially available instruments were able to detect rapid fluctuations in enzymatic activity caused by episodic changes in hydrological conditions. The authors reported seasonal variations in the transport of GLUC activity, which peaked more often and at higher amplitudes in summer, although several of these GLUC activity peaks occurred in absence of rainfall and suspended sediment peaks (Stadler et al. 2019a, Stadler et al. 2016a). Through the screening of GLUC activity in stream water and sediments and using stable isotopes in stream water, the authors suggested that a large portion of the transported GLUC originated from the resuspension of streambed sediments and reflected the existence of a remnant reservoir of GLUC in the catchment (Stadler et al. 2019a). In an urban catchment affected by multiple treated and untreated wastewater discharges, Burnet et al. (2019b) similarly illustrated the large temporal scale of variation in GLUC activity in water. GLUC activity peak episodes occurred exclusively between late fall and early spring and were caused by intense precipitation (24 to 48h prior GLUC activity peak) and/or snowmelt events, which triggered the local discharges of untreated sewage into the river.

Besides the seasonal and event-based fluctuations in GLUC activity, recurrent daily patterns have been reported in various habitats, although the peak activities did not occur at the same time of the day (Burnet et al. 2019a, Burnet et al. 2019b, Ender et al. 2017, Stadler et al. 2016a). The origin of these daily patterns was attributed to the likely temperature dependence of bacterial activity in a small agricultural stream (Stadler et al. 2016a), and in a karst spring (Ender et al. 2017), although the causal link requires further investigations. Another type of daily pattern of GLUC activity was described at an urban drinking water intake and was traced back to the discharge pattern of an upstream wastewater treatment plant (2019b).

Surface water quality mapping. Using a ship-borne instrument, Stadler et al. (Stadler et al. 2019b) recently demonstrated the feasibility of rapid GLUC activity assessment for surface water quality mapping. These first high-resolution spatial data on GLUC activity illustrated the effect of rainfall-induced runoff on surface water quality along urbanization gradients and indicated tributaries and confluences as main fecal pollution hotspots in these large waterbodies.

Recreational water quality assessment. Cazals *et al.* (Cazals et al. 2020) illustrated the usefulness of online GLUC activity monitoring for rapid identification of impaired waters in recreational freshwater bodies. Threshold GLUC activity values were developed to match the regulatory ("gold standard") *E. coli* beach action values while minimizing the rates of failures to act and false alarms. Near-real time monitoring of GLUC activity enabled to identify fecal pollution peaks and determine the exact timing of GLUC activity threshold exceedance.

5.4 What does it tell us? The indicator capacity of GLUC

Relationship to cultivation-based fecal indicator bacteria. All field studies using automated GLUC determination (Table 5.2) performed cross-comparisons with cultivation-based *E. coli* standards (Burnet et al. 2019a, Burnet et al. 2019b, Cazals et al. 2020, Ender et al. 2017, Ryzinska-Paier et al. 2014, Stadler et al. 2019a, Stadler et al. 2016a, Stadler et al. 2019b) and one study reported data also for coliforms (Ryzinska-Paier et al. 2014). Reported correlations between GLUC activity and cultivation-based *E. coli* standards (expressed in linear or non-parametric correlation coefficients r) varied widely among the studied water resources. For freshwaters influenced by urban sewage, r ranged between 0.33-0.84 on non-transformed data (Burnet et al. 2019a, Burnet et al. 2019b, Stadler et al. 2019b) and between 0.10-0.79 on log-transformed data (Cazals et al. 2020), with an apparently strong dependence of hydrometeorology and contamination characteristics (Burnet et al. 2019b, Cazals et al. 2020). Among the studied watersheds influenced by agriculture (manure spreading and/or cattle grazing), r ranged between 0.53-0.56 at karstic springs of remote mountains (Ender et al. 2017, Ryzinska-Paier et al. 2014) while a small brook revealed r = 0.72 (Stadler et al. 2019a, Stadler et al. 2016a). Stronger correlations were found at higher pollution levels (Burnet et al. 2019a, Burnet et al. 2019b) and during events (with the highest r reported being 0.89

(Stadler et al. 2019a)). Notably, GLUC activities often resulted in stronger correlations with environmental parameters than cultivation-based *E. coli* data. For example, correlations up to r = 0.87 with turbidity (2-3 µm particle fraction, karst spring, rain event) (Ender et al. 2017) and r = 0.93 with chlorophyll a (lake, dry weather) were observed (Stadler et al. 2019b).

Table 5.3 Open research topics and future development goals regarding the automated, cultivation-independent determination of enzymatic activities intended for online fecal pollution monitoring of water resources. VNBC: viable but not culturable.

Some open research topics and future development goals
Fecal and health-risk indication capacity of GLUC activity
o What are the limits to use GLUC as a biochemical fecal indicator (i.e. fecal pollution level, age, treatment)?
o Which aquatic habitats are most suitable for GLUC activity monitoring in respect to its fecal indication ability?
o Which habitats or situations are not suitable for GLUC activity monitoring and strong interference or bias from non-fecal sources is to be expected?
o In which situations may GLUC activity become indicative on the occurrence of intestinal pathogen?
o In which situations may GLUC activity become indicative of infection and health risks?
o Can GLUC activity be used as a conservative indicator for pathogen removal during treatment?
GLUC activity of fecal origin: persistence and fate in the (aquatic) environment
o How long does cell-associated enzyme activity of intestinal populations persist?
o How does GLUC activity compare to other cell-viability parameters?
o What are the relative abundances of culturable, VNBC, dead cells/cell debris, free and particle-attached enzymes under various environmental conditions? Do they have a differential persistence?
o Is there a difference in GLUC activities between human *versus* animal sources?
o Which intestinal microbiota contribute to GLUC activity in water?
o Do different microbiota show differential GLUC activity persistence?
o Could the ratio GLUC to cultivation-based fecal indicator standards indicate contamination age?
o Do free enzymes re-attach to abiotic particles, such as to silt-colloids? How does re-attachment influence the enzymatic persistence? Do catchments with high turbidity and GLUC adsorption rates limit the application?
o Which GLUC inhibiting substance may occur in water samples and under what conditions?
GLUC activity of fecal origin: resistance and fate during water treatment and disinfection
o What is the resistance of GLUC activity of fecal origin to the various steps of *wastewater* treatment, including ozonation and chlorination? Do the various GLUC compartments (culturable, VNBC, free enzymes, etc.) have a differential resistance?
o What is the resistance of GLUC activity of fecal origin to the various steps of *drinking water* treatment, including chlorination, UV disinfection and ultrafiltration? Do the various GLUC compartments have a differential resistance?
o How does GLUC activity compare to other cell-viability parameters during the treatment steps?
GLUC activity of non-fecal origin
o Under which conditions does algae-associated GLUC activity become significant?
o Under which conditions does environmental bacteria-associated GLUC activity become significant?
o What are other potential non-fecal associated GLUC sources?
o Is it possible to differentiate or correct for non-fecal associated GLUC activity?
o Does significant GLUC activity occur from “naturalized” (re-grown) intestinal populations in the environment?
o What is the exact nature and origin of daily GLUC fluctuations that are not related to the fecal pollution source dynamics?
Fecal pollution-associated enzymes other than GLUC (questions above are all relevant)
o What are the sources and fate of ß-D-galactosidase? Is it a useful fecal indicator?
o What are the sources and fate of ß-D-glucosidase? Is it a useful fecal indicator?
o Are there any other enzymes or combinations demonstrating enhanced fecal indicator capacity?
o How can enzymatic substrates be improved to increase their sensitivity and specificity for fecal pollution?
Technical realization of automated, online instruments
The field needs…
… Validation guidelines (precision, robustness, specificity, sensitivity)
… Quality control and quality assurance protocols
… Uniform, standardized measurement units
… Strategies to trigger microbiological autosampling, based on online GLUC and/or physicochemical measurements

GLUC does not qualify as a general proxy parameter for cultivation-based E. coli enumeration. The above correlation analysis support previous observations that GLUC activity is not a general proxy for cultivation-based *E. coli* enumeration (Fiksdall and Tryland 2008). Enzymatic activity was demonstrated to be a more persistent biochemical parameter against environmental and treatment (disinfection) stresses as compared to the culturable fraction of FIB in water resources (Fiksdall and Tryland 2008). Indeed, there is evidence that GLUC activity is able to detect culturable cells as well as viable but non-culturable (VBNC) cell populations (Garcia-Armisen et al. 2005). Furthermore, persistent GLUC activity was also reported for damaged or dead *E. coli* cells (Petit et al. 2000) and from the fraction of free enzymes in river water with fecal pollution (Farnleitner et al. 2002). It can be argued that free or particle-associated GLUC activity may be relevant for the detection of low, remote, old or treated (disinfected) fecal pollution.

GLUC activity can also be associated with biotic or abiotic compartments other than E. coli. Without a selective cultivation-based enrichment step for *E. coli*, a significant amount of GLUC activity in water samples may also originate from other microbiota and substances (Fiksdall and Tryland 2008). Recent investigations highlight that microbiome encoded GLUC activities play an important role in the human gastrointestinal system (McIntosh et al. 2012). By genomic and proteomic tools, hundreds of different ß-glucuronidase enzymes, grouped into six distinct categories, could be identified in abundant microbial phyla of Bacteroidetes, Firmicutes, Verrucomicrobia and Proteobacteria in human stool samples (Pollet et al. 2017), confirming previous observations before the genomic era (Frampton and Restaino 1993). However, possible interference from non-intestinal microbiota in water resources was also reported, including environmental bacteria and algae (Baudart et al. 2009, Davies et al. 1994, Fiksdall and Tryland 2008). As a result, GLUC activity is considered to be of fecal origin, especially under the situation of high fecal pollution (culturable cells, VNBC, cell debris and free enzymes), but interfering GLUC activity of non-fecal origin (biotic and abiotic) can also occur (Fiksdall and Tryland 2008).

5.5 Status quo and open questions

Without any doubt, the automated online GLUC activity determination in water resources has been successfully realized during the last decade, offering fascinating new possibilities to support water safety management in the future (Figure 5.1). This technology may not be restricted to GLUC and related enzymes, but could support any type of enzymatic online monitoring (if technically feasible) that can inform about microbial and biochemical water quality issues (Appels et al. 2018, Chróst 1991, Hoppe 2003, Luo et al. 2017). As opposed to the original suggestion almost 20 years ago (Farnleitner et al. 2001), it is now obvious - after the many cross-comparison efforts - that GLUC activity is not a general surrogate for the cultivation-based determination of *E. coli*. Depending on the habitat, fecal pollution characteristics and hydrometeorology, the relationship between culturable *E. coli* concentrations and GLUC activity rates can vary substantially. In cases where the direct comparison with cultivation-based *E. coli* standards is essential, online GLUC determination using automated pre-enrichment procedures by selective growth would be a more suitable approach (Table 5.1, (Angelescu et al. 2019, Brown et al. 2013, Tryland et al. 2016, Tryland et al. 2015), with reported correlation coefficients to standard *E. coli* methods ranging between 0.90-0.94 (Angelescu et al. 2019, Burnet et al. 2019a, Tryland et al. 2016)). However, a trade-off between this stronger relationship and a significantly longer sample-to-result time has to be taken into account (Table 5.1). The rapid online prediction of culturable *E. coli* based on GLUC direct determination may only be possible in special cases: at certain sites and under certain pollution scenarios allowing a sufficiently high statistical relationship. This, however, requires further investigations.

In contrast to the achieved progress in the automated determination of enzymatic hydrolysis rates, the scientific evaluation of the GLUC indication capacity for fecal pollution monitoring has been almost neglected for more than a decade (Fiksdall and Tryland 2008). There is an urgent research need to understand more comprehensively the sources and sinks, the persistence and mobility, and the link of GLUC activity with the actual cellular states. Such investigations should cover all important water resource systems and should also include essential water

treatment and disinfection processes (Table 5.3). Based on the information currently available, we propose *GLUC activity as a conservative biochemical proxy-parameter for bacterial fecal pollution* (not only associated with *E. coli* or fecal coliforms) in water resources. Furthermore, for specific system conditions and exposure scenarios, GLUC activity may also indicate pathogen occurrence and infection risk from fecal pollution and could therefore be part of the strategic management of the given water resource (Table 5.3). However, as highlighted a decade earlier (Fiksdall and Tryland 2008), GLUC activities from non-fecal compartments may interfere with the indented indication capacity especially in the case of low, old or remote fecal pollution. The above-mentioned gaps of knowledge currently limit the (full) application of automated online GLUC activity monitoring in the water management sector and warrant further detailed investigations.

6 Conclusions

This thesis explored how genetic faecal markers can support next generation water safety management, and in particular, how they may be incorporated into health risk assessment. It also assessed the faecal indication capacity and field applicability of a method of rapid microbiology (β-D-glucuronidase, GLUC, activity measurements) for automated, online faecal pollution detection.

Chapter 2 assessed the greatest breakthrough in health-related water microbiology of the past thirty years: the advent and fast spreading of genetic methods that allow now fascinating new possibilities. Through the systematic analysis of over 1,000 scientific articles, we have demonstrated that this emerging scientific field still grows, and in the past decade, the focus of research has shifted from method establishment to the implementation of these methods in scientific field research. Genetic methods have revolutionised research in health-related water microbiology in terms of faecal pollution detection and microbial source tracking, the current core areas of application. Emerging areas of application identified include health and infection risk assessment, (waste)water treatment evaluation and a support role in wastewater surveillance, among others. Genetic faecal parameters are often combined with traditional cultivation-based FIOs and other parameters (environmental parameters, pathogens, etc.) in a toolbox approach. Toolboxes can be adapted for tailored scientific investigations and monitoring campaigns. In combination with bio-banking, new parameters may be measured retrospectively, on stored samples, further expanding the possibilities. Thus, this meta-analysis of the peer-reviewed literature provided the scientific status quo of the ways genetic faecal markers (and other genetic methods) may support next generation water safety management.

Chapter 3 presented a case study where genetic faecal markers were employed to assess the extent and sources of faecal pollution in the Danube River and its floodplain water bodies at Vienna, Austria. The results indicated that the faecal pollution pressures differ between the Danube River and its floodplains. The Danube River is primarily impacted by human faecal pollution, which, in this catchment, is urban wastewater. The impact is low to moderate, occasionally critical. The floodplains are impacted by mixed sources: ruminant, pig, duck and human sources were detected, and the level of faecal pollution is low. This case study is a demonstration of how genetic faecal markers may be used for microbial source tracking, i.e., to identify the key faecal pollution sources at selected water resources and thereby inform water safety management.

Chapter 4 presented a case study where genetic faecal markers were employed to guide the setup and to calibrate a combined catchment microbial fate and transport and QMRA model, to assess future faecal pollution scenarios and their effect on drinking water safety. The study site was the Danube River at Vienna, where the study assumed water abstraction from the river for drinking water supply. The outcomes of Chapter 3 indicated that the model should focus on human faecal pollution: incorporate all relevant urban wastewater sources (wastewater treatment plants and combined sewer overflows, CSOs) into the catchment model. For the same reason, the QMRA was set up for human pathogens. The microbial fate & transport model was further tailored to human faecal pollution through calibration and validation with human-associated genetic faecal markers and human infectious enterovirus data, respectively. The calibrated and validated model was used to simulate future scenarios of climatic and demographic, as well as wastewater infrastructure changes. According to the outcomes, climatic and demographic changes had little impact on drinking water treatment requirements for safe supply in the scenario where 98 % of the pathogen loads stemmed from WWTP discharges. Strong climate change effects were shown in the scenario with enhanced WWTP treatment, where CSOs were the main faecal pollution sources. In the investigation of wastewater infrastructure upgrades, the combination of enhanced wastewater treatment preventing CSOs had the most significant positive effect on the on drinking water treatment requirements. This case study demonstrated how genetic faecal markers can be used in health and infection risk assessment as a decision support tool for evidence-based water safety planning.

Chapter 5 evaluated the fluorescence-based detection of GLUC activity measurements for automated, online faecal pollution detection, based on peer-reviewed literature. New technological adaptations enable now its automated, near-real-time measurement in a robust and analytically precise manner. Large datasets of high temporal or spatial resolution have been reported from a variety of freshwater resources, demonstrating the great potential of this automated method. However, the faecal indication capacity of GLUC activity and the potential link to health risk is still unclear, presenting considerable limitations. This review provided a critical evaluation of automated, online GLUC-based methods and defined open questions concerning this method of rapid microbiology in relation to water safety management.

In summary, genetic faecal markers constitute a new era in water quality assessment. In combination with environmental and other microbiological parameters in a tailored investigation design, they allow deciphering complex faecal pollution patterns for targeted measures and guide and support health and infection risk assessment, among many other roles in water safety assessment and management. This thesis contributed to our understanding of these roles. Additionally, rapid microbiology using online GLUC-based determination is an established method with open questions regarding its interpretation, as the last chapter of this thesis has shown.

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(2020a) Cambridge Dictionary: automated.

(2020b) Keyence Measurement Library: Inline/Offline Measurement.

(2020c) Law Insider: Definition of Surrogate parameter.

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Annex I: Contributions of the author

Chapter 2 of this thesis is based on the manuscript **"Have genetic targets for faecal pollution diagnostics and source tracking revolutionised water quality analysis yet?"** by Demeter, K., Linke, R., Ballesté, E., Reischer, G., Mayer, R., Vierheilig, J., Kolm, C., Stevenson, M., Derx, J., Kirschner, A.K.T., Sommer, R., Shanks, O., Blanch, A.R., Rose, J., Ahmed, W. and Farnleitner, A.H., in revision, *FEMS Microbiological Reviews*. My main contributions to this manuscript were:

- contribution to the design of the systematic analysis (research questions, focus)
- literature retrieval from Scopus and Web of Science using tailored search syntax, development of the syntax
- manual screening of over 5000 articles and selection of approx. 1100 articles for further data extraction
- data extraction from over 800 articles
- data analysis and visualisation
- liaising with co-authors
- manuscript preparation with support from all co-authors

Chapter 3 of this thesis is based on a manuscript in preparation. My main contributions to this manuscript were:

- DNA extraction of a subset of samples
- genetic marker qPCR measurements of selected markers
- data curation and database building (microbiological and environmental parameters)
- data analysis and visualisation
- manuscript preparation with support from Andreas Farnleitner

Chapter 4 of this thesis is based on the publication **“Modelling the interplay of future changes and wastewater management measures on the microbiological river water quality considering safe drinking water production”** by Demeter, K.*, Derx, J.*, Komma, J., Parajka, J., Schijven, J., Sommer, R., Cervero-Aragó, S., Lindner, G., Zoufal-Hruza, C.M., Linke, R., Savio, D., Ixenmaier, S.K., Kirschner, A.K.T., Kromp, H., Blaschke, A.P. and Farnleitner, A.H. 2021, *Science of the Total Environment* 768, 144278, *shared first authors. The published version can be found in ‘Annex II: Published first-authored articles’. My main contributions to this publication were:

- contribution to the study design (research questions, focus)
- curation of dataset used in model calibration
- selection of input data from the literature
- scenario simulations
- visualisation of the results
- manuscript preparation together with Julia Derx, with support from Andreas Farnleitner

Chapter 5 of this thesis is based on the publication "Automated online monitoring of fecal pollution in water by enzymatic methods" by Demeter, K., Burnet, J.-B., Stadler, P., Kirschner, A. K. T., Zessner, M. and Farnleitner, A. H., 2020, *Current Opinion in Environmental Science & Health* 16: 82-91. The published version can be found in 'Annex II: Published first-authored articles'. My main contributions to this manuscript were:

- retrieved and organised the relevant literature
- liaising with co-authors
- manuscript preparation with support from all co-authors

Annex III: Published co-authored article is the publication "Genetic Microbial Source Tracking Support QMRA Modeling for a Riverine Wetland Drinking Water Resource" by Derx, J., Demeter, K., Linke, R., Cervero-Aragó, S., Lindner, G., Stalder, G., Schijven ,J., Sommer, R., Walochnik, J., Kirschner, A. K. T., Komma, J., Blaschke, A. P. and Farnleitner, A. H., 2021, *Frontiers in Microbiology 12* (1914). My main contributions to this manuscript were:

- contribution to the study design (research questions, focus)
- genetic marker qPCR measurements of selected markers
- curation of dataset used in model calibration
- supported Julia Derx with manuscript preparation (structure, writing of parts, edition of several drafts)

Annex II: Published first-authored articles

Annex II-A: Demeter *et al.*, 2021

Modelling the interplay of future changes and wastewater management measures on the microbiological river water quality considering safe drinking water production

Demeter, K.*, Derx, J.*, Komma, J., Parajka, J., Schijven, J., Sommer, R., Cervero-Aragó, S., Lindner, G., Zoufal-Hruza, C.M., Linke, R., Savio, D., Ixenmaier, S.K., Kirschner, A.K.T., Kromp, H., Blaschke, A.P. and Farnleitner, A.H. 2021, *Science of the Total Environment* 768, 144278, *shared first authors.

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Modelling the interplay of future changes and wastewater management measures on the microbiological river water quality considering safe drinking water production

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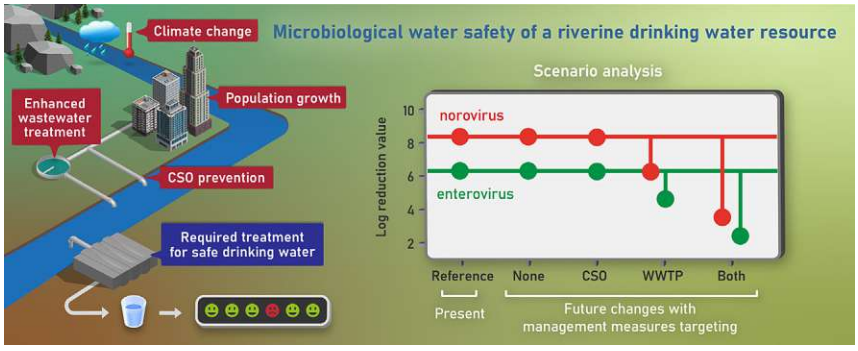
HIGHLIGHTS

- Coupling of hydrologic & QMRA modelling to inform drinking water safety management
- Extended, open-source fate and transport model QMRAcatch calibrated on MST marker
- Dual approach (source apportionment and risk assessment) proved important
- Strong climate change effect if CSOs are major pollutants (enhanced WWTP treatment)
- Effects of future change and pollution control are site-and scenario-specific.

GRAPHICAL ABSTRACT



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ABSTRACT

Rivers are important for drinking water supply worldwide. However, they are often impacted by pathogen discharges via wastewater treatment plants (WWTP) and combined sewer overflows (CSO). To date, accurate predictions of the effects of future changes and pollution control measures on the microbiological water quality of rivers considering safe drinking water production are hindered due to the uncertainty of the pathogen source and transport variables. The aim of this study was to test an integrative approach for an improved understanding of these effects, i.e. climate change and population growth as well as enhanced treatment at WWTPs and/or prevention of CSOs. We applied a significantly extended version of QMRAcatch (v1.0 Python), a probabilistic-

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Climate change
Quantitative microbial risk assessment

deterministic model that combines fate and transport modelling with quantitative microbial infection risk assessment. The impact of climatic changes until the period 2035–2049 was investigated by a conceptual semi-distributed hydrological model, based on regional climate model outputs. QMRAcatch was calibrated and validated using site- and source-specific data (human-associated genetic microbial source tracking marker and enterovirus). The study showed that the degree to which future changes affect drinking water safety strongly depends on the type and magnitude of faecal pollution sources and are thus highly site- and scenario-specific. For example, if the load of pathogens from WWTPs is reduced through enhanced treatment, climate-change driven increases in CSOs had a considerable impact. Preventing CSOs and installing enhanced treatment at the WWTPs together had the most significant positive effect. The simultaneous consideration of source apportionment and concentrations of reference pathogens, focusing on human-specific viruses (enterovirus, norovirus) and cross-comparison with bacterial and protozoan pathogens (*Campylobacter*, *Cryptosporidium*), was found crucial to quantify these effects. While demonstrated here for a large, wastewater-impacted river, the approach is applicable at other catchments and pollution sources. It allows assessing future changes and selecting suitable pollution control measures for long-term water safety planning.

1. Introduction

Rivers are important for drinking water supply worldwide, yet, they are often under pressure from multiple pollution sources. The most widespread health risk associated with drinking water is contamination with pathogens that originate from faecal matter (WHO, 2017b). In densely populated large river catchments, discharges of municipal wastewater treatment plants (WWTP) and combined sewer overflows (CSO) are major contributors to the faecal pollution load (Rickert et al., 2016; WHO, 2017b). Additional faecal pollution sources include wildlife and domestic animal waste. Understanding this plethora of pressures and their future changes, along with their impact on the drinking water source, poses a great challenge for water safety planning (Rickert et al., 2016).

The future climatic and population changes may affect faecal pollution sources, the microbiological quality of surface water, and ultimately drinking water safety. Population growth and the associated increase in (treated) wastewater discharges may result in the deterioration of river water quality, and the concerns may be further aggravated by climatic changes (WHO, 2017a). According to climate projections, the frequency and intensity of extreme rainfall events will increase in many areas (Myhre et al., 2019). This will result in runoff flushes and, in places with a combined sewer system, more frequent and intense CSO events (Bi et al., 2015; Nie et al., 2009; Willems et al., 2012). In addition, the hydrological regimes and temperatures of rivers are likely to change (e.g., Blöschl et al., 2019), affecting their buffering capacities in terms of dilution and inactivation of pathogens. Droughts and the resulting low river discharges would concentrate contaminants in river and groundwater resources (WHO, 2017a), while floods are often accompanied by short-term deteriorations of water quality due to agricultural runoff and CSOs (e.g., Derx et al., 2013). In contrast, higher water temperatures expected due to climate change may facilitate the inactivation of enteric pathogens (Boehm et al., 2019). If the pollution sources are mainly urban wastewater discharges, possible strategies to reduce faecal contamination include enhanced wastewater treatment and CSO prevention. As a final step in wastewater treatment, ozonation and advanced oxidation processes as well as UV-treatment and chlorination allow a considerably reduced pathogen load in the final effluent. Measures to prevent CSOs include reservoirs or any form of green infrastructure affecting runoff water quantity and quality at different spatial scales (Golden and Hoghooghi, 2018).

The sum of climatic and demographic changes were previously found to deteriorate microbiological water quality to a limited degree, with less than 0.5 $\log_{10}$ increase in the mean concentration of faecal indicator bacteria or index pathogens until 2040–2070, as shown for large rivers in Canada (Jalliffier-Verne et al., 2017; Jalliffier-Verne et al., 2015), Bangladesh (Islam et al., 2018a), Pakistan (Iqbal et al., 2019), and a fictive river in the Netherlands (Sterk et al., 2016). The impact of CSOs and WWTPs on the microbiological water quality of rivers has been analysed from various perspectives. Upstream short-term pollution events, such as via CSO discharges (Taghipour et al., 2019) or WWTP bypass and pumping station overflow events (Sokolova et al., 2015) were shown to be less important for drinking water safety if relying on surface water than the optimal treatment performance of the drinking water treatment plant itself. The simultaneous reduction of multiple faecal inputs in two catchments with high human population and livestock numbers was found beneficial under a sustainable future scenario, in comparison with the uncontrolled future scenario (Iqbal et al., 2019; Islam et al., 2018a). Medema and Schijven (2001) calculated that the majority of *Cryptosporidium* oocysts in Dutch rivers originates from treated sewage, while *Giardia* was rather linked to untreated discharges, pointing at the different strategies needed to reduce their concentrations in river water. Sterk et al. (2016) found an elevated infection risk through bathing downstream of the discharge point of a WWTP all-year round, while even higher risks, although intermittent, downstream of a CSO. The various measures that can be taken to reduce the input of pathogens into the river have not yet been analysed systematically. Questions also remain as to how climate and demographic changes would alter the effect of these measures.

Assessing faecal pollution dynamics and their possible future developments at the catchment scale is a complex problem as it involves large uncertainties of the source and transport variables (Cho et al., 2016). Most of these microbial fate and transport models focus on faecal indicator organisms (Islam et al., 2018b; Kim et al., 2017), while some also include microbial source tracking markers (MST) enabling source-specific model calibration (Sokolova et al., 2012) or pathogens allowing the assessment of health risks directly (Dorner et al., 2006; Fauvel et al., 2017). Depending on the purpose, the developments range from physically based models that simulate water currents requiring much computational effort, to process based pathogen fate and transport models requiring a high number of input parameters (e.g., SWAT, Kim et al., 2017). Recently, studies have combined fate and transport models with quantitative microbial risk assessment (QMRA) to estimate the health risk associated with drinking (Sokolova et al., 2015) or bathing (Eregno et al., 2016; Sterk et al., 2016). QMRAcatch was one of the first models of this kind. Its microbial fate and transport module follows a mass balance approach to simulate microbial concentrations in river water and accounts for the uncertainty of model input variables (e.g., concentration of microorganism in raw wastewater) by using a probabilistic approach. The QMRA module of QMRAcatch simulates the infection risks associated with the ingestion of pathogens contained in drinking water as well as the required treatment to produce safe drinking water (Derx et al., 2016; Schijven et al., 2015).

This study aimed to test a new integrative approach for deciphering the interplay between the effects of climate and demographic changes

and wastewater management measures on the microbiological river water quality with regards to the required treatment to produce safe drinking water. We investigated the effects of future climatic and demographic changes up to 2050, as 'no management changes' scenario, as well as these effects combined with measures that aim to reduce the pollution from upstream WWTPs, CSOs, or both. Additionally, we investigated the effects of increased CSOs in a systematic sensitivity analysis. The approach was tested at a Danube River study site in Vienna, representative of large rivers where the dominant source of faecal pollution is human wastewater from upstream point sources. The scenarios were analysed for two viral reference pathogens: enterovirus and norovirus, which are mainly associated with human wastewater and are often used as references for infection risk assessment from water resources (WHO, 2017b). Additionally, the scenarios were extended for *Campylobacter* and *Cryptosporidium* for a cross-comparison between human viral pathogens and bacterial and protozoan reference pathogens. To meet our aim, we significantly extended QMRAcatch (Schijven et al., 2015) (v1.0 Python) now available as open source, which we calibrated and validated based on concentrations of a human-associated genetic MST marker and infectious enteric viruses, measured at the study site monthly over a period of four years. In the scenario analysis, river discharges were simulated using a conceptual semi-distributed hydrological model and four regional climate model projections covering the range of expected climate change pathways.

2. Materials and methods

2.1. Study area

The study site is located at the Danube River, in Vienna, Austria (Fig. 1). The Viennese drinking water supply relies partially on water from the Danube River. The Danube River starts in Germany, approx. 850 km upstream, and shows dynamic variations in river discharge, ranging from 900 to 5300 m^3/s for the characteristic low and high discharges, with a mean discharge of 1900 m^3/s at the study site. The region has a temperate climate where floods are driven by snow melts and heavy rainfall events in the headwater catchment. The Danube River is moderately polluted with faecal matter that originates predominantly from human wastewater (Frick et al., 2017; Kirschner et al., 2017). The catchment upstream of Vienna is home to approx. 11 million inhabitants (Schreiber et al., 2005). Considering that 99% of the human population in the study area is connected to a WWTP (European Commission, 2018), urban wastewater is the main source of human pollution. This site is therefore representative of a large river polluted by upstream discharges of urban wastewater.

2.2. Modelling approach

Two models are applied in this study (Fig. 2). A hydrological model is used to simulate the river discharge in the Danube for a reference period (2003–2017) and a future period (2035–2049) based on regional climate model outputs (Parajka et al., 2016). The hydrological model domain encompasses the entire Danube subcatchment drained by the Danube up to the study site (104,000 km^2). A newly adapted version of the microbial fate and transport and infection risk model QMRAcatch is used to simulate the concentrations of viruses in river water at the study site and the required reduction of human viruses in source water to achieve safe drinking water (log reduction value, LRV). Its model domain is the 190-km-long Danube River section directly upstream of the study site and includes all sources of human wastewater, represented by: i) the effluent of five WWTPs situated 20, 24, 43, 77, and 193 km upstream of the study site and ii) the corresponding CSOs.

2.3. Hydrological model

To simulate the daily river flow rates of the Danube at the study site, we used a conceptual spatially-distributed hydrological model (Blöschl et al., 2008), which we extended for operational river flow forecasting. The structure is similar to that of the HBV model (Bergström, 1976) but several modifications were made including an additional groundwater storage, a bypass flow (Blöschl et al., 2008; Komma et al., 2008), and a modified routing routine. For each raster cell (5 km × 5 km), snow processes, soil moisture processes, and hill slope scale routing are simulated at an hourly time step. In the snow routine, snow accumulation and melt are represented by a simple degree-day concept. Runoff generation and changes in soil moisture storage are calculated by a soil moisture accounting scheme as a nonlinear function of rainfall and evaporation. Runoff is generated as a combination of outflows from three reservoirs representing overland flow, interflow, and deep groundwater flow processes. Runoff routing in the stream network is described by a cascade of linear reservoirs (Szolgay, 2004). More details and application examples are given, e.g., in Blöschl et al. (2008), Komma et al. (2008), and Reszler et al. (2008).

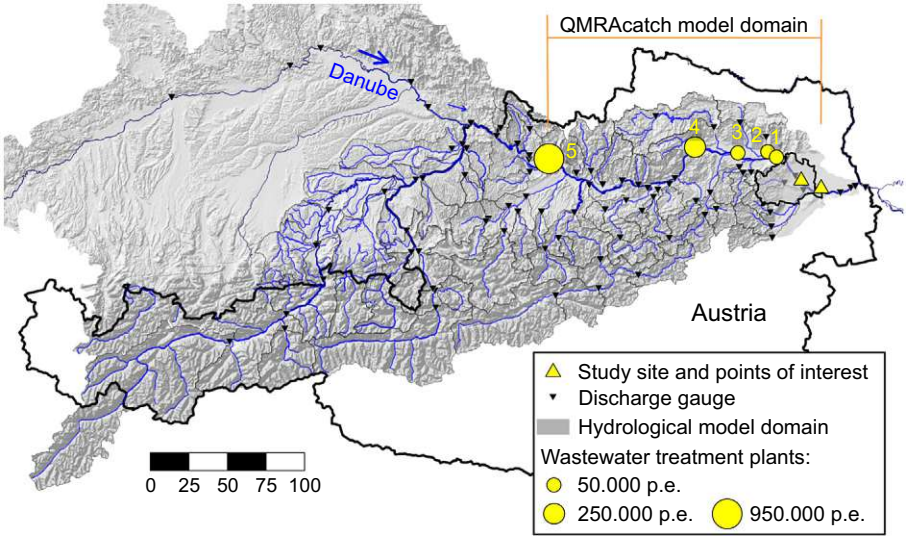


Fig. 1. Map of the study area.

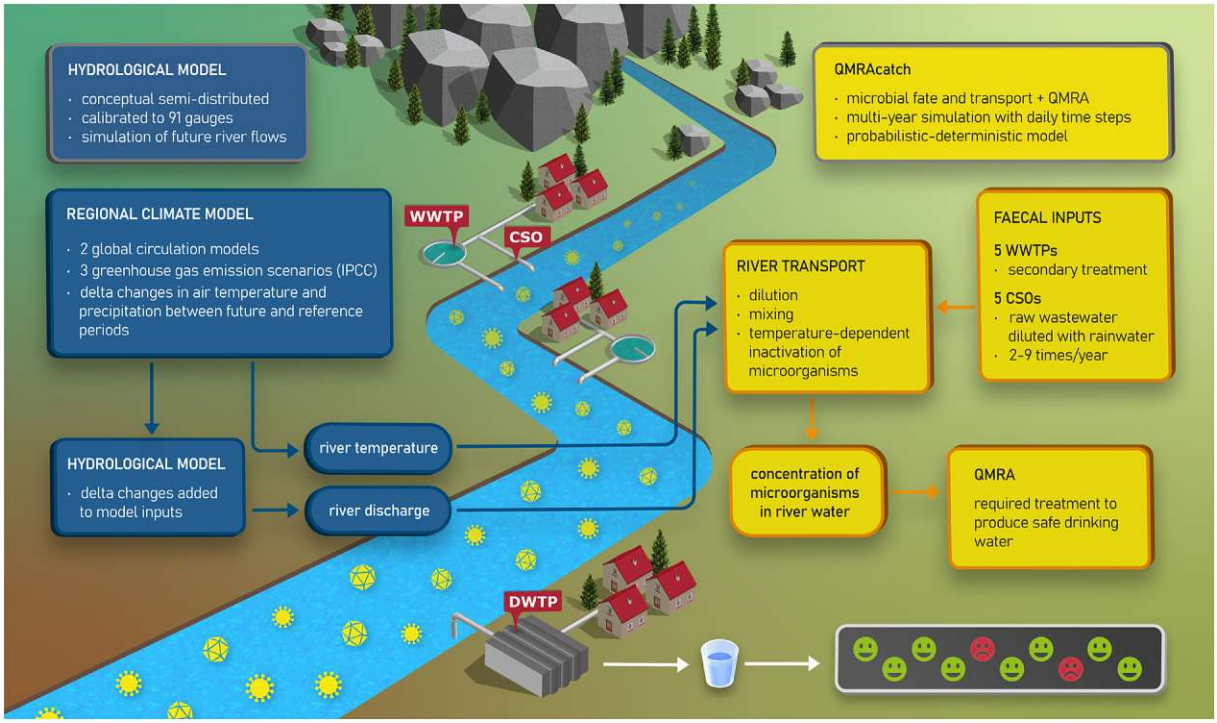


Fig. 2. Overview of model components.

In the extended version, the model input consists of spatially distributed fields of precipitation, air temperature, and potential evaporation. Meteorological and hydrological data were available for the period from 2003 to 2017 (provided by ZAMG and HZB, the central services for meteorology and hydrology in Austria). The data set includes several extreme flow periods, such as the 200-year flood in 2013 or the drought in 2015. The existence of hydro-meteorological extremes in the data set helps to estimate more robust and appropriate model parameters for predictions and extrapolation to future scenarios. For parameter identification, a hydrologic response unit approach based on spatial information about land use, soils, hydrogeology, and topography combined with a manual calibration procedure (Reszler et al., 2008) has been adopted. In this study, 15 different hydrologic response unit types were used to describe the different hydrological response behaviours (Table S1). The parameters have been calibrated and validated against hourly discharge data at 91 discharge gauges at the Danube and its tributaries. The simulation period included a calibration (2012 to 2018) and a validation period (2003 to 2011). The Nash and Sutcliffe coefficient of runoff model efficiency (Nash and Sutcliffe, 1970) at the 91 discharge gauges ranged between 0.72 and 0.84 for the calibration and between 0.66 and 0.85 for the validation period. The model efficiency for the calibration and validation period at the Danube gauge was 0.78 and 0.73, respectively.

2.4. QMRAcatch

2.4.1. Model overview

The probabilistic-deterministic microbial fate and transport and infection risk model QMRAcatch (Schijven et al., 2015) was extended for this study and newly coded as open source (v1.0 Python). QMRAcatch was used to estimate the microbial concentrations (Section 2.4.2) and the required reduction of reference pathogens to produce safe drinking water at the study site (Section 2.4.3). The model comprises the functionality of the original Mathematica version *QMRAcatch06062019.cdf* with the following extensions:

- Simulation time over multiple years in contrast to one year in the previous version.
- All model calculations are repeated for 100 to 1000 Monte Carlo simulations until results remain stable, to account for the natural variability and uncertainty of the model input parameters listed in Tables 1 and 3. In the previous version random values of all stochastic input variables were generated only once for each day in the simulation period.
- Pathogen loads are calculated from the simulated concentrations in river water at the study site.
- One CSO is located at each WWTP, as previously. The CSO discharge volumes and frequencies are now set independently from the continuous WWTP discharges. The days when CSOs occur are set to the days of observed rainfalls (during model calibration and validation) or randomly over the year based on a uniform probability distribution for each Monte Carlo run (scenario and sensitivity analysis).
- The transverse spreading of a continuous point source in a wide river flow was accounted for according to Jirka et al. (2004).
- In contrast to the previous version, the temperature-dependent microbial inactivation coefficients (for which site-specific values are not available) were set during model calibration within constrained limits based on reported persistence data.

2.4.2. Microbial fate and transport module

2.4.2.1. Faecal inputs. In QMRAcatch, the microbial concentration entering the WWTP (C_{raw}) is multiplied by the fraction of pathogens passing the WWTP ($Log_{10}\ F_{wwtp}$) to determine the concentration discharged to the surface water (C_{wwtp}). It is assumed that microbes in water samples at low concentrations are Poisson distributed. In Bayesian inference, the conjugate prior for the rate parameter of the Poisson distribution is the Gamma distribution. C_{raw} is therefore described by a gamma distribution (Table 1 for norovirus and enterovirus, Table S3 for *Cryptosporidium* and *Campylobacter*). The lognormal distribution was used here to describe the treatment efficiency of water (F_{WWTP}). It is convenient in that it describes the skewness of the treatment efficiency well and it is

Table 1
Model input parameters for norovirus and enterovirus (see Table S3 for Cryptosporidium and Campylobacter, as well as Table 3 for the parameters varied in the scenario analysis). Gamma probability distribution function (mean, 95th percentile) of the microbial concentrations in raw wastewater (C_{raw}), normal probability distribution function (mean, 95th percentile) of microbial removal by wastewater treatment (F_{WWTP}), WWTP effluent discharge rate (Q_{WWTP}), inactivation rate coefficients (a_0, a_1), and dose-response parameters α and β.

Parameter	Unit	Distribution	Details	Microorganism	Value	Reference
C_{raw} (mean, 95th perc.)	N/L	Gamma	WWTP 1,2,4,5 WWTP 3	Human MST marker	$(1.84, 5.82) \times 10^9$ $(1.04, 3.52) \times 10^9$	Schijven et al. (2015)
				Enterovirus	$(1.0, 2.0) \times 10^3$	(WHO, 2017b)
				Norovirus	$(1.0, 2.0) \times 10^5$	Katayama et al. (2008) Lodder and Husman (2005)
F_{WWTP} (mean, 95th perc.)	Log_{10}	Normal	WWTP 1,2,4,5 WWTP 3	Human MST marker	2.63, 2.15 2.25, 1.39	Derx et al. (2016)
				Enterovirus	1.8, 0.2	This study (calibrated)
				Norovirus	1, 0.75	Lodder and Husman (2005)
Q_{WWTP}	m^3/s	n.a.	WWTP1 to 5	–	0.30, 0.34, 0.17, 0.67, 7.24	Data provided by the EPA Austria
a_0, a_1	–	n.a.	First order decay in function of water temperature	Human MST marker	0.6, −0.035	This study (calibrated)
				Enterovirus	0.68, −0.036	This study (calibrated)
				Norovirus	2.3, −0.035	Bertrand et al. (2012) (mean value)
α, β	–	n.a.	Dose-response relationship: hypergeometric with beta-distributed parameters	Enterovirus	0.253, 0.422	Teunis et al. (1996)
				Norovirus	0.04, 0.055	Teunis et al. (2008)

easy to interpret. We generated random values for each Monte Carlo run by drawing from these distributions to reflect the temporal concentration variability. A constant discharge of treated wastewater (Q_{WWTP}) was attributed to WWTPs 1–5 based on previous annual discharge measurements at WWTPs 1–5 and in the rest of the QMRAcatch model domain (Fig. 1). The WWTPs provide secondary (conventional biological) treatment without chlorination or other tertiary treatment.

During CSO events, untreated wastewater mixed with rainwater discharges into the river. The microbial concentration in CSO water (C_{CSO}) is therefore a fraction of C_{raw}. The yearly CSO volumes were roughly estimated by Thomas Ertl and Florian Kretschmer (personal communication, Clara et al., 2014) based on the mean annual precipitation, the contributing runoff areas, the proportion of the combined sewer system, and the theoretical fraction of water piped to the WWTPs (ÖWAV, 2007).

2.4.2.2. Dilution in river water. The influx of microorganisms to the river water, through either a WWTP or a CSO, is diluted in river water. The mixing happens gradually as the water flows downstream. To calculate the cross-sectional concentration in the surface waters at X meters downstream of the emission, the transverse spreading of a continuous point source in a wide river flow (W >> h) was accounted for according to Jirka et al. (2004). The distance L_{mh} to the location where complete horizontal mixing over the river cross-section takes place was calculated by

$$L_{mh} = 0.4\frac{UW^2}{E_y} \quad (1)$$

where U is the flow velocity [m/s] calculated according to Manning (1891) and W is the river width [m] (Table A.1). The horizontal diffusivity E_y is calculated according to Fischer et al. (1979).

$$E_y = \alpha_y u^* h \quad (2)$$

where α_y is the diffusivity constant with a possible range of 0.5 ± 0.25 [−] for rivers without strong meanders and lateral dead zones, u^* is the friction velocity [m/s] and h is the river depth [m] (Table A.1). Vertical mixing was computed to be complete after a few hundred meters. The dilutions of microbial loads with river water are then calculated as

$$C_{river} = \sum_{i=1}^{n_{WWTP}} \left[\frac{\left(C_{WWTP_i} Q_{WWTP_i} + C_{CSO_i} Q_{CSO_i}\right)}{Q_{river}} \frac{L_{mh}}{X_i} \right] \quad (3)$$

where Q_{WWTPi} [m^3/s] is the discharge of treated wastewater at $WWTP_i$, Q_{CSOi} [m^3/s] is the CSO discharge, Q_{river} [m^3/s] is the river discharge, X_i is the distance of the point of interest to the pollution source [m], and n_{WWTP} is the number of WWTPs.

2.4.2.3. Inactivation during transport. The degree of removal of pathogens during transport depends on the travel time or flow rate. Inactivation during transport is described as a first order decay reaction, where the decay rate in water (μ_w [d^{-1}]) during transport is a function of the water temperature (T [°C]):

$$\mu_w(t) = \frac{ln\,10}{10^{a_0 + a_1 T}} \quad (4)$$

where a_0 [$\log_{10}$ day] and a_1 [$\log_{10}$ day °C^{-1}] are microorganism and pathogen-specific inactivation rate parameters (Bertrand et al., 2012). After a travel time of *m* days to the study site, microorganism concentrations ($C_{m,T}$ [m^{-3}]) are calculated as:

$$C_{m,T} = C_0\, exp\left(-\sum_{i=1}^{m} \mu_{W_i}\right) \quad (5)$$

where C_0 is the initial concentration [m^{-3}] and T_i is the temperature [°C] on the i^{th} day. The parameters a_0 and a_1 were determined using linear regression between reported times to first $\log_{10}$ reduction (TFL) versus water temperature. Inactivation rates that were reported in the literature were reviewed and summarized in Fig. B.1 and Table S2.

2.4.3. QMRA module

Daily probabilities of infection for enterovirus and norovirus can be estimated using a hypergeometric dose-response relation (Teunis and Havelaar, 2000):

$$P_{inf} = 1 - {}_1F_1(\alpha, \alpha+\beta, D) \quad (6)$$

$$D = C_{RW} \times 10^{LRV} \times I \times V \quad (7)$$

where P_{inf} is the daily probability of infection for a person per exposure, α, β are the parameters of dose-response models (Table 1), D is the dose of ingested microorganisms, C_{RW} is the microorganisms' concentration in the river water, LRV is the required treatment reduction of viruses, I is the infectious fraction, and V is the consumed unboiled water volume (1 L/person/day (WHO, 2017b)). As the daily health-based target (hbt), $1 \cdot 10^{-6}$ infections/person/day was adopted in this study (Signor and Ashbolt, 2009). LRV was estimated iteratively until the criterion $P_{inf} \leq$ hbt according to Eq. (6) was fulfilled for both the mean (μ) and 95th percentile values of P_{inf}.

$$LRV = \max\left[log_{10}\left(\frac{\mu^{P_{inf}}}{hbt}\right), log_{10}\left(\frac{95\%^{P_{inf}}}{hbt}\right)\right] \quad (8)$$

The dose-response model parameters for enterovirus were based on results from a human challenge study conducted with rotavirus where the minimum infectious dose for enterovirus was 1 focus forming unit (Ward et al., 1986). We used enterovirus data based on a cell culture method, detecting infectious enterovirus. The fraction of infectious to total viral particles is unknown for norovirus, as their enumeration method is based on PCR methods. However, the frequent occurrence of these viruses in outbreaks suggests high infectivity (Teunis et al., 2008). As the same authors pointed out, the resulting risk estimates might still be unbiased if the ratio of total to infectious numbers of viruses is constant because the exposure estimates in our scenarios are based on the same enumeration methods as in the human challenge study.

2.4.4. Microbial characterization of wastewater and river water

2.4.4.1. Microbiological analyses of wastewater. Values for C_{raw} and C_{wwtp} of the human-associated MST marker at WWTP2 and 3 were available from single 1-L samples collected from 2010 to 2013 (n=72, Schijven et al., 2015, Table 1). Additionally, values for C_{wwtp} of enterovirus at WWTP2 were isolated from 1-L samples between 2011 and 2017 (n=73) according to the inorganic flocculation and ultracentrifugation method of Walter and Rüdiger (1981) and enumerated following an MPN method (Chang et al., 1958) on Buffalo green monkey kidney cells (Dahling and Wright, 1986). For the concentrations of microorganisms in wastewater, QMRAcatch estimates the parameters of a Gamma distribution based on mean and 95th percentile values. The enterovirus concentrations were MPN values, i.e., maximum likelihood estimates, assuming Poisson distributed count observations. Given that these are concentration estimates already, concentration estimates of zero could be included in the calculation of the mean and 95th percentile values. We assumed a mean and 95th percentile of 1×10^3 and 2×10^3 MPN/L for C_{raw} of enterovirus, respectively (WHO, 2017b). F_{wwtp} was adjusted until the mean and 95th percentile of generated random values of C_{wwtp} matched the respective observed values (Table 1). Mean C_{raw} and F_{wwtp} values of 1×10^5 gene copies (gc)/L and 1-$\log_{10}$ for norovirus were assumed, respectively, according to Katayama et al. (2008) and Lodder and Husman (2005) (Table 1). The microbiological input parameters for *Cryptosporidium* and *Campylobacter* are described in Section 2 of the Supplementary information.

2.4.4.2. Microbiological analyses of river water. Surface water samples were collected from the Danube riverbank as grab samples at two sampling points (Fig. 1) alternately on a monthly basis over four years, resulting in a bi-weekly dataset of independent values (2013–2017, n=87–94). Because of the vicinity of the two points, they were treated as one point of interest for drinking water production, called hereafter 'the study site' (Fig. 1). The samples were analysed for the human-associated MST marker HF183/BacR287 as well as for infectious enterovirus. The human-associated MST marker was quantified in 500–600-mL water samples using quantitative PCR as described previously (Green et al., 2014; Mayer et al., 2018). The human-associated MST marker was detected in all samples with a median concentration of 1.1×10^4 ME/L (marker equivalent per litre) and a range of 4.0×10^2 to 1.4×10^6 ME/L (n=87, Fig. B.2). The filtration volume, the use of 2.5-μL of undiluted DNA extract in qPCR, and the minimal theoretically detectable marker concentration per reaction defines the detection threshold (3.0×10^2 ME/L) (Reischer et al., 2008). Infectious enteroviruses were enumerated from 10-L water samples according to the method described above. Enteroviruses were detected in 42% of the samples with a maximum concentration of 11.3 CU-MPN/L (cytopathic unit, most probable number, n=94). The limit of detection was 0.1 CU-MPN/L.

2.4.5. Calibration, validation, and application of QMRAcatch

Due to the limited availability of pathogen data in river water, we took a three-step approach for the calibration, validation and application of QMRAcatch: (1) model calibration and validation using the source-specific and highly abundant human-associated MST marker by adjusting the calibration parameters; (2) model calibration and validation using the reference pathogen enterovirus by adjusting just the microorganism- and virus-specific calibration parameters (the others taken over from step one); and (3) application of the calibrated and validated model to various pathogens (enterovirus, norovirus, *Campylobacter* and *Cryptosporidium*) by using measured data or values assumed from the literature as model inputs (overview in Table B.1, data in Tables 1 and S3).

The datasets of observed concentrations of the human-associated MST marker and enterovirus (n=87 and 94, respectively) were split into two time periods. Data for the period from July 2013 to June 2015 was used for calibration, and for the period from July 2015 to June 2017 for validation, for both microorganisms (Table B.1). Non-detects were set to the limit of detection in the calculations.

The mean absolute error (MAE) was used as a performance metric (Willmott and Matsuura, 2005). $\log_{10}$ transformed concentrations were used in the MAE computations because microorganisms typically follow a lognormal distribution, and the use of logarithms minimizes the influence of outliers present in the data (Hong et al., 2018). The Mann-Whitney test was used for the distribution comparisons of the simulated and observed datasets and the p-value of the Mann-Whitney statistic was a metric of model performance. During model calibration, the calibration parameters were adjusted to minimize the objective function (OF).

$$OF = MAE + (1-p) \quad (9)$$

The calibration parameters can be grouped into (i) not faecal microorganism and pathogen-specific and (ii) faecal microorganism and pathogen-specific parameters. The parameters of the first group describe the discharge and mixing processes which are assumed to be the same for faecal indicators and pathogens in river water. These are the constant diffusion coefficient α_y (Table A.1, Eqs. (1) and (2)), the frequency and discharge volumes of CSOs 1–5, and the microbial concentration in CSO water as a fraction of the concentration in raw wastewater (Table 3). The parameters of the second group are the microorganism- and virus-specific inactivation parameters a_0 and a_1 (Table 1). In the first step (calibration with the human-associated MST marker), calibration parameters of both groups were adjusted, and the best combination used for the validation. In the second step (calibration with enterovirus), the calibrated values of the parameters of the first group were taken over from step 1 and kept constant, and only the parameters of the second group were adjusted. The best combination of the calibration parameter settings was used for the validation. The CSO volumes per year were constrained to ±25% of the yearly estimates (Section 2.4.2). The CSO events were constrained to days when the daily amount of rainfall exceeded 13 mm/d. Recorded precipitation data were used at gauges Vienna Hohe Warte (at WWTPs 1 and 2), Langenlebarn (at WWTP 3), Krems (at WWTP 4), and Linz-City (at WWTP 5, data provided by the Austrian Meteorology Survey, ZAMG). Inactivation rates of the human-associated MST marker, enterovirus, and norovirus that were reported in the literature were collected and summarized in Fig. B.1 and Table S2. Boehm et al. (2019) conducted an extensive literature review on inactivation studies for viruses and conducted a multiple linear regression analysis between environmental variables and first-order decay rates. Enterovirus inactivation rates showed a statistically significant relationship with temperature, method and sunlight, therefore we restricted our selection to studies conducted with cell

culture (so that they are comparable to our results, see Section 2.4.4) and in natural or artificial sunlight. The coefficient 'water type' was not significant in the multiple linear regression, therefore we included all water types (marine and estuarine – no study was conducted in freshwater in sunlight). Norovirus only showed a significant relationship with temperature so we included all studies listed by Boehm et al. (2019) (Table S2). An ordinary least square method was used to fit the time-to-first-log (TFL) as a function of water temperature (dashed lines in Fig. B.1). During the adjustment of intercept a_0 and slope a_1 (used in Eq. (4), solid lines in Fig. B.1), it was ensured that the inactivation as function of temperature obtained through the calibration lay within the prediction interval of the ordinary least square regressions (Fig. B.1, shaded area, left and centre).

2.5. Scenario analysis

We defined a reference scenario and the following future scenarios: i) climate and demographic changes and ii) three scenarios of wastewater management measures that aim to reduce the faecal load from WWTPs and CSOs. Table 2 provides an overview of the scenarios. Additionally, we conducted a sensitivity analysis to investigate the impact of CSO changes. For all scenarios and the sensitivity analysis, the concentrations of enterovirus, norovirus, *Cryptosporidium* and *Campylobacter* in the Danube were simulated using the input settings as described in Tables 1, 3 and S3. Subsequently, a QMRA was conducted for assessing the required LRV to achieve the health-based target.

2.5.1. Reference scenario

We simulated the concentrations of norovirus and enterovirus in river water at the study site, as well as the required LRVs to produce safe drinking water for the reference period from 2003 to 2017. This period included hydrologically extreme years and was therefore deemed a robust basis for the scenario analysis (Tables 2 and 3).

2.5.2. Future scenarios

2.5.2.1. Climate and demographic changes: 'No management changes' scenario. In this study, flow projections of future climate scenarios were modelled using a hydrological model forcing from a delta change approach as described in detail by Parajka et al. (2016). In a first step, regional climate model (RCM) outputs were used to estimate monthly differences in air temperature and precipitation between reference (control) and future periods (2035–2049). These differences (delta changes) were then added to the observed precipitation and air temperature data and used as model inputs to simulate future hydrologic changes (Fig. C.1). The daily precipitation was scaled by the relative delta changes for each month, and the frequency of rainy days was kept as in the reference period. The daily air temperature was changed by the mean daily delta changes each month. To obtain future delta changes in water temperature, the delta changes of daily air temperature were multiplied by seasonal conversion factors derived from the observed changes in air and water temperatures of the Danube from 1900 to 2010 (Standhartinger and Godina, 2013, p. 46, Fig. 11). The conversion factors for December, January, and February resulted in 1.22; for March, April, and May they resulted in 0.52; for June, July, and August they resulted in 0.76; and for September, October, and November they resulted in 1.5.

The RCM scenarios used in this study are based on the results of the reclip:century project (Loibl et al., 2011; Parajka et al., 2016). The ensemble climate projections are represented by COSMO-CLM RCM runs forced by the ECHAM5 and HADCM3 global circulation models for three different Intergovernmental Panel on Climate Change (IPCC) emission scenarios (A1B, B1, and A2; Nakicenovic et al., 2000). These represent a large spread of different emission pathways based on no change in greenhouse gas emission practices (A2), a scenario with a moderate decline in emissions after 2050 (A1B), and a scenario indicating considerably reduced emissions from the present onwards (B1). For this study, the projections by the ECHAM5 model were selected for the three emission scenarios (A1B, A2, B1), as well as the projections by the HADCM3 model for one emission scenario (A1B). Although these scenarios are meanwhile replaced by the Representative Concentration Pathways (RCPs, van Vuuren et al. (2011)) these older scenarios are still comparable to the newer RCPs with respect to their climate change signals. Moreover, the model setup for RCM simulations of reclip:century are specifically tailored for the complex terrain of the Alpine Region and therefore provide more robust estimates of the future climate change in the Alps and surrounding areas (Blöschl et al., 2018; Blöschl et al., 2017).

The reclip:century scenarios project, for the study area, changes in air temperature and precipitation between the future period 2035–2049 and the reference period 2003–2017. Precipitation projections show that winters will become 5% (HADCM3 A1B) to 22% (ECHAM5 A2) wetter and extreme precipitation quantities will increase. Predictions of future precipitation changes for summer range from 4% (HADCM3 A1B) to −21% (ECHAM5 A2). These changes are generally in line with the newer generation of RCM simulation from the EURO-CORDEX initiative where an ensemble of simulations for RCP4.5 and 8.5 are downscaled for the Austrian domain. Only the EURO-CORDEX ensemble mean summer precipitation change signal for Austria is +3% for RCP8.5, showing somewhat different results compared to ECHAM5 A2, however, the ensemble spread in EURO-CORDEX is rather large pointing towards higher uncertainties during the summer season.

For the study site of the Danube, all climate scenarios project a general decrease of river flows during the low flow period (summer) and a slight increase or no change of river flow during the high flow period (end of winter/spring). The river flow in the Danube study catchment is expected to decrease on average by 14% (ECHAM5 A1B) to 25% (ECHAM5 A2), with a slight increase of about 10%–15% in January and February for the ECHAM5 A1B scenario and an almost 50% decrease in August for the ECHAM5 A2 scenario (Fig. C.1).

Population growth will result in a corresponding increase in urban wastewater discharges into the Danube. An increase in wastewater discharge volumes by 14% until 2050 was considered at WWTPs 1–5, according to the projected population growth of Lower Austria, the state covering the majority of the model domain (Austria, 2017, Tables 2 and 3).

2.5.2.2. Scenarios of wastewater management measures

2.5.2.2.1. 'Enhanced wastewater treatment' scenario. The current EU regulations for WWTPs require a reduction of organic carbon, nitrogen,

Table 2
Overview of the scenario analysis. +: taken into account, −: not applicable/not applied.

Scenario	Climate change (river discharge and temperature)	Population growth (WWTP discharge)	Enhanced wastewater treatment	CSO prevention
Reference	−	−	−	−
No management changes	+	+	−	−
CSO prevention	+	+	−	+
Enhanced wastewater treatment	+	+	+	−
CSO prevention and enhanced wastewater treatment	+	+	+	+

Table 3
Model input parameters for the reference scenario (2003–2017) and future scenarios (2035–2049) as well as for the sensitivity analysis, for the study site in the Danube.

Parameter	Dimension	Reference scenario	Description of future change	Future scenarios/sensitivity analysis
Population growth and climate change				
Effluent discharge at WWTPs 1–5	m^3/s	See Table 1	Population growth	+14% (Austria, 2017)
Daily river discharge at study site	m^3/s	Hydrological modelling for period 2003–2017	Climate scenarios ECHAM5-A1B, A2, B1, HADCM3-A1B (Loibl et al., 2011, Parajka et al., 2016) (Section 2.5.2.1)	Hydrological modelling from 2035 to 2049
Daily river water temperature	°C	Data records at Danube gauge Greifenstein from 2003 to 2018 (viadonau, 2019)		Delta changes in air temperature and season-specific conversion factors (Section 2.5.2.1)
Changes due to wastewater management measures				
Log_{10} reduction by wastewater treatment (F_{wwtp})	Log_{10}	See Table 1	Additional treatment	+ 4 (Campos et al., 2016, Francy et al., 2012, Gerrity et al., 2012, Owens et al., 2000, Paraskeva and Graham, 2002)
CSO frequency	N/year	At WWTP 1: 2 At WWTP 2: 2.5 At WWTP 3: 9.5 At WWTP 4: 5.5 At WWTP 5: 4 (calibrated)	Complete prevention of CSOs through, e.g., reservoirs	0
CSO discharge at WWTPs 1–5	m^3/s	At WWTP 1: 0.89 At WWTP 2: 1.01 At WWTP 3: 0.52 At WWTP 4: 2.00 At WWTP 5: 21.73 (calibrated)	Complete prevention of CSOs through, e.g., reservoirs	0
Sensitivity to changes in CSOs				
CSO frequency at WWTPs 1–5	N/year	Calibrated (see above)	More frequent extreme rainfall events	Up to 3-fold increase
CSO discharge at WWTPs 1–5	m^3/year	Calibrated (see above)	More frequent extreme rainfall events	Up to 3-fold increase
Concentration of pathogens in CSO water	–	0.1 (calibrated)	Fraction of the concentration in raw wastewater	0.1 and 1.0 (de Man et al., 2014, Sterk et al., 2016)

and phosphorous, but there are no microbiological requirements or obligations for disinfection (European Commission, 1998). A possible strategy to reduce the load of pathogens from WWTPs is to include ozonation and/or UV irradiation as tertiary wastewater treatment. According to previous reports, the efficiency of disinfection during wastewater treatment on reducing virus concentrations can remarkably vary, depending on the dose of chemicals or the UV fluence, from 1.5 to 4 log_{10} particles/L by ozonation (Gerrity et al., 2012; Owens et al., 2000; Paraskeva and Graham, 2002) or UV irradiation (Campos et al., 2016; Francy et al., 2012). We considered an additional treatment step at at WWTPs 1–5 that reaches a reduction of entero- and norovirus by 4 log_{10} in the scenarios (Tables 2 and 3).

2.5.2.2.2. 'CSO prevention' scenario. A further possible measure is to prevent CSO events using, for example, stormwater reservoirs, retention basins, rain barrels, green roofs, permeable patios, or grassed swales (Demuzere et al., 2014; Lewellyn et al., 2016; Pazwash, 2016). We assumed that the measures are capable of completely preventing CSOs (Tables 2 and 3).

2.5.2.2.3. 'CSO prevention and enhanced wastewater treatment' scenario. A combination of the above two wastewater management measures was considered in the fourth future scenario.

2.5.3. Sensitivity analysis to investigate the effects of increased storm events

The extreme storm event frequency is thought to increase with warming at a rate similar to the water vapour holding capacity of the air, the so-called Clausius-Clapeyron rate, at ~7%/°C (Molnar et al., 2015). CSOs are therefore likely to happen more frequently, but their reaction to changes in rainfall is highly non-linear (Willems et al., 2012). Considering this high uncertainty, we did not include an increased rate or intensity of CSOs in the future scenarios (they were kept the same as in the reference scenario) but conducted a sensitivity analysis to investigate how changes in CSO discharge volumes and frequencies would modulate the future scenarios. We took the 'no management changes' and 'enhanced wastewater treatment' scenarios as baselines. The above-listed two variables were varied individually, while the settings for all other parameters were kept the same as those for the baselines (Table 3).

3. Results

3.1. Model calibration and validation

The QMRAcatch model was calibrated and validated in two consecutive steps: First, against data on the human-associated MST marker and second, against data on enterovirus measured at the study site (Table B.1). From the calibration parameters, the model proved to be the most sensitive to the microorganism and virus-specific inactivation rate parameters a_0 and a_1 during the manual calibration. The parameters were constrained so that the resulting time to first log reduction versus water temperature function remained within the 95% prediction interval of the regression line fit to experimental values for both microorganisms (Fig. B.1, Tables 1 and S2).

The Mann-Whitney tests indicated that the simulated and observed concentrations were not significantly different for the human-associated MST marker and enterovirus ($p>0.05$, Table 4). The cumulative distribution plot of the simulated and observed concentrations is shown in Fig. B.2.

The model errors within the 5-95th percentiles ranged from −1.3 to 1.3 log_{10} N/L for the human-associated MST marker and from −1.1 to 1.5 log_{10} N/L for enterovirus (Figs. B.2 and B.3). The error distributions were very similar for the calibration and validation periods. The OF values were almost the same in the validation period as in the calibration period for the human-associated MST marker. The OF values for

Table 4
Model performance for simulated microbial concentrations at the study site after 1000 Monte Carlo runs.

	Parameter	Time period	n (n of detects)	Mann-Whitney test, p-value	Mean absolute error [$\log_{10}$ (N/L)]	Objective function (Eq. (9))
Calibration	Human MST marker	2013–2015	44 (44)	0.61	0.54	0.9
	Enterovirus	2013–2015	46 (17)	0.75	0.62	0.9
Validation	Human MST marker	2015–2017	43 (43)	0.59	0.63	1.0
	Enterovirus	2015–2017	48 (22)	0.13	0.64	1.5

enterovirus were the same as for the human-associated MST marker for the calibration period, but slightly higher for the validation period.

3.2. Scenario analysis: virus concentration and required LRV

QMRAcatch was applied to simulate the reference and four future scenarios using the calibrated settings but with river flows and temperatures as simulated by the regional climate and hydrological models (Fig. 2, Tables 2 and 3). For these scenarios, we simulated pathogen concentrations in river water at the study site and calculated the required treatment reduction (LRV) of pathogens from river water for the production of safe drinking water. The viral reference pathogens norovirus and enterovirus were the primary focus of the analysis. For cross-comparisons, the scenario analysis was additionally performed for *Cryptosporidium* and *Campylobacter* (results reported in Section 2 of the Supplementary information).

3.2.1. Reference

For the reference period, the median and range of concentrations of enterovirus at the study site were −0.55 (−2.84 to 2.54) $\log_{10}$ N/L, while they were 3 orders of magnitude higher for norovirus: 2.30 (1.23 to 3.23) $\log_{10}$ N/L (Fig. 3, top, Table C.1). The required LRV was 6.3 and 8.4 $\log_{10}$ for enterovirus and norovirus, respectively (Fig. 3, bottom).

3.2.2. Future climate, population, and no management changes

Four regional climate scenarios were tested, covering the entire range of expected climate pathways. They showed similar results in terms of the simulated concentrations of enterovirus and norovirus in river water as well as the LRVs (Fig. C.2, Table C.1). The climate scenario ECHAM5 A2, based on no efforts taken to reduce greenhouse gas emissions, was chosen as the basis for all future scenarios. Based on this climate scenario, the discharge of the Danube River at the study site will be up to 50% lower during low flows (summer-autumn), while only

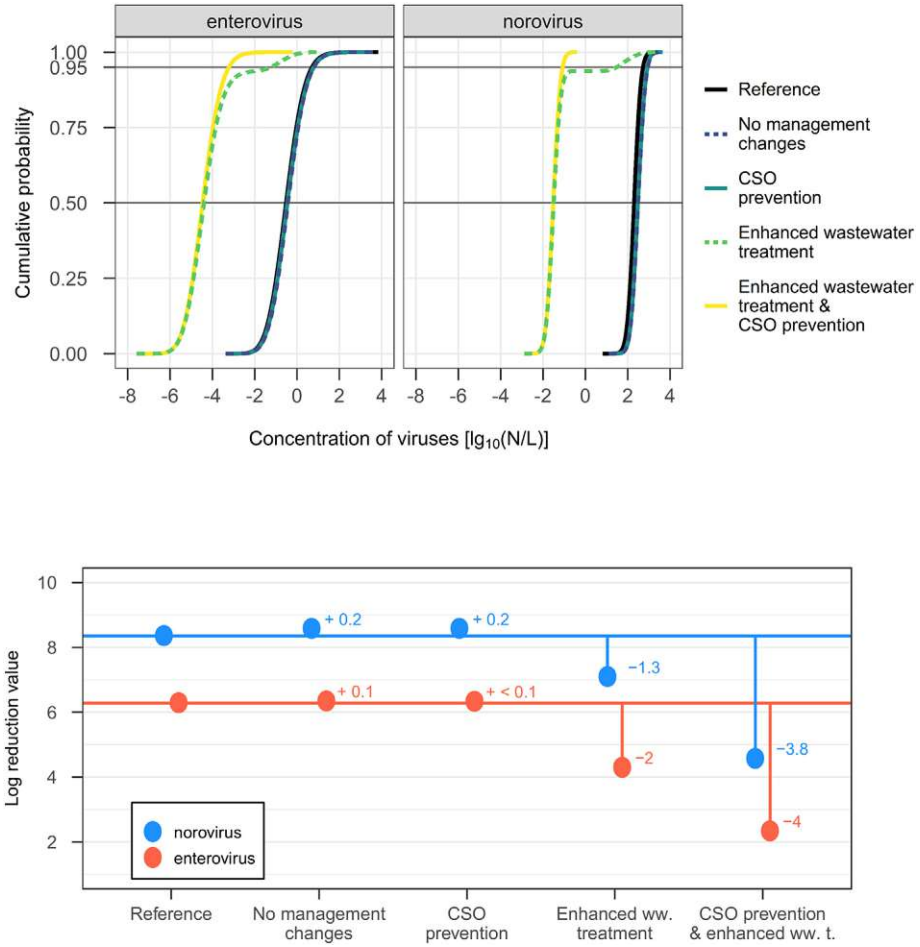


Fig. 3. Scenario simulations for norovirus and enterovirus (see Fig. S1 for the results for Cryptosporidium and Campylobacter). Upper panel: Simulated concentrations in river water. The 'CSO prevention' scenario entirely overlaps with the 'no management changes' one. Bottom panel: Required virus log reduction values to achieve safe drinking water.

slightly higher during high flows (late winter-spring). The temperature of river water will be 2 °C higher on average (Figs. C.1 and C.3).

In comparison to the reference period, the projected climatic and demographic changes showed a negligible effect on the concentrations of enterovirus and norovirus in river water as well as on the required LRVs (Fig. 3, Table C.1).

3.2.3. Future climate, population, and prevention of CSOs

While preventing CSO events precluded a few peaks of virus concentration in the river, it did not affect the overall distribution of concentrations, which remained similar to the reference and 'no management changes' scenarios (Fig. 3, top, Table C.1). The LRVs were not affected either (Fig. 3, bottom).

3.2.4. Future climate, population, and enhanced wastewater treatment

The installation of a tertiary treatment step at the five WWTPs (assumed effect: 4 $\log_{10}$) reduced the median concentrations of enterovirus and norovirus in river water by 3.9 and 3.8 $\log_{10}$, respectively, compared to the reference scenario. However, the maximum concentrations were only slightly reduced (1.9 and 0.1 $\log_{10}$ lower), and a batch of virus concentration peaks 3 to 5 $\log_{10}$ higher than the median remained (Fig. 3, top and Table C.1). The LRVs for enterovirus and norovirus were 2.0 and 1.3 $\log_{10}$ lower than in the reference scenario (Fig. 3, bottom).

3.2.5. Future climate, population, and combination of enhanced wastewater treatment and prevention of CSOs

The measure reduced both the median and maximum virus concentrations at the study site by approximately 4 $\log_{10}$ compared to the reference scenario (Fig. 3, top, Table C.1). The LRVs were 3.9 and 3.8 $\log_{10}$ lower than in the reference scenario (Fig. 3, bottom).

The observed pattern of the scenario analysis was found to be principally the same for all four reference pathogens (Fig. 3 for norovirus and enterovirus, Fig. S1 for *Campylobacter* and *Cryptosporidium*).

3.3. Sensitivity of future scenarios to uncertainties in CSO predictions

Considering the lack of information regarding the effect of climate change on CSOs, we conducted a sensitivity analysis to see how changes in CSOs would modulate the two future scenarios with CSO events ('no management changes' and 'enhanced wastewater treatment'). We varied two factors: the frequency of CSO events and the volume of CSO events (up to a 3-fold increase compared to the reference). We calculated the LRVs for enterovirus and norovirus.

In the 'no management changes' scenario, varying the frequency and the volume of CSO events had no or very little effect on the LRVs. In contrast, in the 'enhanced wastewater treatment' scenario, the same variations in the frequency and volume of CSO events had a considerable effect on the LRVs. An increase in the frequency of CSOs had a more pronounced effect on the LRVs, with 0.35 to 0.40 $\log_{10}$ higher LRVs for a 100% increase in CSO frequency, compared to 0.23 $\log_{10}$ higher LRVs for a 100% increase in CSO volumes (Fig. 4).

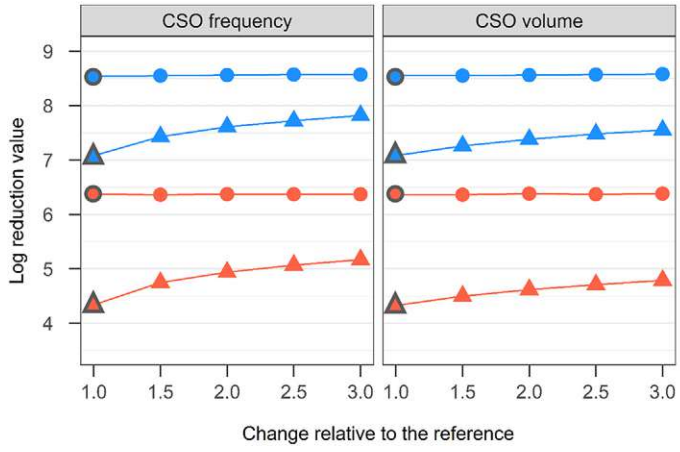
The above results show that the 'no management changes' scenario is not sensitive to an increase in CSOs. However, in the 'enhanced wastewater treatment' scenario, the required LRV for enterovirus and norovirus could be up to 1.03 and 0.74 $\log_{10}$ higher, respectively, depending on how the frequency and volumes of CSOs are affected by climate change (Fig. 4).

Additionally, we assessed the effect of the virus concentration in CSO water on the LRV. In the two baseline scenarios, the virus concentration in CSO water is assumed to be 10% of that of raw wastewater. Increasing it to equal raw wastewater did not cause changes in the predictions for the 'no management changes' case; however, it increased the LRV values by 1 $\log_{10}$ for the 'enhanced wastewater treatment' case (results not shown).

3.4. Scenario analysis: source apportionment of the load of viruses at the study site

The above results of the scenario analysis raise some intriguing questions about the effect of the wastewater management measures targeting WWTPs and/or CSOs (Section 3.2) and their interplay with climate and population changes (Section 3.3). To better understand this effect, we conducted a source apportionment of the load of pathogens at the study site originating from WWTPs and from CSOs. We did this by running each scenario twice: once by setting all pathogen inputs from WWTPs to zero and once by setting all inputs from CSOs to zero. We then calculated the daily load of pathogens at the study site by multiplying the simulated daily concentrations at the study site by the daily river flows. Fig. 5 displays the sum of loads from WWTPs and CSOs, i.e., the entire load of viruses in each scenario together with the percentage contribution of WWTPs and CSOs (Fig. S2 shows the same for *Cryptosporidium* and *Campylobacter*).

The analysis revealed that under the current situation and in the 'no management changes' scenario, WWTPs discharging secondary treated wastewater were the major contributors to the load of viruses at the study site (10^{10} N/d enterovirus and 10^{13} N/d norovirus, 97–99% of the total load). The rest, 1–3%, originated from CSOs that discharge



Legend and regressions

Virus	Enhanced treatment	CSO frequency: Slope (sign.)	CSO frequency: Std. error of slope	CSO frequency: R^2	CSO volume: Slope (sign.)	CSO volume: Std. error of slope	CSO volume: R^2
norovirus	0	0.02 (**)	<0.01	0.94	0.02 (**)	<0.01	0.94
norovirus	4	0.35 (**)	0.06	0.92	0.23 (**)	0.02	0.97
enterovirus	0	<0.01 (n.s.)	<0.01	0.13	0.01 (n.s.)	<0.01	0.63
enterovirus	4	0.40 (**)	0.07	0.91	0.23 (**)	0.02	0.97

Fig. 4. Effect of various future CSO changes on the required log reduction value (LRV). The black contour shows the 'no management changes' (circle) and 'enhanced wastewater treatment' (triangle) scenarios. The table shows the results of linear regression analyses for enhanced treatment achieving an additional reduction of viruses of 0 or 4 $\log_{10}$; n.s., not significant; **, $p < 0.01$.

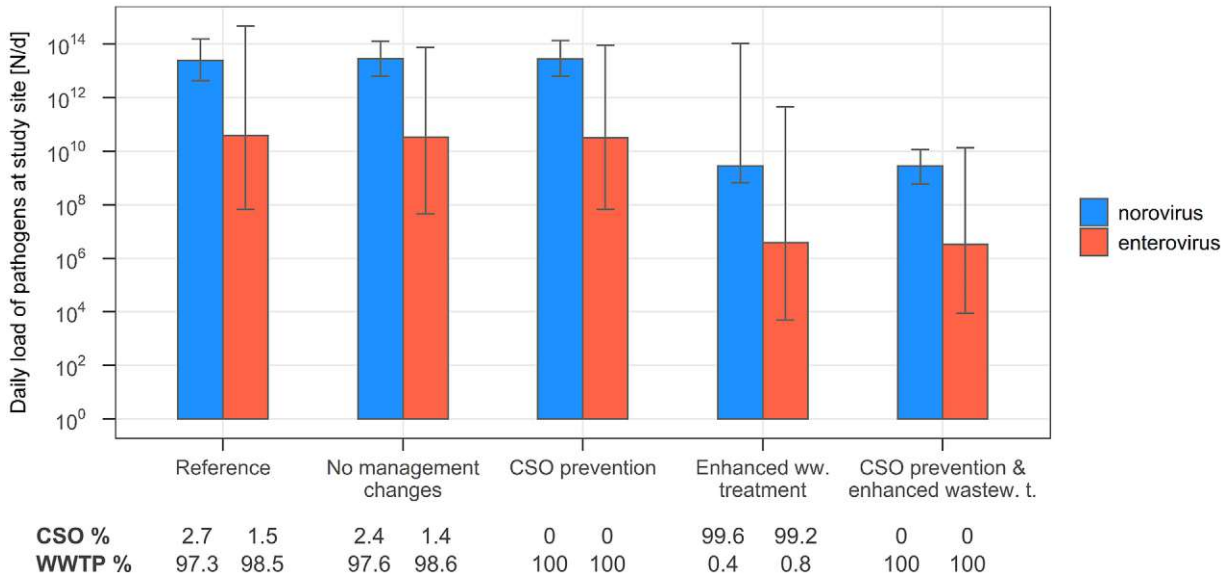


Fig. 5. Scenarios: Median daily load of norovirus and enterovirus at the study site and relative source apportionment. The whiskers show the range of simulated daily loads. See Fig. S2 for the results for *Cryptosporidium* and *Campylobacter*.

raw wastewater diluted with rainwater (Fig. 5). This explains why changes in CSOs (neither their prevention nor their increase due to climate change) did not affect virus concentration distributions and LRVs (Figs. 3 and 4). It also explains why enhanced wastewater treatment was effective at improving river microbial water quality (LRVs reduced by 1.3 and 2 $\log_{10}$, for norovirus and enterovirus, Fig. 3): It addressed the main contributor to the pollution load.

This source contribution relationship between WWTPs and CSOs flipped once enhanced wastewater treatment was in place: the main contributors were then the CSOs, with 10^6 N/d enterovirus and 10^9 N/d norovirus contributing over 99% to the total load. The median daily load was reduced by 4 $\log_{10}$; however, the maximum daily loads were reduced by only 0.2 and 3.1 $\log_{10}$ for norovirus and enterovirus, respectively (Fig. 5). This means a highly unequal distribution of the pollution load over time: While on days with no CSO events the daily load is relatively low, showing the beneficial effect of enhanced wastewater treatment, on days with CSOs the load is up to 5 $\log_{10}$ higher. The same pattern is visible in the virus concentration results (Fig. 3, top). Also, this predominance of CSOs explains why a climate-change-driven increase in CSOs affects LRVs so remarkably if enhanced wastewater treatment is in place (Fig. 4), and why the prevention of CSOs ('CSO prevention and enhanced wastewater treatment ' scenario) resulted in such a pronounced additional decrease of LRVs (an additional 2.5 and 1.9 $\log_{10}$ reduction of viruses, Fig. 3, bottom).

This pattern observed at the viral reference pathogens proved to be similar in the case of the bacterial and protozoan reference pathogens (*Campylobacter* and *Cryptosporidium*, Fig. S2).

4. Discussion

In this study, we tested an integrative modelling approach that combines CO_2 emission scenarios of the IPCC, a regional climate model, a conceptual hydrological model of the catchment as well as the significantly extended version of the microbial fate, transport and infection risk model QMRAcatch. The latter was used to estimate the source apportionment and the pathogen concentration in river water for QMRA. The combination of these methodological aspects was the key in gaining insights into the effects of future climatic and demographic changes and their interplay with possible upgrades in wastewater infrastructure on the microbiological water quality of rivers.

4.1. Implications of the assumptions and uncertainties of the modelling approach

Here, we discuss the model assumptions in this study, the uncertainty in the choice of input parameters, and their implications on the results. To assess the effects of climate change, monthly differences of air temperature and precipitation between simulations for a reference (2003–2017) and a future period (2035–2049) were calculated based on regional climate model outputs and used as input to a hydrological model. To investigate the changes in intense rainfall events and the impact on CSOs, however, methods that account for the spatial and temporal variability of rainfall would be needed (Muller-Thomy et al., 2018). Hydraulic modelling of the sewer system, e.g., by using the urban stormwater model SWMM, would enable the studying of these effects on CSOs. For example, Bi et al. (2015) found a 15–500% increase in the volume discharged by CSOs in 2050 as compared to 2013 in Canada. This highlights that the relationship between changes in precipitation and CSO variables is not linear. How the contaminant concentration in CSO water will change in the future is also very much specific to the urban area and the sewer system drained by the CSO. An in-depth and location-specific analysis of urban sewer systems and their response to climate change was beyond the focus of this study.

As a wastewater upgrade for WWTPs, we assumed that enhanced treatment achieves an additional 4-log reduction of enterovirus and norovirus concentrations. While we added this value to the mean and 95th percentile of the assumed normal probability distribution of secondary treatment, a more realistic approach would be to apply distributions of microorganism- and process-specific values. The concentration of pathogens in WWTP effluent was assumed to be the same in the future as it is now. However, there may be differences in the disease burden in the future (Levy et al., 2016).

Our study focuses on two pathogenic human viruses, enterovirus and norovirus, and their source, human wastewater. As an extension, we compare these results with a bacterial and a protozoan reference pathogen, *Campylobacter* and *Cryptosporidium*. Since these latter two may basically also originate from reservoirs other than humans, it is important to note that the current study focussed on the human-wastewater-associated fraction of these pathogens for direct cross-comparisons to the viral refence pathogens, assuming human communal waste water as the dominating pollution source.

The above-listed uncertainties and assumptions affect the absolute LRVs to achieve safe drinking water for all scenarios likewise. We aimed to study the effects of various changes of the system on microbiological drinking water safety requirements, not absolute LRV values.

4.2. Accurateness of the pathogen fate and transport predictions

In order to accurately predict the reference pathogen concentrations and loads, the model calibration and validation of the fate and transport model based on site- and source-specific data are seen as an essential step. To evaluate QMRAcatch, we used measured MST and enterovirus concentrations collected at the study site over four years.

Our literature survey on reported MST marker and virus inactivation rates revealed a current lack of studies conducted in real-world and natural light conditions, in particular for norovirus, which creates a source of model uncertainty. In order to overcome this limitation, we set the microbial inactivation coefficients during calibration within constrained limits based on reported persistence data for the human-associated MST marker and enterovirus.

The use of human-associated MST data allowed for source-targeted calibration and validation of the model (Mayer et al., 2018; Zhang et al., 2019). The human-associated MST marker thus provides a better basis for calibration and validation in the context of our research questions (on point sources of human wastewater) than a standard faecal indicator organism, such as *E. coli* would do, since *E. coli* may, for example, also originate from other non-faecal sources (Frick et al., 2018). Integrating pathogen data in the calibration-validation process is an essential confirmation, and it allows to assess health risks directly (Boehm and Soller, 2013; Lodder et al., 2015). However, pathogens can hardly serve as the basis for a comprehensive calibration on their own, since their concentrations in environmental waters are often very low, and the required large sample volumes and processing efforts render the establishment of large data series unfeasible. Still, a smaller pathogen dataset may complement the calibration process. The human-associated MST marker has approx. 4–6 $\log_{10}$ higher concentrations in raw wastewater than most pathogens, and often maintains high concentrations in environmental waters. Therefore, the model calibration followed a nested approach to make optimal use of both the host-associated MST marker and pathogen data. Discharge and mixing processes of faecal pollution associated microorganisms and pathogens in river water could be robustly calibrated using sufficiently abundant source-targeted MST marker data. The pathogen data were then used to adjust the model to the pathogen-specific inactivation rate coefficients. The calibrated and validated model was then able to simulate the low, non-detectable but significant ranges of the enteric pathogens in question or even simulate new pathogens, where only information on the concentrations in sewage and environmental persistence is available (Table B.1). Despite the advantages of this systematic approach, only a few microbial fate and transport modelling studies have used it so far (Derx et al., 2016; Schijven et al., 2015).

4.3. Deciphering the interplay of future changes and wastewater management measures

Several studies investigated the effects of future changes in climate, population or wastewater infrastructure on the microbiological river water quality so far, but the controlling factors remain yet unclear (Iqbal et al., 2019; Islam et al., 2018a; Jalliffier-Verne et al., 2017; Sterk et al., 2016). This study brought new insights into this question by integrating source apportionment, concentrations of reference pathogens and risk assessment into a modelling analysis. Source apportionment was previously used to identify the dominant sources of faecal indicator bacteria (Soller et al., 2014; Stapleton et al., 2011) or to study the effects of sociodemographic and climate changes on faecal indicator bacteria loads into a river (Iqbal et al., 2019). Our study identified source apportionment together with the other methodological aspects of the integrative modelling approach as the key for understanding for the first time the interplay of future changes and wastewater management measures. We showed how this interplay affects pathogen loads into rivers, and the pathogen concentrations in rivers considering safe drinking water production.

For the scenario with no management changes at our example study site, changes in river flows and water temperatures were shown to have a minor negative effect on the microbiological river water quality, in line with the predictions for other regions (Iqbal et al., 2019, Islam et al., 2018a, Jalliffier-Verne et al., 2017, Sterk et al., 2016). According to the reference scenario, WWTPs discharging secondary treated sewage are the major contributors to the pathogen loads, not CSOs. Therefore, neither potential increases in CSO events due to climate change nor their prevention affected the drinking water safety requirements. In contrast, if enhanced wastewater treatment was in place at the WWTPs, CSOs suddenly became the major contributors to the pathogen loads. Here, climate-change driven increases in CSO events resulted in significantly higher treatment requirements. While this issue was addressed earlier by Sterk et al. (2016) in the context of bathing water infection risks, our modelling results for the first time identify the conditions and extent to which increased CSOs affect the microbiological river water quality in the context of safe drinking water production.

The greatest improvement in the microbiological water quality of the riverine water intake was achieved with measures targeting both WWTPs and CSOs. The Austrian Waste and Wastewater Association estimates that an additional yearly investment of 150 million Euros is needed to tackle upcoming problems in this sector in the coming years (ÖWAV, 2020). Since the current regulatory standard is secondary treatment at the WWTPs, it is very important to evaluate the benefits enhanced wastewater treatment would bring, which is currently discussed in the context of micropollutant abatement. Furthermore, it is important to estimate the impact of urban soil sealing, an important yet often neglected aspect in city development, with respect to the microbiological water quality of the receiving waters.

5. Conclusions

- The pathogen fate and transport and infection risk model QMRAcatch (v1.0 Python) was significantly extended and is now available as open source.
- Climatic and demographic changes had little impact on the microbiological river water quality considering safe drinking water, where 98% of the pathogen loads stemmed from WWTP discharges. Strong climate change effects are shown in the scenario with enhanced WWTP treatment, where CSOs are the major faecal pollution sources.
- The required log reduction value (LRV) to produce safe drinking water was 6.3, 8.4, 4.9 and 5.1 $\log_{10}$ for enterovirus, norovirus, *Campylobacter* and *Cryptosporidium* in the scenario with secondary WWTP treatment. Enhanced wastewater treatment led to a reduction of LRVs by 0.5 to 2.0 $\log_{10}$. This measure combined with preventing CSOs had the most significant positive effect with a reduction of LRVs by up to 4 $\log_{10}$.
- The integrative modelling framework is demonstrated at a large, wastewater-impacted river, and is applicable at other catchments and types of pollution sources for long-term water safety planning.

Software availability

The source code for QMRAcatch v1.0 python is available upon request. The original Mathematica version is available at www.waterandhealth.at.

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CRediT authorship contribution statement

Katalin Demeter: Data curation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Julia Derx:** Methodology, Software, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Jürgen Komma:** Formal analysis, Investigation, Writing – original draft. **Juraj Parajka:** Formal analysis, Investigation, Writing – original draft. **Jack Schijven:** Software, Writing – original draft. **Regina Sommer:** Conceptualization, Funding acquisition. **Silvia Cervero-Aragó:** Investigation, Writing – original draft. **Gerhard Lindner:** Investigation. **Christa M. Zoufal-Hruza:** Investigation, Writing – original draft. **Rita Linke:** Investigation, Writing – original draft. **Domenico Savio:** Investigation. **Simone K. Ixenmaier:** Investigation. **Alexander K.T. Kirschner:** Conceptualization, Funding acquisition. **Harald Kromp:** Conceptualization. **Alfred P. Blaschke:** Conceptualization, Funding acquisition, Supervision. **Andreas H. Farnleitner:** Conceptualization, Writing – original draft, Writing – review & editing, Funding acquisition, Supervision.

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. QMRAcatch model details

Table A.1
River dimensions and intermediate calculations for dilution of microbial concentrations with river water.

River geometry			
W	Width of the river	250	[m]
h	Depth of the river	4	[m]
s	Slope of the river bed	0.0004	[−]
Constants			
n	Manning coefficient	0.024	[m $s^{-0.3}$]
α_y	Diffusivity constant, calibrated	0.35	[−]
g	Gravitation constant	9.81	[m s^{-2}]
Intermediate calculations			
R_h	Hydraulic radius	$=\frac{W \cdot h}{W+2h}$ =3.9	[m]
U	Average flow velocity (Manning, 1891)	$=\frac{R_h^{2/3}\sqrt{s}}{n}$=2.1	[m/s]
u^*	Friction velocity	$=\sqrt{g \cdot h \cdot s}$=0.1	[m/s]

Appendix B. Additional information to the calibration and validation

Table B.1
A tiered approach to the application of the model to various targeted microorganisms and pathogens. n.a.: not applied.

Microorganism/pathogen	QMRAcatch calibration	QMRAcatch validation	Scenario simulations
Human-associated MST marker	Yes Dataset 2013–2015	Yes Dataset 2015–2017	n.a.
Enterovirus	Yes Dataset 2013–2015	Yes Dataset 2015–2017	Yes, input data partially measured, partially from literature
Norovirus	n.a.	n.a.	Yes, input data from literature
Campylobacter	n.a.	n.a.	Yes, input data from literature
Cryptosporidium	n.a.	n.a.	Yes, input data measured

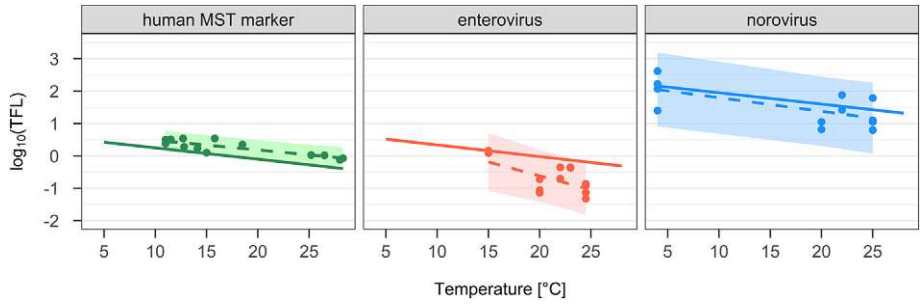

Fig. B.1. Inactivation of the human-associated MST marker, enterovirus, and norovirus as applied in this study (solid lines) and as reported in experimental studies (dots, Table S2), plotted as time to first $\log_{10}$ reduction (TFL, days) values $\log_{10}$-transformed ($\log_{10}$(TFL)) in function of the temperature. Ordinary-least-square regressions (dashed lines) were fitted to the literature values, shown with their 95% prediction intervals (shaded). The intercept and the slope of the solid lines were the result of the model calibration for the human MST marker and enterovirus, and are the reported values of Bertrand et al. (2012) for norovirus. These values were used as model input parameters a_0 and a_1 in Eq. (4) (Table 1).

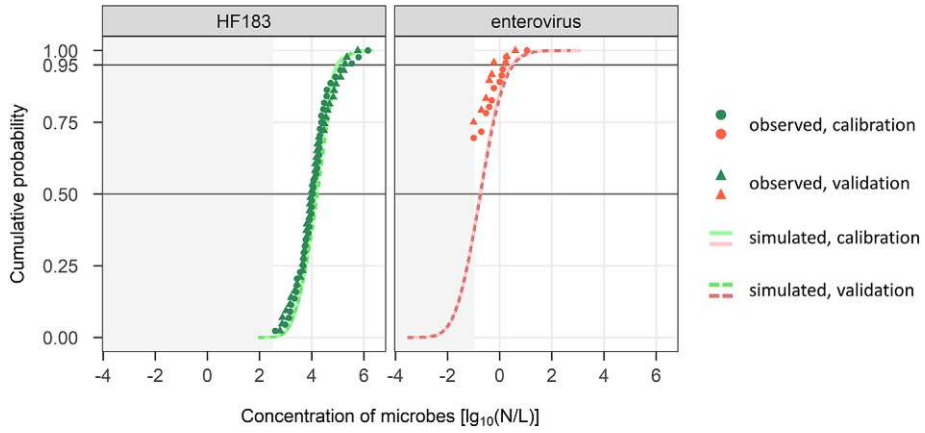

Fig. B.2. Simulated and observed concentrations of the human-associated MST marker and of enterovirus during calibration and validation. The light grey area marks values under the detection threshold for the human-associated MST marker (qPCR) and under the limit of detection for enterovirus (MPN method).

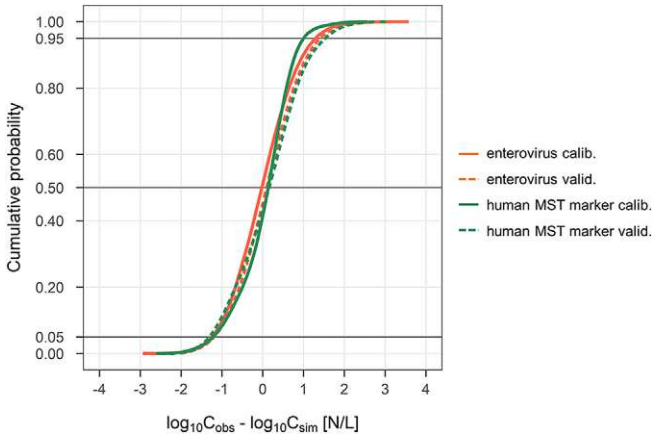

Fig. B.3. Calibration and verification. Cumulative distribution plot of the difference between the simulated and measured concentrations ($\log_{10}$-transformed) of the human-associated MST marker and enterovirus for the calibration and validation periods.

Appendix C. Additional information to the scenario analysis

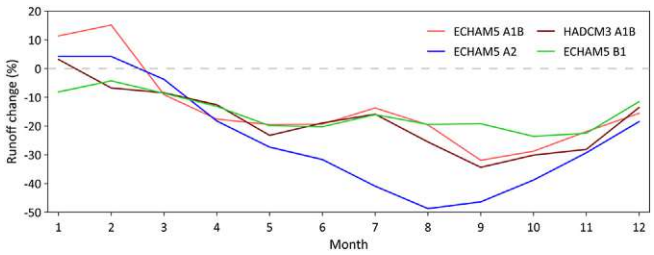

Fig. C.1. Mean monthly change of the river flow for the Danube River in Vienna, estimated according to four climate projections: ECHAM5 A1B, ECHAM5 A2, ECHAM5 B1 and HADCM3 A1B, where A1B, B1, and A2 represent three Intergovernmental Panel on Climate Change emission scenarios, and ECHAM 5 and HADCM3 are two global climate models. The change represents the relative change between the reference period 2003–2017 and the future period 2035–2049.

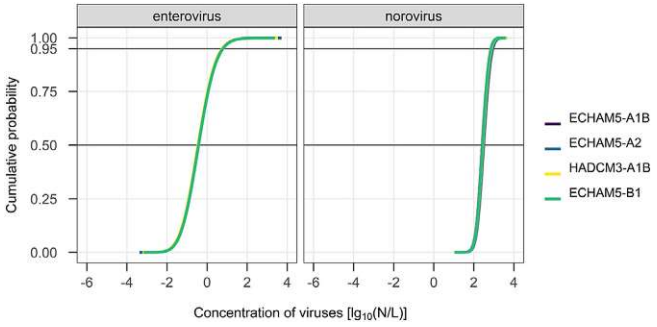

Fig. C.2. Simulated concentrations of norovirus and enterovirus in river water according to the four climate scenarios considered in this study.

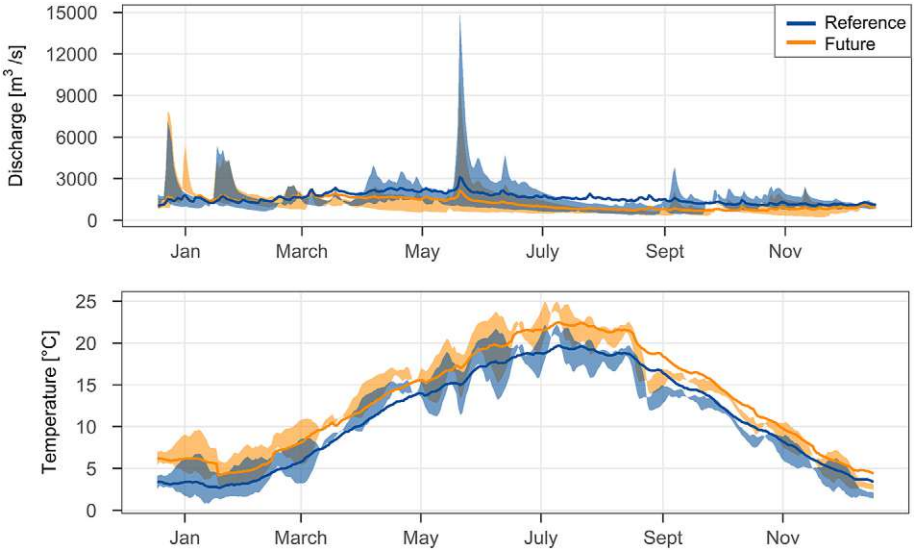

Fig. C.3. Discharge (upper panel) and water temperature (lower panel) of the Danube in the study area for the reference period (2003–2017) and the future period (2035–2049). The projections are based on the ECHAM5 A2 climate scenario. The line shows the mean across the 15 years, while the ribbon shows the range between the year with the lowest yearly mean discharge/temperature and the year with the highest one.

Table C.1
Simulated concentration of enterovirus and norovirus in river water at the study site in the scenario analysis. For each scenario, 100 Monte Carlo runs were simulated. Each statistic is given by the median and range across the 100 runs.

Virus	Scenario	Simulated concentration [$\log_{10}$(N/L)]		
		Median (range of median)	Min (range of min)	Max (range of max)
Scenarios of global change and wastewater infrastructure upgrades				
Enterovirus	Reference	−0.55 (−0.58 to −0.51)	−2.84 (−3.33 to −2.61)	2.54 (1.78 to 3.58)
	No management changes	−0.47 (−0.50 to −0.44)	−2.83 (−3.54 to −2.48)	2.59 (2.05 to 3.53)
	CSO prevention	−0.49 (−0.51 to −0.45)	−2.84 (−3.52 to −2.55)	2.61 (1.98 to 3.73)
	Enhanced ww. treatment	−4.43 (−4.45 to −4.40)	−6.84 (−7.53 to −6.51)	0.68 (0.44 to 1.25)
	Enhanced ww. treatment & CSO prevention	−4.49 (−4.51 to −4.46)	−6.86 (−7.32 to −6.59)	−1.45 (−2.10 to −0.61)
Norovirus	Reference	2.30 (2.29 to 2.31)	1.23 (0.81 to 1.46)	3.23 (3.07 to 3.50)
	No management changes	2.49 (2.48 to 2.50)	1.40 (1.10 to 1.57)	3.47 (3.34 to 3.66)
	CSO prevention	2.48 (2.47 to 2.49)	1.42 (1.11 to 1.56)	3.44 (3.30 to 3.65)
	Enhanced ww. treatment	−1.50 (−1.51 to −1.49)	−2.61 (−2.89 to −2.41)	3.15 (2.94 to 3.61)
	Enhanced ww. treatment & CSO prevention	−1.52 (−1.53 to −1.51)	−2.61 (−2.83 to −2.41)	−0.57 (−0.71 to −0.40)
Climate scenarios				
Enterovirus	ECHAM5-A1B	−0.44 (−0.47 to −0.42)	−2.74 (−3.31 to −2.47)	2.57 (2.08 to 3.46)
	ECHAM5-A2	−0.47 (−0.50 to −0.44)	−2.83 (−3.54 to −2.48)	2.59 (2.05 to 3.53)
	HADCM3-A1B	−0.45 (−0.47 to −0.42)	−2.75 (−3.22 to −2.44)	2.54 (2.08 to 3.53)
	ECHAM5-B1	−0.43 (−0.45 to −0.41)	−2.74 (−3.17 to −2.43)	2.62 (2.05 to 3.39)
Norovirus	ECHAM5-A1B	2.42 (2.41 to 2.43)	1.33 (1.06 to 1.54)	3.38 (3.26 to 3.65)
	ECHAM5-A2	2.49 (2.48 to 2.50)	1.40 (1.10 to 1.57)	3.47 (3.34 to 3.66)
	HADCM3-A1B	2.44 (2.44 to 2.45)	1.32 (1.01 to 1.55)	3.37 (3.25 to 3.66)
	ECHAM5-B1	2.43 (2.42 to 2.44)	1.34 (1.04 to 1.56)	3.34 (3.23 to 3.59)

Appendix D. Supplementary data

Supplementary data to this article providing more details on model calibration and validation as well as all results of the scenario simulations for *Cryptosporidium* and *Campylobacter* can be found online at https://doi.org/10.1016/j.scitotenv.2020.144278.

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Supplementary Information

Modelling the interplay of future changes and wastewater management measures on the microbiological river water quality considering safe drinking water production

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Section 1. Model calibration and validation

Table S1. *Calibration parameters of the hydrological model. D, snow melt factor (mm/day/°C); T_s, threshold temperature for snow fall (°C), T_r, threshold temperature for rain (°C); T_m, melt temperature (°C); L_{by}, bypass flow (mm/day); L_P, limit for potential evaporation (-); L_S, maximum soil moisture storage (mm); beta, non-linearity parameter (-); k_0, surface runoff storage coefficient (h); k_1, interflow storage coefficient (h); f_{k1}, scaling parameter (-); k_2, lower soil storage coefficient (h); f_{k2}, scaling parameter (-); k_3, groundwater storage coefficient (h); L_1, threshold for surface runoff (mm); L_{cp}, maximum percolation rate (mm/day); gamma, non-linearity parameter (-); f_3, deep percolation factor (-) (Blöschl et al. 2008).*

		D	T_S	T_r	T_m	L_{by}	L_P	L_S	beta	k_0	k_1	f_{k1}	k_2	f_{k2}	k_3	L_1	L_{CP}	gamma	f_3
Urban areas	Min	1.7	-1.0	0.5	0.1	0.1	0.5	116.1	1.3	11.1	107.0	0.8	185.0	1.0	800.0	36.9	0.5	0.0	0.9
	Mean	1.9	-0.9	0.6	0.2	0.1	0.6	128.1	1.3	14.6	133.4	1.0	223.5	1.0	1130.0	39.9	4.2	0.0	0.9
	Max	2.2	-0.8	0.7	0.3	0.6	0.7	131.1	1.3	15.5	140.0	1.0	255.0	1.5	1400.0	40.6	6.0	0.0	0.9
Water bodies	Min	1.9	-0.8	0.7	0.3	0.0	0.6	81.0	1.1	9.3	93.2	1.0	150.0	1.0	500.0	25.8	1.0	0.0	0.5
	Mean	2.1	-0.6	0.9	0.6	0.0	0.6	114.4	1.3	13.6	125.8	1.0	255.0	1.1	1400.0	41.2	4.4	0.0	0.9
	Max	2.4	-0.2	1.3	1.0	0.1	0.6	143.0	1.6	16.7	148.8	1.0	500.0	1.5	3500.0	50.0	6.5	0.0	1.0
Broad-leaved forest	Min	1.4	-1.0	0.5	0.1	0.0	0.4	108.6	1.3	10.4	101.5	0.8	150.0	0.3	500.0	29.4	2.0	0.0	0.5
	Mean	1.9	-0.9	0.6	0.3	0.4	0.6	156.3	1.9	16.3	146.3	1.0	232.7	1.0	1209.1	47.0	4.4	0.0	0.9
	Max	2.3	-0.6	0.9	0.5	0.6	0.7	270.1	2.2	27.8	233.5	1.0	500.0	1.6	3500.0	74.2	7.0	0.0	1.0
Coniferous forest	Min	1.5	-1.0	0.5	0.1	0.0	0.4	78.7	1.5	7.5	79.5	0.7	150.0	0.5	500.0	23.2	2.0	0.0	0.5
	Mean	2.0	-0.6	0.9	0.5	0.2	0.6	150.2	1.9	15.8	142.4	1.0	360.7	1.1	2305.8	45.1	5.3	0.0	0.7
	Max	2.5	-0.3	1.2	0.9	0.6	0.7	255.2	2.2	23.5	200.5	1.4	500.0	3.0	3500.0	70.4	30.0	0.0	1.0
Mixed forest	Min	1.4	-1.0	0.5	0.1	0.0	0.4	78.7	1.4	7.5	79.5	0.7	150.0	0.3	500.0	23.2	2.0	0.0	0.5
	Mean	2.0	-0.6	0.9	0.5	0.4	0.6	144.0	1.9	15.1	137.1	1.0	263.4	1.1	1472.2	42.7	4.6	0.0	0.8
	Max	2.5	-0.3	1.2	0.9	0.6	0.7	240.2	2.2	22.4	192.2	1.4	500.0	3.0	3500.0	66.7	10.0	0.0	1.0
Artifical non-agric. veg.	Min	1.8	-1.0	0.5	0.1	0.1	0.6	131.1	1.3	15.5	140.0	0.8	220.0	1.0	1100.0	40.6	4.5	0.0	0.9
	Mean	2.0	-0.8	0.7	0.3	0.1	0.7	131.1	1.3	15.5	140.0	0.9	228.8	1.1	1175.0	40.6	5.3	0.0	0.9
	Max	2.2	-0.8	0.7	0.3	0.1	0.7	131.1	1.3	15.5	140.0	1.0	255.0	1.5	1400.0	40.6	6.0	0.0	0.9
Pastures	Min	1.4	-1.0	0.5	0.1	0.0	0.4	90.7	1.3	10.1	99.3	0.8	150.0	0.3	500.0	29.4	2.0	0.0	0.5
	Mean	2.1	-0.8	0.7	0.4	0.3	0.6	151.5	1.8	16.2	145.2	1.0	280.5	1.1	1618.8	46.6	4.5	0.0	0.8

	Max	2.4	-0.4	1.1	0.8	0.6	0.7	240.2	2.0	24.9	211.5	1.4	500.0	3.0	3500.0	68.0	10.0	0.0	1.0
on-irrigated arable land	Min	1.4	-1.0	0.5	0.1	0.0	0.4	78.7	1.1	7.6	80.1	0.8	150.0	0.3	500.0	26.8	2.0	0.0	0.5
	Mean	1.9	-0.9	0.6	0.2	0.2	0.6	173.7	1.8	19.0	166.3	1.0	284.4	1.0	1652.3	51.7	4.1	0.0	0.8
	Max	2.3	-0.7	0.8	0.4	0.6	0.7	270.1	2.2	27.8	233.5	1.0	500.0	1.5	3500.0	74.2	6.0	0.0	1.0
Complex cultivation patterns	Min	1.4	-1.0	0.5	0.1	0.0	0.4	113.1	1.5	11.5	109.8	0.8	150.0	0.3	500.0	36.1	2.0	0.0	0.5
	Mean	2.0	-0.8	0.7	0.3	0.4	0.6	158.6	1.8	16.7	149.0	1.0	307.8	1.0	1852.4	47.8	4.8	0.0	0.8
	Max	2.3	-0.5	1.0	0.6	0.6	0.7	270.1	2.2	27.8	233.5	1.4	500.0	3.0	3500.0	74.2	30.0	0.0	1.0
Vineyards	Min	1.4	-1.0	0.5	0.1	0.0	0.4	131.1	1.7	13.0	121.3	1.0	255.0	1.0	1400.0	40.8	2.0	0.0	0.5
	Mean	1.7	-1.0	0.5	0.1	0.3	0.5	158.0	1.8	16.7	148.9	1.0	319.2	1.1	1950.0	48.1	4.3	0.0	0.8
	Max	2.1	-0.9	0.6	0.2	0.6	0.7	183.4	1.9	20.9	181.2	1.0	500.0	1.5	3500.0	54.7	6.0	0.0	0.9
Fruit trees	Min	1.9	-0.8	0.7	0.3	0.1	0.6	168.4	1.9	16.6	148.2	0.8	255.0	0.5	1400.0	50.9	5.0	0.0	0.9
	Mean	2.0	-0.8	0.7	0.3	0.1	0.6	175.9	1.9	18.8	164.8	0.9	255.0	0.8	1400.0	52.8	5.4	0.0	0.9
	Max	2.2	-0.8	0.7	0.3	0.1	0.7	183.4	1.9	20.9	181.2	1.0	255.0	1.0	1400.0	54.7	5.8	0.0	0.9
Natural grassland, moors	Min	1.9	-0.6	0.9	0.5	0.0	0.4	69.0	1.1	5.3	63.0	0.8	150.0	0.9	500.0	20.8	3.5	0.0	0.5
	Mean	2.2	-0.3	1.2	0.9	0.3	0.6	115.9	1.7	12.3	116.0	1.0	315.3	1.1	1916.7	35.8	6.4	0.0	0.8
	Max	2.7	0.0	1.5	1.2	0.6	0.7	155.7	1.9	15.5	140.0	1.4	500.0	1.6	3500.0	44.2	30.0	0.0	1.0
Bare rocks	Min	1.9	-0.5	1.0	0.6	0.0	0.4	46.6	1.1	4.3	54.8	0.8	150.0	0.9	500.0	12.7	2.0	0.0	0.5
	Mean	2.2	-0.2	1.3	1.0	0.3	0.5	76.5	1.3	8.3	85.4	1.0	275.0	1.1	1571.4	23.2	2.6	0.0	0.8
	Max	2.5	0.0	1.5	1.2	0.6	0.7	155.7	1.6	15.1	137.2	1.4	500.0	1.6	3500.0	41.9	6.5	0.0	1.0
Glaciers	Min	2.2	-0.2	1.3	1.0	0.0	0.5	81.0	1.4	9.3	93.2	1.0	500.0	1.5	3500.0	25.8	0.5	0.0	0.5
	Mean	2.3	-0.1	1.4	1.1	0.0	0.5	81.0	1.4	9.3	93.2	1.2	500.0	1.5	3500.0	25.8	0.5	0.0	0.5
	Max	2.4	0.0	1.5	1.2	0.0	0.6	81.0	1.4	9.3	93.2	1.4	500.0	1.6	3500.0	25.8	0.5	0.0	0.5

Table S2. *Inactivation rates of the human-associated MST marker, enterovirus and norovirus from the literature (plotted on Figure 3).*

(Sero)type	Strain	Lighting condition	Water type	Temp (°C)	Enumeration method	Cell line / Host bacteria	k (d^{-1})	R^2	Reference
Human-associated MST marker									
		Natural light	Fresh, in situ	15.8	qPCR		-0.29		(Ahmed et al. 2014)
		Natural light	Fresh, in situ	28	qPCR		-3.03		(He et al. 2016)
		Natural light	Fresh, in situ	12.7	qPCR		-0.66		(He et al. 2016)
		Natural light	Fresh, in situ	26.5	qPCR		-2.19		(Balleste et al. 2018)
		Natural light	Fresh, in situ	11.5	qPCR		-0.71		(Balleste et al. 2018)
		Dark	Fresh, in lab	25.2	qPCR		-2.13		(Dick et al. 2010)
		Artificial sunlight	Fresh, in lab	28.3	qPCR		-2.69		(Dick et al. 2010)
		Artificial sunlight	Fresh, in lab	15.0	qPCR		-1.82		(Dick et al. 2010)
	DNA	Natural light	Fresh	11 (4-18)	qPCR		-0.74		(Liang et al. 2012)
	RNA	Natural light	Fresh	11 (4-18)	qPCR		-0.96		(Liang et al. 2012)
		Dark	Fresh	18.5	qPCR		-1.03		(Jeanneau et al. 2012)
		Natural sunlight	Fresh	12.8	qPCR		-1.2		(Green et al. 2011)
		Natural light	Fresh, in situ	14.1	qPCR		-1.36		(Korajkic et al. 2014)
Enterovirus (Boehm et al. 2019)									
Poliovirus 1	Sabin type 1	Natural light	Marine	24.5	Cell culture	BGM	48.3	1.0	(Fujioka and Yoneyama 2002)
Echovirus 7	Sewage isolate	Natural light	Marine	24.5	Cell culture	BGM	30.2	0.98	(Fujioka and Yoneyama 2002)
Coxsackievirus B5		Natural light	Marine	24.5	Cell culture	BGM	31.9	0.98	(Fujioka and Yoneyama 2002)
Poliovirus 1	Sabin type 1	Natural light	Marine	24.5	Cell culture	BGM	16.77	0.92	(Fujioka and Yoneyama 2002)
Echovirus 7	Sewage isolate	Natural light	Marine	24.5	Cell culture	BGM	16.89	0.89	(Fujioka and Yoneyama 2002)
Coxsackievirus B5		Natural light	Marine	24.5	Cell culture	BGM	18.87	0.94	(Fujioka and Yoneyama 2002)
Poliovirus 3	ATCC VR-300	Solar simulator	Estuarine	20	Cell culture	HeLa	12.0	0.99	(Silverman et al. 2013)
Poliovirus 3	ATCC VR-300	Solar simulator	Estuarine	20	Cell culture	HeLa	31.68	0.95	(Silverman et al. 2013)
Poliovirus 3	ATCC VR-300	Solar simulator	Estuarine	20	Cell culture	HeLa	26.16	0.99	(Silverman et al. 2013)
Poliovirus 3	ATCC VR-300	Solar simulator	Estuarine	20	Cell culture	HeLa	25.68	0.97	(Silverman et al. 2013)
Poliovirus 3	ATCC VR-300	Solar simulator	Estuarine	20	Cell culture	HeLa	12.0	0.98	(Silverman et al. 2013)
Poliovirus 1	LSc	Natural light	Marine	23	Cell culture	BGM	5.36		(Johnson et al. 1997)
Poliovirus 1	LSc	Natural light	Estuarine	23	Cell culture	BGM	5.11		(Johnson et al. 1997)
Poliovirus 1	LSc2ab	Natural light	Estuarine	22	Cell culture	Hep-2	5.14	0.83	(Akin et al. 1971)
Poliovirus 1	LSc2ab	Natural light	Estuarine	22	Cell culture	Hep-2	11.57	0.88	(Akin et al. 1971)
		Natural light	Marine	15	Cell culture	MA104	1.58		(Girones et al. 1989)
		Natural light	Marine	15	Cell culture	BGM	1.85	0.97	(Jofre et al. 1986)
Norovirus (Boehm et al. 2019)									
Murine norovirus	MNV-1	Dark	Fresh	22	Cell culture	RAW 264.7	0.087	0.98	(Moresco et al. 2015)

Murine norovirus	MNV-1	Dark	Fresh	22	RT-qPCR		0.03	0.73	(Moresco et al. 2015)
Murine norovirus	MNV-1	Dark	Fresh	4	Cell culture	RAW 264.7	0.02	0.98	(Moresco et al. 2015)
Murine norovirus	MNV-1	Dark	Fresh	4	RT-qPCR		0.01	0.54	(Moresco et al. 2015)
Human norovirus GII	GII.4	Not reported	Fresh	4	RT-qPCR		0.01	0.99	(Ngazoa et al. 2008)
Human norovirus GII	GII.4	Not reported	Fresh	25	RT-qPCR		0.04	0.93	(Ngazoa et al. 2008)
Human norovirus GI	GI.1-8fIIb	Dark	Fresh	25	RT-qPCR		0.18		(Bae and Schwab 2008)
Human norovirus GI	GI.1-8fIIb	Dark	Fresh	4	RT-qPCR		0.09		(Bae and Schwab 2008)
Murine norovirus	MNV-1	Dark	Fresh	25	RT-qPCR		0.21		(Bae and Schwab 2008)
Murine norovirus	MNV-1	Dark	Fresh	25	Cell culture	RAW 264.7	0.37		(Bae and Schwab 2008)
Murine norovirus	MNV-1	Natural light	Fresh	20	Cell culture	RAW 264.7	0.35		(Elmahdy et al. 2018)
Murine norovirus	MNV-1	Dark	Fresh	20	Cell culture	RAW 264.7	0.21		(Elmahdy et al. 2018)

Section 2. Scenario analysis for *Cryptosporidium* and *Campylobacter*

Table S3. *Model input parameters for Cryptosporidium and Campylobacter (see also Tables 1 and 3). Gamma probability distribution function (mean, 95th percentile) of the microbial concentrations in raw wastewater (C_{raw}), normal probability distribution function (mean, 95th percentile) of microbial removal by wastewater treatment (F_{WWTP}), inactivation rate coefficients (a_0, a_1), and dose-response parameters α and β.*

Parameter	Unit	Distribution	Details	Microorganism	Value	Reference
C_{raw} (mean, 95th perc.)	N/L	Gamma	WWTP 1-5	*Cryptosporidium*	770, 1800	This study
				Campylobacter	1400, 7000	(World Health Organisation 2017)
F_{WWTP} (mean, 95th perc.)	Log_{10}	Normal	WWTP 1-5	*Cryptosporidium*	2.2, 1.5	This study
				Campylobacter	1, 0.8	(World Health Organisation 2017)
a_0, a_1	-	n.a.	first order decay in function of water temperature	*Cryptosporidium*	3.1, -0.078	(Schijven et al. 2013)
				Campylobacter	0.53, -0.017	(Schijven et al. 2013)
α, β	-	n.a.	dose-response relationship: hypergeometric with beta-distributed parameters	*Cryptosporidium*	0.3, 1.1	(Schijven et al. 2015, Schijven et al. 2011)
				Campylobacter	0.1453, 8.007	(Murphy et al. 2016)

Microbiological analysis for *Cryptosporidium spp.*

WWTP influent and effluent samples for *Cryptosporidium* spp. were collected at WWTP1-3 between June 2018 and March 2019 (n=10 per WWTP). Oocysts were isolated from influent samples by directly centrifuging 13 mL at 3,000 ×g for 10 min and from effluent samples by filtering 1 L using the flat membrane method described in ISO 15553(ISO 2006) and centrifuging it at 1,550 ×g for 15 min. Supernatants were discarded and pellets were resuspended in 2 mL of ultrapure water. One mL of the suspension was used for the immunomagnetic separation of *Cryptosporidium* using the Dynabeads GC Combo kit (Thermo Fisher, UK). Concentrates were stained with the EasyStain kit (BTF Pty. Ltd., Biomerieux, Australia) and quantified as described by Stevenson et al. (2015).

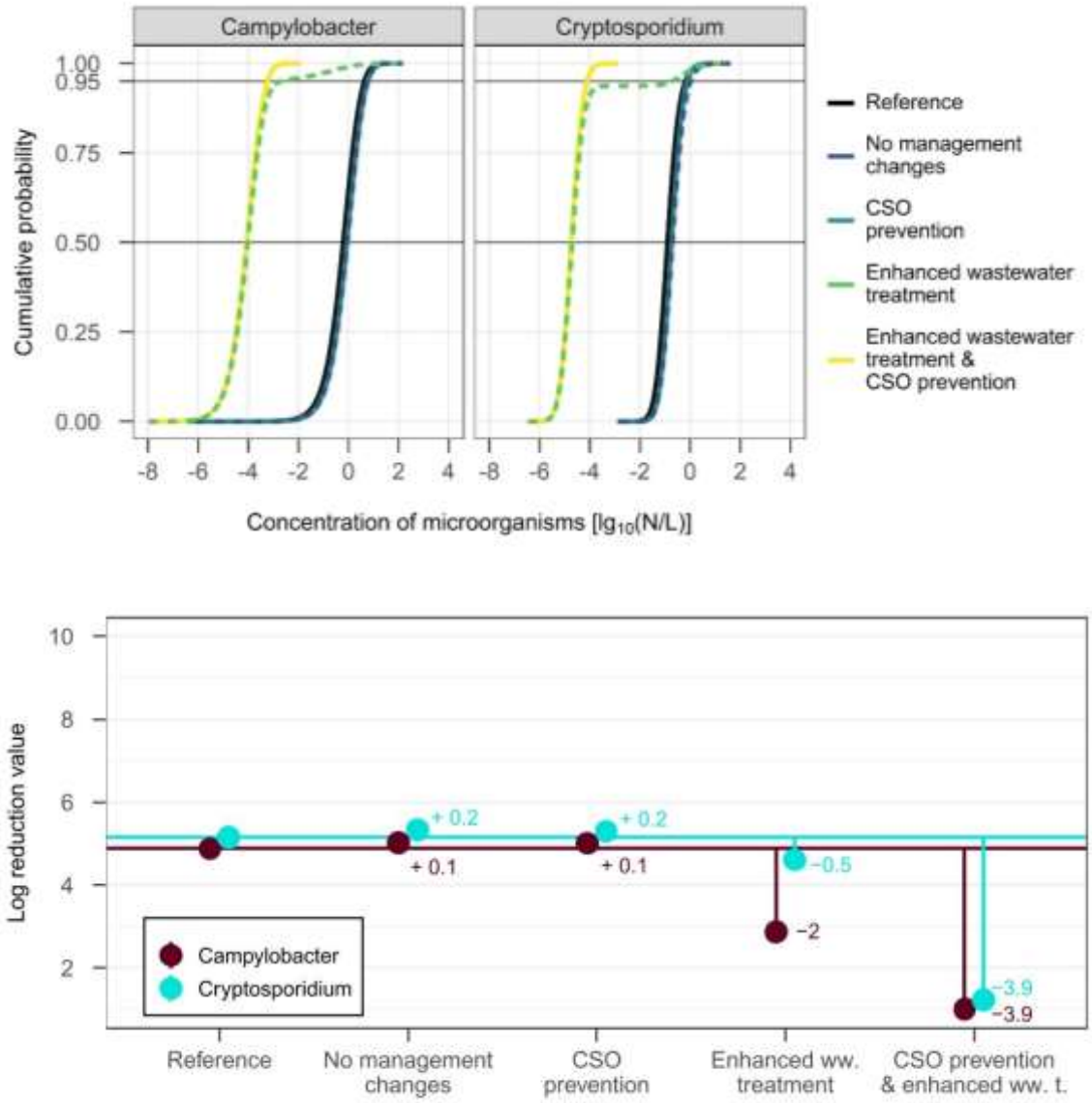

Figure S1. *Scenario simulations for Cryptosporidium and Campylobacter (see Figure 2 for norovirus and enterovirus). Upper panel: Simulated concentrations in river water. The no-CSO scenario entirely overlaps with the no management changes one. Bottom panel: Required log reduction values to achieve safe drinking water.*

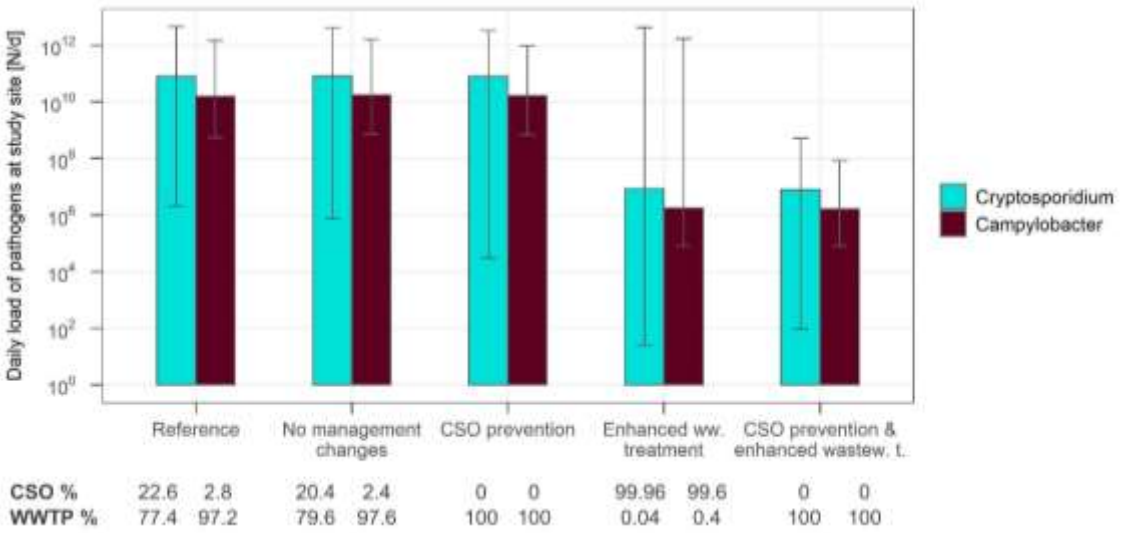

Figure S2. *Scenarios: Median daily load of viruses at the study site and relative source apportionment for Cryptosporidium and Campylobacter (see Figure 4 for norovirus and enterovirus). The whiskers show the range of simulated daily loads.*

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Annex II-B: Demeter *et al.*, 2020

Automated online monitoring of fecal pollution in water by enzymatic methods

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Automated online monitoring of fecal pollution in water by enzymatic methods

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Abstract

To facilitate the prompt management of public health risks from water resources, the fluorescence-based detection of the enzymatic activity of β-D-glucuronidase (GLUC) has been suggested as a rapid method to monitor fecal pollution. New technological adaptations enable now its automated, near-real-time measurement in a robust and analytically precise manner. Large data sets of high temporal or spatial resolution have been reported from a variety of freshwater resources, demonstrating the great potential of this automated method. However, the fecal indication capacity of GLUC activity and the potential link to health risk is still unclear, presenting considerable limitations. This review provides a critical evaluation of automated, online GLUC-based methods (and alternatives) and defines open questions to be solved before the method can fully support water management.

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Edited by **Warish Ahmed** and **Kerry Hamilton**

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Keywords

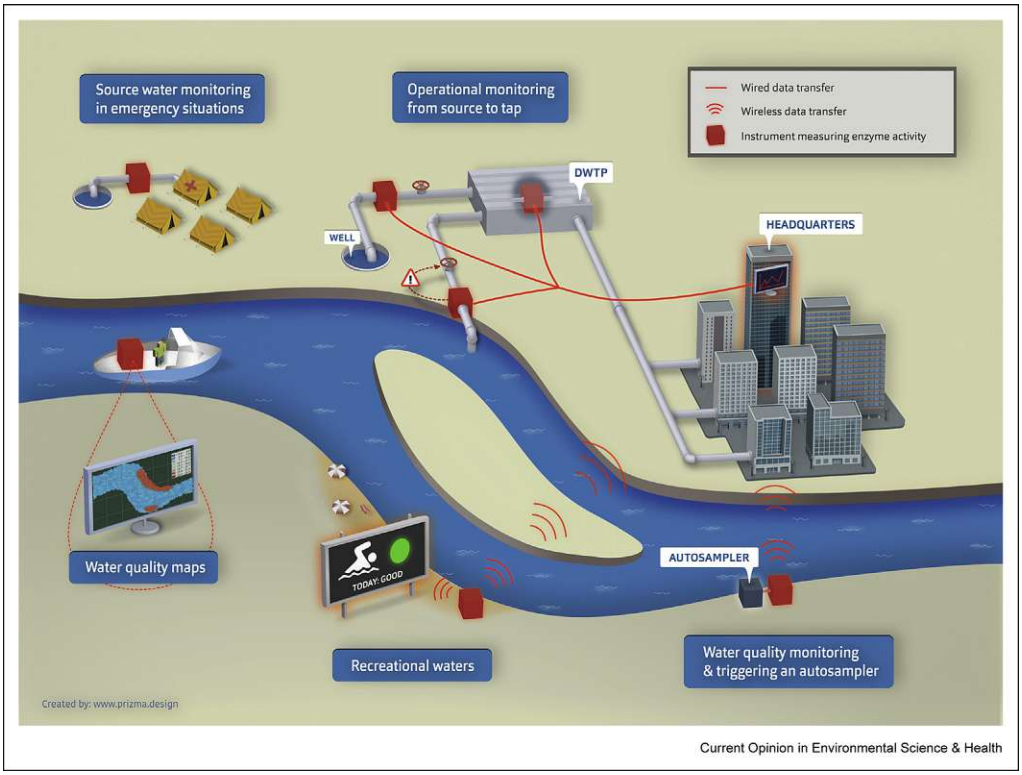
Fecal pollution, Rapid enzymatic methods, β-d-Glucuronidase, Fecal indicator bacteria, *E. coli*, Water safety.

Introduction

The prevention of waterborne diseases requires a systemic framework including water quality monitoring, pollution characterization, and health risk assessment [3]. The monitoring of fecal pollution is a key element in this approach. Fecal pollution patterns in water may vary greatly on short temporal and spatial scales [4,5]. However, culture-based monitoring standards using fecal indicator bacteria (FIB, such as *Escherichia coli*, intestinal enterococci) only provide a result after 18–24 h and grab samples are collected at large intervals (often >>1 day). Pollution peaks might be missed, or if caught, the result is only available retrospectively. Therefore, there is a need for continuous and (near-)real-time monitoring of fecal pollution in water. Such devices may be applied for monitoring and strategic management throughout the water sector, from drinking water supply to recreational waters (Figure 1). Wired or wireless data transmission enables remote control and thus the method may become an integral part of an increasingly digitalized water industry.

Methods based on the fluorometric measurement of the enzymatic activity of β-D-galactosidase (GAL) and β-D-glucuronidase (GLUC) in water were suggested over two decades ago as rapid surrogates for the culture-based determination of coliforms (GAL) and fecal coliforms or *E. coli* (GLUC) [8–11]. Fluorogenic and chromogenic enzymatic substrates had been well known for a long time as diagnostic supplements in bacterial media (e.g. Ref. [13] included now in ISO 9308-2:2012 for the detection of *E. coli* [14]). During the last decade, fluorogenic substrate technologies were incorporated into online instruments enabling the automated and

Figure 1



Potential applications of rapid online enzymatic methods for the detection of fecal pollution in water. The instruments may be placed at various monitoring points in natural waters or at critical control points along the drinking water supply chain. Connection with existing infrastructure allows the instrument triggering the action of another instrument, such as an autosampler to allow cross-comparison with laboratory-based standard microbiological assays. Connection to the headquarters and/or to cell phones allows data management and central monitoring. DWTP, drinking water treatment plant.

rapid determination of specific enzymatic hydrolysis rates in water [15–17].

Here, we provide an update and extension of the milestone review of Fiksdal and Tryland [9] by focusing on online, automated enzyme measurement platforms intended for fecal pollution monitoring in water resources. The emphasis lies on the direct determination of enzymatic hydrolysis rates in water (not involving a culture step) because the short time to result supports near-real-time monitoring applications. The focus is on GLUC activity rates, because the studies available to date in peer-reviewed literature cover almost exclusively this parameter.

Does the automation work? *The technical realization of rapid-automated GLUC measurement*

Device principles

The technical developments necessary for the enzymatic assay to be operated remotely and fully automated have been achieved and are well documented [15–18] (Table 1, upper panel). The devices typically consist of a sample intake, reagent stocks, a temperature-controlled reaction chamber, a UV emitter and optical sensor as well as a control unit and a user interface (for references,

Definitions

Rapid detection: there is no widely accepted definition, Noble and Weisberg suggest 'methods that provide results in less than 4 h' [1].

Online measurement: continuous and automatic monitoring of a parameter. Intranet and/or Internet connection allows controlling the results remotely [2].

Proxy or surrogate parameter: a parameter that is used as an indicator of the presence of another parameter in the absence of a direct measure [6,7].

Automated: carried out by machines or computers without needing human control [12].

Table 1

Published methods for the laboratory-independent measurement of enzymatic activities intended for the monitoring of fecal pollution.

Enzyme	Method	Measurement principle	Substrate	Time to result	Automated or manual	Literature reference: method	Literature reference: field applications	Commercial realization
Automated devices for the direct measurement of enzymatic activity								
GLUC (GLU, GAL)	Fluorometric	Direct enzymatic	4-Methylumbelliferyl-β-d-glucuronide	15 min	Automated online	[16,19]	[19,21,22,25,27–29]	ColiMinder (VWMS, Austria)
GLUC (GAL)	Fluorometric	Direct enzymatic	4-Methylumbelliferyl-β-D-glucuronide	75 min[a]	Automated online	[15,17,20]	[17,19]	BACTcontrol (microLan, The Netherlands), previously ColiGuard (mbOnline, Austria)
Alternative methods for laboratory-independent monitoring of fecal pollution based on enzymatic activities								
GLUC	Fluorometric (ColiSense)	Enzymatic after lysis	6-Chloro-4-methyl-umbelliferyl-beta-D-glucuronide (6-CMUG)	75 min	Manual, field-portable	[26,46,47]	[48]	–
GAL	Fluorometric	Enzymatic after selective culture	GAL: 4-methylumbelliferyl-D-galactoside	15–120 min	Manual, field-portable	[42]	[42]	Colifast Field kit (Colifast AS, Norway)
GLUC, GAL	Fluorometric	Enzymatic after selective culture	GAL: 4-methylumbelliferyl-D-galactoside GLUC: not disclosed	2.5–15 h	Automated online	[42,43]	[42,43]	Colifast ALARM, Colifast CALM (Colifast AS, Norway)
GLUC, GAL	Fluorometric	Enzymatic after selective culture	Pyrene-glucuronide and anthracene-galactoside	2–18 h	Automated online	[45]	[21]	Tecta B16 (ENDETEC, Canada)
GLUC	Fluorometric	Enzymatic after selective culture	4-Methylumbelliferyl-β-D-glucuronide	2–12 h	Automated field-deployable and manual field-portable	[44]	–	ALERT System, ALERT Lab (Fluidion SAS, France)
GLUC	Voltammetric (EcoStat)	Enzymatic after selective culture	Methyl-β-D-glucuronide sodium salt	≤10 h	Automated	[49]	–	–

GLU, β-D-glucosidase.
[a] Including a sample concentration step.

Table 2

Applications of automated GLUC enzymatic activity measurement devices.

Enzyme	Intended application	Duration	Water resource type (mean discharge)	Location	Land use (major fecal pollution sources)	Meteorological conditions	Literature reference
GLUC	Automated near-real-time monitoring of source water quality	2 years	Karst aquifer spring (5 m^3/s)	Northern Alps, Austria	Forested and summer pastures (domestic and wildlife ruminants)	Dry weather, rainfall	[17]
			Alluvial aquifer	Danube River, Vienna, Austria	Protected wetland and floodplain forest (wildlife ruminants)	Dry weather, rainfall	
GLUC	Comparison of two automated online technologies for investigation of catchment-based transport of *E. coli*	1 year	Stream (2.7 10^{-3} m^3/s)	Hydrological Open-Air Laboratory (HOAL) catchment, Austria	Agricultural cropland (swine manure)	Dry weather, rainfall	[19]
GLUC	Automated near-real-time monitoring of source water quality in remote and resource-limited settings	10 days	Karst spring (0.6–0.9 m^3/s)	Seo Ho River, Vietnam	Agricultural (livestock, manure, untreated domestic sewage)	Dry weather, rainfall	[22]
GLUC	Ship-borne automated surface water quality mapping at various spatial scales	3 h–1 day	Lake	Yahara lakes, Wisconsin, USA	Predominantly agricultural with urban areas (diffuse agricultural pollution, leaks from sanitary sewers, urban stormwater outfalls, birds)	Dry weather, rainfall	[28]
		5 days	River (5700 m^3/s)	Lower Columbia River, Oregon/Washington, USA	Agricultural and urban	Dry weather	
		1 day	River (1300 m^3/s)	Upper Mississippi River, Wisconsin, USA	Predominantly agricultural with urban areas(diffuse agricultural sources, wastewater treatment plant effluents)	Dry weather	
GLUC	Investigation of catchment microbial dynamics at seasonal to hourly time scales	2 years	Stream (2.7 10^{-3} m^3/s)	Hydrological Open-Air Laboratory (HOAL) catchment, Austria	Agricultural cropland (swine manure)	Dry weather, rainfall	[25]
GLUC	Identification of dominant fecal pollution sources in an urban drinking water supply	1.5 years	River (300 m^3/s)	Greater Montreal Area, QC, Canada	Predominantly urban, small agricultural tributaries (treated and untreated sewage discharges, diffuse agricultural sources)	Dry weather, rainfall, and snowmelt, spring flood	[27]
GLUC	Automated near-real-time monitoring of recreational water quality	2 months	River (7500 m^3/s)	Greater Montreal Area, QC, Canada	Combined sewer overflows	Dry weather, rainfall	[29]
		4 months	River (300 m^3/s)	Greater Montreal Area, QC, Canada	Predominantly urban, small agricultural tributaries	Dry weather, rainfall	

(*continued on next page*)

Table 2. (*continued*)

Enzyme	Intended application	Duration	Water resource type (mean discharge)	Location	Land use (major fecal pollution sources)	Meteorological conditions	Literature reference
					(treated and untreated sewage discharges, diffuse agricultural sources)		
		2 months	River (12,600 m^3/s)	Quebec City, QC, Canada	Mixed (Combined sewer overflow discharges, diffuse agriculture runoff, gulls)	Dry weather, rainfall	
		3 months	River (150–450 m^3/s)	Waikato River, Hamilton, New Zealand	Agricultural (diffuse runoff from livestock grazing, effluent spreading and wildlife, stormwater outfalls)	Dry weather, rainfall	

see Table 1). Technical applications (casing, power supply, etc.) have been reported for operation in buildings, remotely as a stationary device or as a mobile outdoor device [17,19,20]. Reported sample volumes range from 6 to 5000 mL, with the possibility to concentrate large sample volumes [15–17]. Measurement intervals between 15 and 180 min have been described [17,19,20].

Analytical performance

The available evaluations have indicated high analytical precision for the automated GLUC activity measurements with coefficients of variation below 5% [21]. Widely used cultivation-based FIB standards achieved a lower analytical precision with coefficients of variation between 16% and 31% [21]. The general performance of GLUC activity measurements was reported to be comparable with manually performed analysis and the simultaneous determination of the limit of quantification can be integrated into the automated data analysis by the instrument [17]. It should be noted that the reported units differ among manufacturers and studies (hydrolysis rate *versus* Fishman units per volume), although conversions can be achieved.

The *robustness* of the automated GLUC activity measurements in freshwater types having a wide range of physicochemical and microbiological characteristics was demonstrated by recent studies in pristine waters [17], surface waters with elevated suspended solid loads [19,22], and waters impacted by treated and/or untreated municipal sewage [21,23]. However, marine waters were only tested so far using laboratory-based direct GLUC assays [10,24]. Reported environmental factors influencing measurement accuracy and error-free running time are ambient temperature and suspended organic matter [9,19]. Both factors are now managed well by specific adaptations of the devices, including the specific design of the reaction chamber, sample pre-filtration, adapted cleaning procedures, and data-correction algorithms [20]. Such devices were successfully operated outdoors *in situ* for up to 2 years (e.g. Ref. [17,25]).

Alternative laboratory-independent methods based on GLUC activity

A portable device has been developed based on the direct measurement of GLUC activity after cell lysis ([26]; Table 1, lower panel). In addition, automated devices based on enrichment in selective growth media before the measurement of enzymatic activity have been successfully realized, with several fluorometric and one voltammetric method based on this principle. Some instruments have been designed for online monitoring others as field-deployable devices (Table 1, lower panel).

Where has it been used? *Field studies using automated GLUC measurement*

Automated GLUC activity measurement devices have been deployed with the aim to characterize the temporal and spatial patterns of GLUC activity and describe the relationship to cultivation-based standard *E. coli* detection methods in various water resources (Table 2).

Vulnerability assessment of water resources

The first demonstration of the technical feasibility of near-real-time monitoring of GLUC and GAL activities was provided by Ryzinska-Paier et al. [17] at an alpine karst spring and an alluvial aquifer in Austria over a period of 2 years. The seasonal dynamics of GLUC activity at a karstic spring environment were described for the first time (>5000 successful automated measurements). In a freshwater resource for urban drinking water supply in Canada, Burnet et al. [27] used a 1.5 year long GLUC activity time series to identify the dominant fecal pollution source among multiple wastewater discharges and to uncover the hydraulic connection between an upstream wastewater treatment plant and the drinking water treatment plant. Ender et al. [22] demonstrated the feasibility of automated near-real-time monitoring of GLUC activity in a remote karst spring in Northern Vietnam using a portable instrument designed to operate under limited resources settings.

Catchment microbial/biochemical dynamics

The automated near-real-time monitoring of GLUC activity as biochemical indicator has a considerable potential. Stadler et al. [19] first demonstrated that two different commercially available instruments were able to detect rapid fluctuations in enzymatic activity caused by episodic changes in hydrological conditions. The authors reported seasonal variations in the transport of GLUC activity, which peaked more often and at higher amplitudes in summer, although several of these GLUC activity peaks occurred in absence of rainfall and suspended sediment peaks [19,25]. Through the screening of GLUC activity in stream water and sediments and using stable isotopes in stream water, the authors suggested that a large portion of the transported GLUC originated from the resuspension of streambed sediments and reflected the existence of a remnant reservoir of GLUC in the catchment [25]. In an urban catchment affected by multiple treated and untreated wastewater discharges, Burnet et al. [27] similarly illustrated the large temporal scale of variation in GLUC activity in water. GLUC activity peak episodes occurred exclusively between late fall and early spring and were caused by intense precipitation (24–48 h before GLUC activity peak) and/or snowmelt events, which triggered the local discharges of untreated sewage into the river.

Besides the seasonal and event-based fluctuations in GLUC activity, recurrent daily patterns have been reported in various habitats, although the peak activities did not occur at the same time of the day [19,21,22,27]. The origin of these daily patterns was attributed to the likely temperature dependence of bacterial activity in a small agricultural stream [19], and in a karst spring [22], although the causal link requires further investigations. Another type of daily pattern of GLUC activity was described at an urban drinking water intake and was traced back to the discharge pattern of an upstream wastewater treatment plant [27].

Surface water quality mapping

Using a ship-borne instrument, Stadler et al. [28] recently demonstrated the feasibility of rapid GLUC activity assessment for surface water quality mapping. These first high-resolution spatial data on GLUC activity illustrated the effect of rainfall-induced runoff on surface water quality along urbanization gradients and indicated tributaries and confluences as main fecal pollution hotspots in these large waterbodies.

Recreational water quality assessment

Cazals et al. [29] illustrated the usefulness of online GLUC activity monitoring for rapid identification of impaired waters in recreational freshwater bodies. Threshold GLUC activity values were developed to match the regulatory ('gold standard') *E. coli* beach action values while minimizing the rates of failures to act and false alarms. Near-real-time monitoring of GLUC activity enabled to identify fecal pollution peaks and determine the exact timing of GLUC activity threshold exceedance.

What does it tell us? *Indicator capacity of GLUC*

Relationship to cultivation-based FIB

All field studies using automated GLUC determination (Table 2) performed cross-comparisons with cultivation-based *E. coli* standards [17,19,21,22,25,27–29] and one study reported data also for coliforms [17]. Reported correlations between GLUC activity and cultivation-based *E. coli* standards (expressed in linear or non-parametric correlation coefficients r) varied widely among the studied water resources. For freshwaters influenced by urban sewage, r ranged between 0.33 and 0.84 on non-transformed data [21,27,28] and between 0.10 and 0.79 on log-transformed data [29], with an apparently strong dependence of hydrometeorology and contamination characteristics [27,29]. Among the studied watersheds influenced by agriculture (manure spreading and/or cattle grazing), r ranged between 0.53 and 0.56 at karstic springs of remote mountains [17,22], whereas a small brook revealed r = 0.72 [19,25]. Stronger correlations were found at higher pollution levels [21,27] and during events (with the highest r reported being 0.89 [25]). Notably, GLUC activities often resulted in stronger correlations with

Table 3

Open research topics and future development goals regarding the automated, cultivation-independent determination of enzymatic activities intended for online fecal pollution monitoring at water resources.

Some open research topics and future development goals
Fecal and health-risk indication capacity of GLUC activity
• What are the limits to use GLUC as a biochemical fecal indicator (i.e. fecal pollution level, age, treatment)? • Which aquatic habitats are most suitable for GLUC activity monitoring in respect to its fecal indication ability? • Which habitats or situations are not suitable for GLUC activity monitoring and strong interference or bias from non-fecal sources is to be expected? • In which situations may GLUC activity become indicative of the occurrence of intestinal pathogens? • In which situations may GLUC activity become indicative of infection and health risks? • Can GLUC activity be used as a conservative indicator for pathogen removal during treatment?
GLUC activity of fecal origin: persistence and fate in the (aquatic) environment
• How long does cell-associated enzyme activity of intestinal populations persist? • How does GLUC activity compare to other cell-viability parameters? • What are the relative abundances of culturable, VNBC, dead cells/cell debris, free and particle-attached enzymes under various environmental conditions? Do they have a differential persistence? • Is there a difference in GLUC activities between human versus animal sources? • Which intestinal microbiota contribute to GLUC activity in water? • Do different microbiota show differential GLUC activity persistence? • Could the ratio GLUC to cultivation-based fecal indicator standards indicate contamination age? • Do free enzymes re-attach to abiotic particles, such as to silt-colloids? How does re-attachment influence the enzymatic persistence? Do catchments with high turbidity and GLUC adsorption rates limit the application? • Which GLUC inhibiting substance may occur in water samples and under what conditions?
GLUC activity of fecal origin: resistance and fate during water treatment and disinfection
• What is the resistance of GLUC activity of fecal origin to the various steps of wastewater treatment, including ozonation, UV disinfection and chlorination? Do the various GLUC compartments (culturable, VNBC, free enzymes, etc.) have a differential resistance? • What is the resistance of GLUC activity of fecal origin to the various steps of drinking water treatment, including chlorination, UV disinfection and ultrafiltration? Do the various GLUC compartments have a differential resistance? • How does GLUC activity compare to other cell-viability parameters during the treatment steps?
GLUC activity of non-fecal origin
• Under which conditions does algae-associated GLUC activity become significant? • Under which conditions does environmental bacteria-associated GLUC activity become significant? • What are other potential non-fecal associated GLUC sources? • Is it possible to differentiate or correct for non-fecal associated GLUC activity? • Does significant GLUC activity occur from 'naturalized' (re-grown) intestinal populations in the environment? • What is the exact nature and origin of daily GLUC fluctuations that are not related to the fecal pollution source dynamics?
Fecal pollution-associated enzymes other than GLUC (questions above are all relevant)
• What are the sources and fate of β-D-galactosidase? Is it a useful fecal indicator? • What are the sources and fate of β-D-glucosidase? Is it a useful fecal indicator? • Are there any other enzymes or combinations demonstrating enhanced fecal indicator capacity? • How can enzymatic substrates be improved to increase their sensitivity and specificity for fecal pollution?
Technical realization of automated, online instruments
The field needs … Validation guidelines (precision, robustness, specificity, sensitivity) … Quality control and quality assurance protocols … Uniform, standardized measurement units … Strategies to trigger microbiological autosampling, based on online GLUC and/or physicochemical measurements

VNBC, viable but not culturable.

environmental parameters than cultivation-based *E. coli* data. For example, correlations up to r = 0.87 with turbidity (2–3 μm particle fraction, karst spring, rain event) [22] and r = 0.93 with chlorophyll a (lake, dry weather) were observed [28].

GLUC does not qualify as a general proxy parameter for cultivation-based *E. coli* enumeration

The above correlation analysis supports previous observations that GLUC activity is not a general proxy for cultivation-based *E. coli* enumeration [9]. Enzymatic activity was demonstrated to be a more persistent biochemical parameter against environmental and treatment (disinfection) stresses as compared to the culturable fraction of FIB in water resources [9]. Indeed, there is evidence that GLUC activity is able to detect culturable cells as well as viable but non-culturable cell populations [30]. Furthermore, persistent GLUC activity was also reported for damaged or dead *E. coli* cells [31] and from the fraction of free enzymes in river water with fecal pollution [32]. It can be argued that free or particle-associated GLUC activity may be relevant for the detection of low, remote, old, or treated (disinfected) fecal pollution.

GLUC activity can also be associated with biotic or abiotic compartments other than *E. coli*

Without a selective cultivation-based enrichment step for *E. coli*, a significant amount of GLUC activity in water samples may also originate from other microbiota and substances [9]. Recent investigations highlight that microbiome-encoded GLUC activities play an important role in the human gastrointestinal system [33]. By genomic and proteomic tools, hundreds of different β-glucuronidase enzymes, grouped into six distinct categories, could be identified in abundant microbial phyla of Bacteroidetes, Firmicutes, Verrucomicrobia, and Proteobacteria in human stool samples [34], confirming previous observations before the genomic era [35]. However, possible interference from non-intestinal microbiota in water resources was also reported, including environmental bacteria and algae [9,36,37]. As a result, GLUC activity is considered to be of fecal origin, especially under the situation of high fecal pollution (culturable cells, viable but non-culturable, cell debris, and free enzymes), but interfering GLUC activity of non-fecal origin (biotic and abiotic) can also occur [9].

Status quo and open questions

Without any doubt, the automated online GLUC activity determination in water resources has been successfully realized during the last decade, offering fascinating new possibilities to support water safety management in the future (Figure 1). This technology may not be restricted to GLUC and related enzymes, but could support any type of enzymatic online monitoring (if technically feasible) that can inform about microbial and biochemical water quality issues [38–41]. As opposed to the original suggestion almost 20 years ago [11], it is now obvious—after the many cross-comparison efforts—that GLUC activity is not a general surrogate for the cultivation-based determination of *E. coli*. Depending on the habitat, fecal pollution characteristics and hydrometeorology, the relationship between culturable *E. coli* concentrations and GLUC activity rates can vary substantially. In cases where the direct comparison with cultivation-based *E. coli* standards is essential, online GLUC determination using automated pre-enrichment procedures by selective growth would be a more suitable approach (Table 1, [42–45], with reported correlation coefficients to standard *E. coli* methods ranging between 0.90 and 0.94 [21,42,44]). However, a trade-off between this stronger relationship and a significantly longer sample-to-result time has to be taken into account (Table 1). The rapid online prediction of culturable *E. coli* based on GLUC direct determination may only be possible in special cases: at certain sites and under certain pollution scenarios allowing a sufficiently high statistical relationship. This, however, requires further investigations.

In contrast to the achieved progress in the automated determination of enzymatic hydrolysis rates, the scientific evaluation of the GLUC indication capacity for fecal pollution monitoring has been almost neglected for more than a decade [9]. There is an urgent research need to understand more comprehensively the sources and sinks, the persistence and mobility, and the link of GLUC activity with the actual cellular states. Such investigations should cover all important water resource systems and should also include essential water treatment and disinfection processes (Table 3). Based on the information currently available, we propose *GLUC activity as a conservative biochemical proxy-parameter for bacterial fecal pollution* (not only associated with *E. coli* or fecal coliforms) in water resources. Furthermore, for specific system conditions and exposure scenarios, GLUC activity may also indicate pathogen occurrence and infection risk from fecal pollution and could therefore be part of the strategic management of the given water resource (Table 3). However, as highlighted a decade earlier [9], GLUC activities from non-fecal compartments may interfere with the indented indication capacity, especially in the case of low, old, or remote fecal pollution. The above-mentioned gaps of knowledge currently limit the application of automated online GLUC activity monitoring in the water management sector and warrant further detailed investigations.

Declaration of Competing Interest

Nothing declared.

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Annex III: Published co-authored article

Genetic Microbial Source Tracking Support QMRA Modeling for a Riverine Wetland Drinking Water Resource

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Genetic Microbial Source Tracking Support QMRA Modeling for a Riverine Wetland Drinking Water Resource

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Riverine wetlands are important natural habitats and contain valuable drinking water resources. The transport of human- and animal-associated fecal pathogens into the surface water bodies poses potential risks to water safety. The aim of this study was to develop a new integrative modeling approach supported by microbial source tracking (MST) markers for quantifying the transport pathways of two important reference pathogens, *Cryptosporidium* and *Giardia,* from external (allochthonous) and internal (autochthonous) fecal sources in riverine wetlands considering safe drinking water production. The probabilistic-deterministic model QMRAcatch (v 1.1 python backwater) was modified and extended to account for short-time variations in flow and microbial transport at hourly time steps. As input to the model, we determined the discharge rates, volumes and inundated areas of the backwater channel based on 2-D hydrodynamic flow simulations. To test if we considered all relevant fecal pollution sources and transport pathways, we validated QMRAcatch using measured concentrations of human, ruminant, pig and bird associated MST markers as well as *E. coli* in a Danube wetland area from 2010 to 2015. For the model validation, we obtained MST marker decay rates in water from the literature, adjusted them within confidence limits, and simulated the MST marker concentrations in the backwater channel, resulting in mean absolute errors of < 0.7 $\log_{10}$ particles/L (Kruskal–Wallis $p > 0.05$). In the scenarios, we investigated (i) the impact of river discharges into the backwater channel (allochthonous sources), (ii) the resuspension of pathogens from animal fecal deposits in inundated areas, and (iii) the pathogen release from animal fecal deposits after rainfall (autochthonous sources). Autochthonous and allochthonous human and animal sources

resulted in mean loads and concentrations of *Cryptosporidium* and *Giardia* (oo)cysts in the backwater channel of 3–13 × 10^9 particles/hour and 0.4–1.2 particles/L during floods and rainfall events, and in required pathogen treatment reductions to achieve safe drinking water of 5.0–6.2 $\log_{10}$. The integrative modeling approach supports the sustainable and proactive drinking water safety management of alluvial backwater areas.

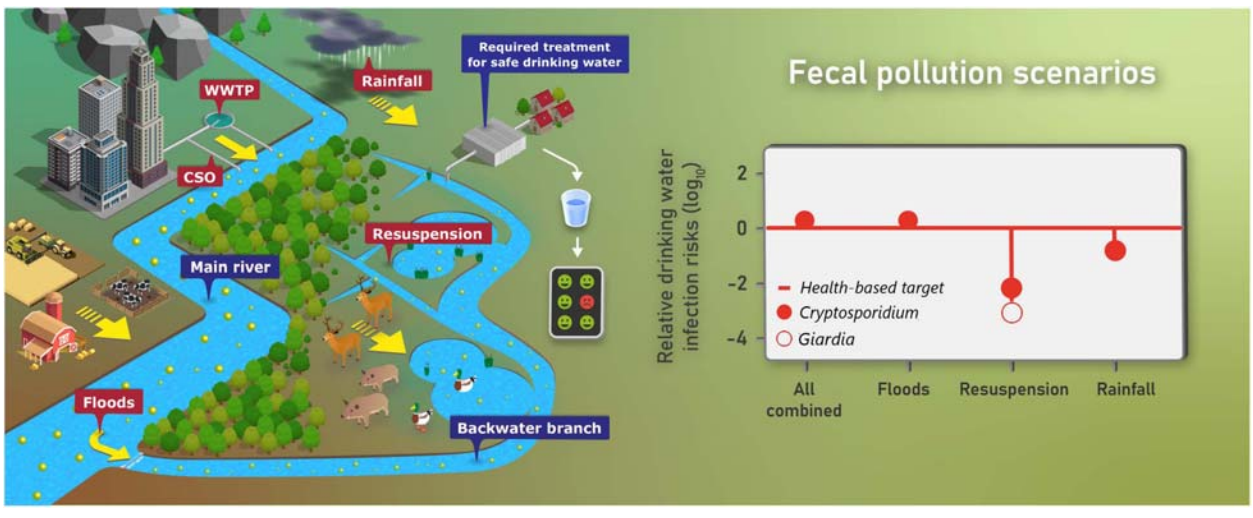
Keywords: genetic microbial source tracking markers, microbial fate and transport model, hydrodynamic model, *Cryptosporidium*, *Giardia*, QMRA, microbial decay in environment

INTRODUCTION

Alluvial backwater areas along large rivers are important as natural habitats, for flood protection, and contain valuable resources for drinking water production. The impacts of urban wastewater discharges, and livestock and wildlife fecal deposits pose substantial hazards to these ecosystems concerning water supply (Hogan et al., 2012; Frick et al., 2020). River water impacted by human wastewater and/or diffusive animal sources may transport fecal pathogens into the backwater area from outside (allochthonous sources). Inside the backwater area, pathogens may be released from animal fecal deposits following rainfall events, or may be resuspended in inundated areas during floods (autochthonous sources). Several authors have studied the impact of allochthonous and anthroponotic pathogens, such as human-specific viruses, on the microbiological quality of wetland water resources considering safe drinking water production (Sanders et al., 2005; Daniels et al., 2014; Liu et al., 2015; Derx et al., 2016). In this respect, however, there is little known about the relative contribution of animal versus human and autochthonous versus allochthonous sources of fecal pollution.

The microbial transport and removal mechanisms in wetlands have been primarily studied for fecal indicator organisms (FIO) (Sanders et al., 2005; Liu et al., 2015). Solely relying on FIO, however, does not allow differentiating the impact of different sources, as FIO occur in all human and animal sources. Microbial source tracking markers (MST) provide therefore immensely valuable information to identify fecal pollution sources for wetlands. This was demonstrated, e.g., by Frick et al. (2020), who linked FIO data with host-associated MST data and river connectivity in an alluvial Danube wetland. If combined with pathogen data, host-associated microbial source tracking markers of sufficient specificity and sensitivity can greatly support the source-targeted calibration of microbial fate and transport models and support a health risk assessment, as shown by Derx et al. (2016) and Demeter et al. (2021). So far, the spectrum of available animal and human MST marker assays has not yet been exploited in this context. In combination with FIO and zoonotic pathogen data, host-associated MST markers could significantly support microbial fate and transport modeling and microbial infection risk assessments in wetlands.

Important transport processes in wetlands are the advection, release and resuspension, decay and settling of microbial particles or chemical substances (Pavlik et al., 1999; Grant et al., 2001; Hogan et al., 2012; Peterson and Hanna, 2016). Further influencing factors for the retention mechanisms are water temperature, turbidity, salinity, and vegetation cover (Daniels et al., 2014). The flow patterns driving these transport processes in riverine or coastal wetlands vary in space and time, and are commonly simulated based on multi-dimensional, hydrodynamic flow and transport models (Sanders et al., 2005;



GRAPHICAL ABSTRACT

Liu et al., 2015). Due to the uncertainty of the source and transport variables, several studies conducted in wetlands used probabilistic-deterministic approaches to model microbial fate and transport. Daniels et al. (2014) developed a deterministic pathogen transport model for wetlands within a Bayesian statistical framework. Schijven et al. (2015) developed the probabilistic-deterministic microbial fate, transport and infection risk model QMRAcatch. This model was later applied by Derx et al. (2016) for evaluating the impact of human fecal pollution in a large riverine wetland. Daniels et al. (2014), Schijven et al. (2015), and Derx et al. (2016), however, did not account for the complex spatiotemporal variations of the flow and transport processes in wetlands.

The primary aim of this study was to develop a new integrative modeling approach supported by MST markers for quantifying the impact of human and animal, as well as, autochthonous and allochthonous fecal sources on the microbiological quality of an alluvial backwater channel considering safe drinking water production. The model should be able to account for the transient spatiotemporal variations of flow patterns and for the uncertainty of the source and transport variables. The secondary aim was to test the developed model at a Danube backwater area supported by MST markers and *E. coli* for quantifying the concentrations and loads of *Cryptosporidium* and *Giardia* (oo)cysts considering safe drinking water. These are important reference protozoa occurring ubiquitously in human and animal fecal sources (Stalder et al., 2011). *Cryptosporidium* and *Giardia* (oo)cysts are highly persistent in the environment and infectious at low dose (de Regnier et al., 1989; Boyer et al., 2009), making them suitable to address our research question. To meet our aims, we modified and extended QMRAcatch (v 1.1 python backwater). We defined event-driven scenarios of microbial transport into the backwater channel, (i) via the river entering the backwater during floods (allochthonous sources), (ii) via the resuspension from fecal deposits during flooding and inundation of the backwater area, and, (iii) via the release and runoff from fecal deposits during rainfall within the backwater area (autochthonous sources). These were assumed to be the most important transport pathways, and human wastewater, ruminants, wild boar (pigs) and birds were considered as the most important fecal pollution sources, based on the findings of Frick et al. (2020) in our study area. To validate this assumption, we used a comprehensive 6-year monthly monitoring dataset of human and animal MST markers and *E. coli*.

MATERIALS AND METHODS

Study Area

The investigated alluvial model backwater area is situated at the Danube at the downstream end of the city of Vienna, in Austria (**Figure 1**, see Frick et al., 2020 for a detailed description). The catchment upstream of Vienna is home to approximately 11 million inhabitants (Schreiber et al., 2005). Considering that 99% of the human population in the study area is connected to a WWTP (European Commission, 2018), urban wastewater is the main source of human pollution. Due to the regulation of the Danube, the backwater area has been almost completely disconnected from the main stream. The backwater presently consists of a channel network, whose main lateral branch has a surface connection with the Danube River at its lowermost end through a levee opening ('entry point during floods', **Figure 1**). Floods that enter the floodplain via the backflow connection move upstream along the main lateral branch, creating a distinct gradient in hydrologic connectivity in the various waterbodies with distance to the inflow (Reckendorfer et al., 2013). The backwater channel connects with the Danube River if the discharge exceeds 2200 m^3/s (mean discharge: 1900 m^3/s). Danube water enters the backwater channel and its lateral branches during the rising limb of flood events, causing inundation of parts of the surface area (**Figure 1**). When the flood peak is reached, the flow direction reverses, and the water flows back toward the Danube River.

The backwater area is 14 km^2 in size, and represents an important water resource for drinking water supply for Vienna (Hein et al., 2006). There are five drinking water wells (riverbank filtrate) situated in the 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 is no livestock in the considered backwater area, but there is a considerable population of wild animals, such as ruminants, wild boars and birds (Frühauf and Sabathy, 2006a,b; Parz-Gollner, 2006; Arnberger et al., 2009). Population sizes estimated for 2010 resulted in 180 red deer (*Cervus elaphus*), 44 roe deer (*Capreolus capreolus*), 17 fallow deer (*Dama dama*), 20 European mouflon (*Ovis orientalis musimon*) and 150 wild boars (*Sus scrofa*) (Government of the City of Vienna, Alexander Faltejsek, personal communication). Hunting is allowed in the area. The bird abundance is nearly 2500 at maximum in total (Frühauf and Sabathy, 2006a,b; Parz-Gollner, 2006; Schulze and Schütz, 2013). In addition, 600,000 visitors visit the national park area every year (Hinterberger et al., 2000). Bathing is prohibited at 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).

Modeling Approach

For this paper, we used a modified and extended version of the probabilistic-deterministic microbial fate and transport and infection risk model QMRAcatch (Schijven et al., 2015; Demeter et al., 2021; **Figure 2**). We took a two-step modeling approach: (1) To test the assumption of the most relevant fecal sources and transport processes, we performed a source-targeted model validation using measured concentrations of host-associated MST markers and *E. coli* at the study site (Section "Validation of the Microbial Fate and Transport Model"). (2) To evaluate the importance of animal versus human, or autochthonous versus allochthonous sources in terms of potential health relevance, we then simulated various pollution scenarios (Section "Scenario Load and Infection Risk Assessment"). In these scenarios, we simulated the concentrations and loads of *Cryptosporidium* and *Giardia* in the backwater channel, and the required pathogen treatment reduction and daily drinking

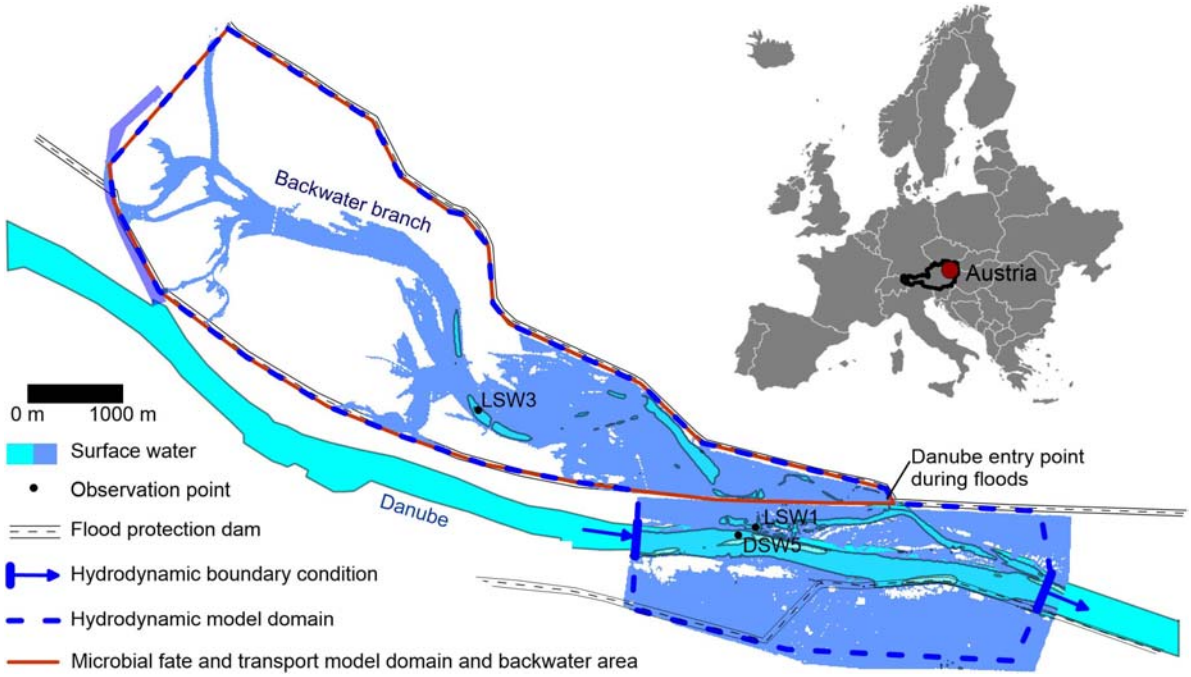


FIGURE 1 | Study area with observation points DSW5 (Danube), LSW1 (affected by Danube discharge and inundation) and LSW 3 (affected by rainfall-release from fecal deposits) (Frick et al., 2020), QMRAcatch and hydrodynamic model domain; the surface water area simulated during the peak of the flood event in January 2011 is indicated in blue.

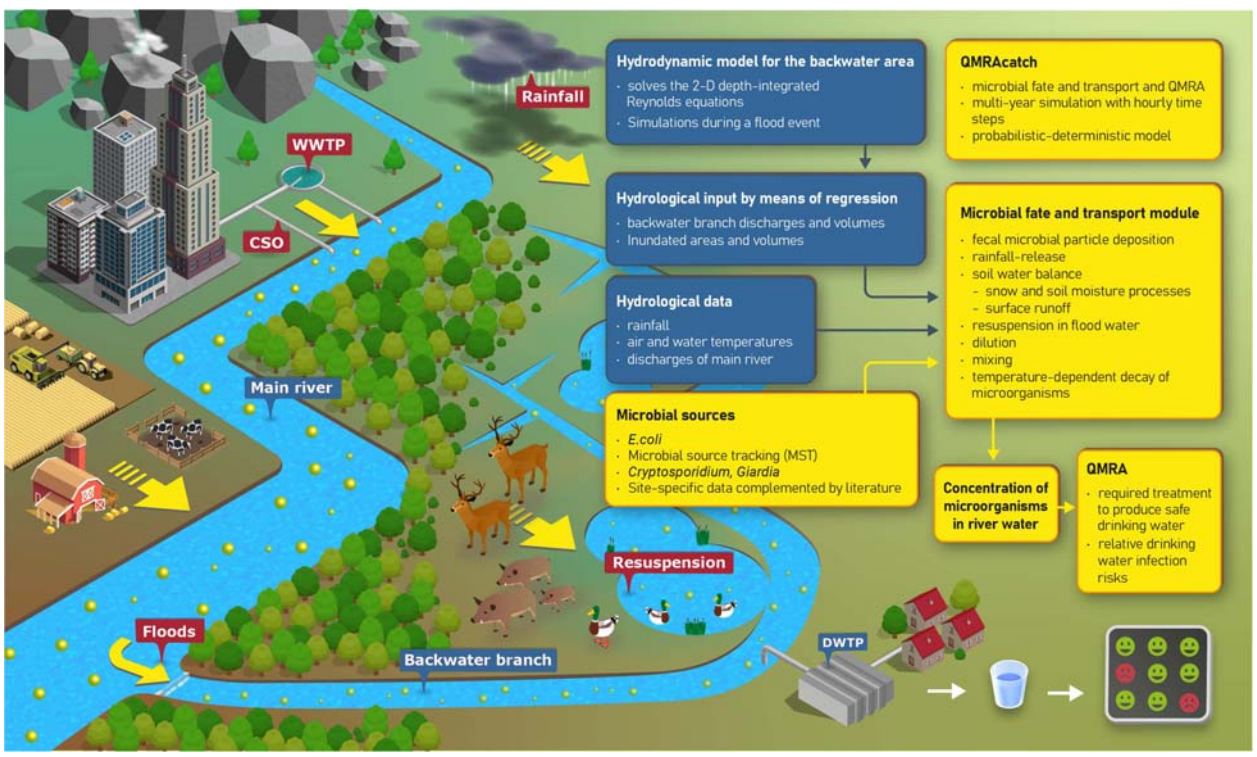


FIGURE 2 | Microbial sources and transport processes in the alluvial backwater area (graphics). Components and data flows of the integrative modeling approach (flow chart).

water infection risks relative to a health-based benchmark. The model domain of QMRAcatch encompasses the total backwater area, delimited by the Danube along the southern boundary and a flood protection dam on its northern end (**Figure 1**). Hydrological monitoring data together with 2-D hydrodynamic flow simulations during a flood event (Section "Hydrological and Hydrodynamic Flow Situation") as well as measured microbial source data complemented with literature data (Section "Microbiological Source Characterization") served as input to the model.

QMRAcatch

Model Overview

The probabilistic-deterministic microbial fate and transport and infection risk model QMRAcatch (Schijven et al., 2015) was extended for this study and coded as open source (v1.1 Python backwater). QMRAcatch was used to simulate the microbial concentrations of the backwater channel (Sections "Microbial Fate and Transport Module" to "Microbiological Source Characterization") and the daily drinking water infection risks after further treatment of the source water (Section "Quantitative Microbial Risk Assessment Module"). The model comprises the functionality of the version QMRAcatch 1.0 Python (Demeter et al., 2021) with the following extensions:

- Simulation time steps of 1 h in contrast to 1 day in the previous version.
- The concentration of MST markers, fecal indicators and pathogens in the main river (Danube) water is described by a statistical distribution according to the observed data set.
- Microbial particle release from animal fecal deposits is described as a function of precipitation and elapsed time since the start of precipitation according to Bradford and Schijven (2002).
- Hourly discharges and volumes of the backwater channel, flooded areas and floodplain volumes are additional input variables.
- Model equations were included to calculate surface runoff based on transient evaporation and soil moisture processes using air temperature and rainfall as input variables.
- Microbial decay in animal feces is described based on a uniformly distributed first order rate coefficient μ_f. In the earlier version, the decay rates in water and feces were not differentiated.
- The prevalence of the reference pathogens *Cryptosporidium* and *Giardia* in animal waste is described by a mixture of beta distributions from reported studies following the methodology of Dorner et al. (2004).
- Data on times of consumption and consumed volumes of unboiled drinking water per person per day by the Dutch National Food Consumption Survey 2007–2010 (DNFCS) (Van Rossum et al., 2011) are used to calculate the daily cumulative dose and the daily drinking water infection risks.

Microbial Fate and Transport Module

Microbial contamination of the backwater channel occurs from the following three reservoirs: Danube, non-flooded and flooded area. Microbial particles are transported via inflows of Danube water during floods, by resuspension from animal deposits in inundated areas, and during rainfall causing release and runoff from animal fecal deposits in non-flooded areas.

Transport via the Danube entering the backwater during floods

Microorganisms carried by Danube water are subjected to mixing with the backwaters and temperature-dependent decay. Assuming steady-state conditions and complete mixing on each hour, the analytical solution for the microbial concentration in the backwater channel, $C_{r\rightarrow bw}$ [particles/L] at time step t is (Schijven et al., 2015):

$$C_{r\rightarrow bw}(t) = \frac{Q_{bw}}{Q_{bw}+\mu_w(T_{bw})V_{bw}}C_r + \left(C_{r\rightarrow bw}(t-1) - \frac{Q_{bw}}{Q_{bw}+\mu_w(T_{bw})V_{bw}}C_r\right)\exp\left(-\frac{Q_{bw}+\mu_w(T_{bw})V_{bw}}{V_{bw}}t\right) \quad (1)$$

where C_r [particles/L] is the microbial particle concentration of the Danube river, Q_{bw} and V_{bw} are the discharge and volume of the backwater channel (Section "Hydrological and Hydrodynamic Flow Situation," **Supplementary Table 2**). The degree of reduction of microorganisms during the transport depends on the travel time or flow rate. Decay during transport is described as a first order reaction, where the decay rate in water (μ_w [1/d]) is a function of the water temperature (T_{bw} [°C]):

$$\mu_w(t) = \frac{ln10}{10^{a_0+a_1 T_{bw}}} \quad (2)$$

where a_0 [$\log_{10}$ day] and a_1 [$\log_{10}$ day/°C] are microorganism-specific decay rate parameters (Bertrand et al., 2012). We conducted an extensive literature review on the microorganism-specific decay rates in water and adjusted the values within prediction intervals (**Supplementary Table 4**, **Figure 4**). The rates implicitly included additional removal or regrowth processes, or effects of other environmental factors, such as temperature, pH, TOC (van Elsas et al., 2011; Ahmed et al., 2019).

Microbial particle deposition

Microbial particle loadings from animals were determined using the method described by **Dorner et al. (2004)** and **Sterk et al. (2016)**. For pathogens, the fraction of animals infected by *Cryptosporidium* or *Giardia* was derived by random sampling from several (equally weighted) beta-distributions, describing the probability that an animal is positive (prev):

$$prev \sim \beta(\alpha, \beta) \quad (3)$$

Parameters a and b for each of the b-distributions are based upon prevalence studies (see **Supplementary Table 1**). In the selection process, studies were prioritized based on their recentness, number of samples and location. Studies conducted in temperate, high-income regions were given priority.

TABLE 1 | Microorganism-specific model input parameters in QMRAcatch.

Parameter	Dimension	Details	Distribution type	Microorganism	Value	References
Concentrations of feces C_f (mean, 95^{th} percentile)	N/g	Deer sources	Gamma	*E. coli*	(3, 16) × 10^7	Farnleitner et al., 2010
				Human MST	(3, 11) × 10^3	Farnleitner et al., 2014
				Ruminant MST	(2, 6) × 10^9	Farnleitner et al., 2014
				Pig MST	(3, 20) × 10^3	Farnleitner et al., 2014
				Duck MST	(7, 52) × 10^3	Farnleitner et al., 2014
				C. bovis + *C. ryanae*	103, 225	Garcia-Presedo et al., 2013
				Giardia duodenalis A-II	89, 320	Garcia-Presedo et al., 2013
		Pig sources	Gamma	*E. coli*	(0.04, 2.5) × 10^8	Frick et al., 2018
				Human MST	(7, 33) × 10^3	Farnleitner et al., 2014
				Ruminant MST	0, 0	Farnleitner et al., 2014
				Pig MST	(2, 10) × 10^{10}	Farnleitner et al., 2014
				Duck MST	0, 0	Farnleitner et al., 2014
				Cryptosporidium	70, 133	Castro-Hermida et al., 2011
				Giardia	8, 10	Castro-Hermida et al., 2011
		Bird sources	Gamma	*E. coli*	(0.001, 3.2) × 10^8	Frick et al., 2018
				Human MST	(2, 6) × 10^3	Farnleitner et al., 2014
				Ruminant MST	(1, 9) × 10^3	Farnleitner et al., 2014
				Pig MST	(8, 30) × 10^4	Farnleitner et al., 2014
				Duck MST	(1.3, 4.5) × 10^7	Farnleitner et al., 2014
				Cryptosporidium parvum	288, 686	for Canada migratory geese (Graczyk et al., 1998)
				Giardia	405, 786	for Canada migratory geese (Graczyk et al., 1998)
a_0, a_1	log_{10} day, log_{10} day/ °C	Parameters to describe first order decay in water as function of water temperature	Constant	Human MST marker	0.78, −0.03	This study (best fit)
				Ruminant MST marker	0.79, −0.037	This study (best fit)
				Pig MST marker	1.7, −0.039	This study (best fit)
				Bird MST marker	1.85, −0.039	This study (best fit)
				E. coli	1.04, −0.017	Franz et al., 2014
				Cryptosporidium	3.3, −0.076	Ives et al., 2007
				Giardia	2.16, −0.07	de Regnier et al., 1989
μ_f minimum, maximum	1/d	first order decay in feces in all animal sources based on reported values in bovine feces	Uniform	Ruminant MST marker	−0.13, −0.11	Oladeinde et al., 2014
				E. coli	−0.3, +0.1	Oladeinde et al., 2014
				Cryptosporidium	−0.05, −0.03	Olson et al., 1999
				Giardia	−0.38, −0.11	Olson et al., 1999
a, β	1/mm, -	release parameters		All microorganisms	0.1, 2.0	Bradford and Schijven, 2002; Guber et al., 2015
Dose-response model parameters						
α, β	-	hypergeometric		*Cryptosporidium*	0.3, 1.1	Schijven et al., 2011, 2015
r	-	exponential		*Giardia*	0.02	Regli et al., 1991

Decay rate coefficients in fresh water and in feces (Equations 2 and 9), release parameters (Equation 4), and dose-response parameters α and β (Equation 13).

For each infected animal, the microbial particle numbers shed per hour were determined by multiplying the mass of feces per dropping m_f (kg, normally distributed), the number of droppings per hour per animal (Poisson distributed) and the microbial concentration (**Tables 1**, **2**). The microbial concentration in feces is described by a gamma distribution based on mean and 95th percentile values reported in the literature (**Table 1**). Fecal deposition only takes place in the non-flooded area of the floodplain.

Transport in non-flooded area

Rainfall-induced microbial particle release was modeled iteratively using the function for the release of *Cryptosporidium*

TABLE 2 | Input parameter settings for the animal sources in QMRAcatch.

Parameter	Unit	Value	References	Value	References	Value	References
		Deer		*Wild boar*		*Birds*	
Population size	N	240	Vierheilig et al., 2013; Böhm, 2016	200	Vierheilig et al., 2013, MA 49, personal communication	2,500	Frühauf and Sabathy, 2006a,b; Parz-Gollner, 2006; Schulze and Schütz, 2013
Weight (Mean, 95%)	g	15,30	von Oheimb et al., 2005	10, 20	Schmidt et al., 2004	0.5, 1	Hahn et al., 2007
Defecation rate	1/h/animal	0.63	von Oheimb et al., 2005	0.2	Schmidt et al., 2004	2.1	Hahn et al., 2007

and *Giardia* from dairy cattle manure of Bradford and Schijven (2002). The release rate is given by:

$$\omega(t) = aP(t)[1 + a\beta P\,(t)\,t_{rain}\,(t)]^{-(1+\frac{1}{\beta})} \quad (4)$$

according to Guber et al. (2015), where P (mm/h) is the amount of rainfall at time step t, t_{rain} is the time passed since the start of the rainfall event, a (1/mm) controls the initial release rate, and β (–) determines the shape of the release curve (**Table 1**). Release from the deposits occurs in the non-flooded area (A_{dep} [m^2]). First, the available number of microbial particles in animal fecal deposits N_{deptot} is determined from the newly deposited numbers (N_{dep}) plus the residual deposits of the previous time step (N_{depres}, Equation 9):

$$N_{deptot}\,(t) = N_{dep}\,(t) + N_{depres}(t-1)\triangle A_{dep}(t) \quad (5)$$

where

$$\triangle A_{dep}(t) \;=\; Min[1, A_{dep}(t)/A_{dep}(t-1)]. \quad (6)$$

If the size of the non-flooded area decreases, $\triangle A_{dep}$ is smaller than one, otherwise it is one. The number of microbial particles that are released in the non-flooded area are then calculated from the total number of microbial particles in animal fecal deposits (N_{deptot}):

$$N_{rel,nonflooded}(t) = \begin{cases} P_Q = 0 \rightarrow 0 \\ P_Q > 0 \;\rightarrow \omega N_{deptot}\,(t) \cdot \exp[\mu_w\,(t)] \end{cases} \quad (7)$$

where μ_w is determined according to Equation 2. We calculated the surface water runoff volume P_Q [m^3/h] as function of precipitation (P, including rain water and snow melt), evaporation and soil moisture processes according to Blöschl et al. (2008), Equations 1 – 4. To calculate the changes in soil moisture we used Equations 5, 7 and 8 given by Blöschl et al. (2008), with parameter settings according to Demeter et al. (2021) for the study site (**Supplementary Table 3**).

Transport in flooded area

Animal fecal deposits are completely resuspended in floodwater. The number of resuspended microbial particles, $N_{rel,flooded}$, is:

$$N_{rel,flooded}(t) = \begin{cases} \triangle A_{dep} = 1 \rightarrow 0 \\ \triangle A_{dep} \;< 1 \;\rightarrow N_{depres} \cdot (1 - \triangle A_{dep}) \cdot \exp[\mu_w\,(t)] \end{cases} \quad (8)$$

where μ_w and $\triangle A_{dep}$ are determined according to Equations (2) and (6). The number of released microbial particles is then subtracted from the total deposited numbers and reduced by first-order decay in feces, μ_f [1/h]:

$$N_{depres}\,(t) = [N_{deptot}\,(t) - \omega(t)N_{deptot}\,(t)] \cdot \exp[\mu_f\,(t)] \quad (9)$$

For μ_f, we took ranges of reported values for bovine feces as the boundaries of a uniform distribution according to Wu et al. (2020). This distribution was assumed to evenly represent the varying μ_f values with environmental conditions and time (**Table 1**). The number of microbial particles running off to the backwater channel in non-flooded and flooded areas are then added:

$$N_{vro}(t) = N_{rel,nonflooded}(t) + N_{rel,flooded}(t) \quad (10)$$

The numbers of microbial particles from each animal group are summed. The microbial particle concentrations in the backwater channel are then calculated:

$$C_{bw}\,(t) = \begin{cases} V_{fl}(t) + P_Q(t) > 0 \rightarrow \frac{C_{r\rightarrow bw}(t)\cdot V_{fl}(t) + N_{vro}(t)}{V_{fl}(t) + P_Q(t)} \\ V_{fl}(t) + P_Q(t) = 0 \;\rightarrow C_{r\rightarrow bw}\,(t) \end{cases} \quad (11)$$

where V_{fl} [m^3] is determined by means of regression (Section "Hydrological and Hydrodynamic Flow Situation," **Supplementary Table 2**).

Hydrological and Hydrodynamic Flow Situation

We selected the years 2010–2015 as study period. The lowest Danube discharges in this period occurred in 2011 (Q_{95}: 2400 m^3/s), and the highest discharges in 2013 (Q_{95}: 4100 m^3/s, **Figure 3**). Hourly discharge data of the Danube were available at gauge Wildungsmauer, which is located 12 km downstream of the study site. The annual precipitation was 452 mm in 2011 and 659 mm in 2013. Hourly precipitation data (mm/h) and air temperature was available at station Groß Enzersdorf, located at 6 km distance from the Danube along the upstream model boundary, **Figure 1**), the latter ranging from −20°C to 38°C (mean: 11.2°C, standard deviation: 8.9°C). The water temperature of the Danube (gauge Greifenstein, 41 km upstream of the study area) ranged from 0 to 23°C (mean: 11.2°C, standard deviation: 5.9°C).

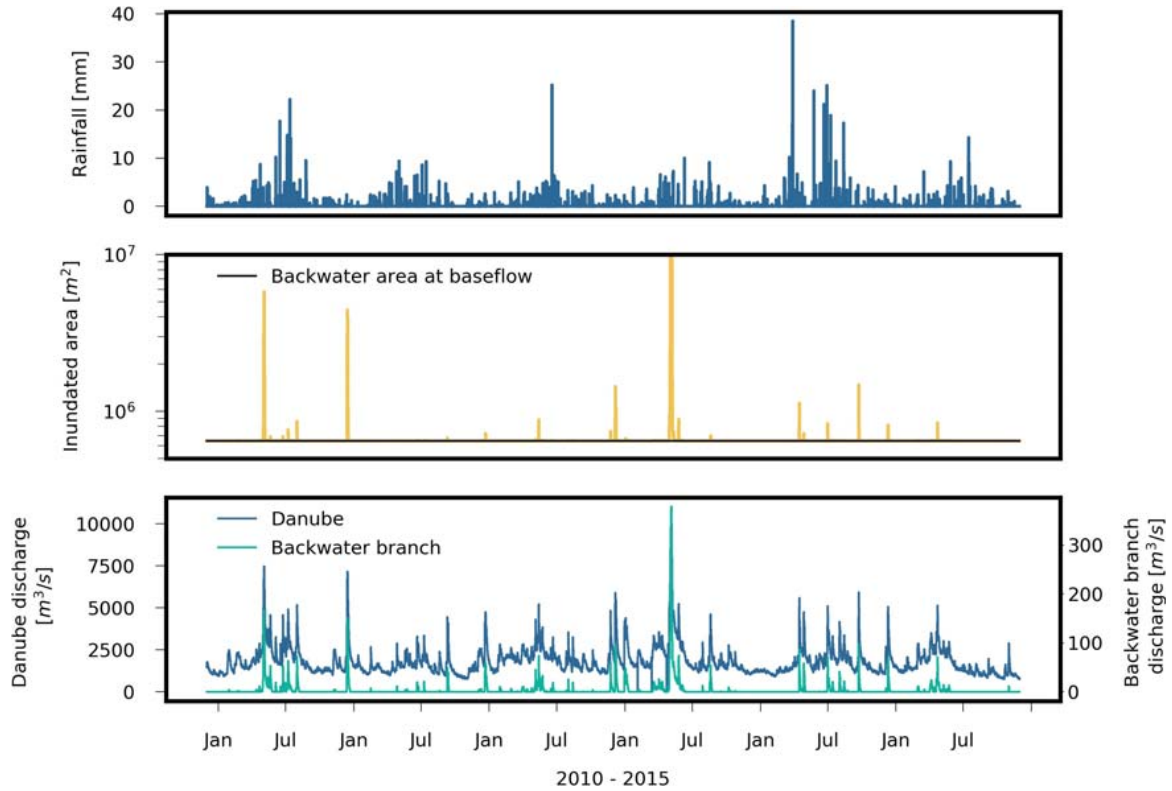


FIGURE 3 | Observed hourly rainfall and Danube discharge, and, simulated hourly discharge of the backwater channel and inundated area by means of regression (**Supplementary Table 2**) during the investigation period.

A 2-D hydrodynamic surface water model (CCHE2D Version 2.0, National Center for Computational Hydroscience and Engineering, University of Mississippi) was used to simulate the flow velocities and water levels of the backwater channel on an hourly basis during a flood event in January 2011 with an approximate 10-year return period. The model is described in detail by Gabriel et al. (2014) and Frick et al. (2020). In short, the model solves the two-dimensional formulation of the shallow water equations and uses depth integrated Reynolds equations. 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 km^2 (**Figure 1**). The spatial distribution of the Manning roughness values were based on a detailed land use and vegetation classification. During model calibration the Manning roughness values were fine-adjusted to floods of the River Danube during August 2008 and June 2009, ranging from 0.024 to 0.125 $s \cdot m^{-0.3}$ within the model domain. The Nash and Sutcliffe coefficient of runoff model efficiency (Nash and Sutcliffe, 1970) at several water level gauges along the backwater branch from the inlet point of the Danube to LSW 3 (**Figure 1**) ranged from 0.92 to 0.98 for both calibration periods, and from 0.93 to 0.96 for the validation period during the flood event in January 2011.

The transient water quantities were determined by means of polynomial regression based on the hydrodynamic flow simulations during the rising limb of the flood event (**Supplementary Table 2**). The following variables were calculated and served as input to the microbial fate and transport model: hourly discharges and volumes of the backwater channel, and hourly volumes and areas of inundation (see **Supplementary Section** "Detailed Model Information" for more details). The shortest and longest period when the Danube discharged into the backwater channel were 16 days in 2011, and 91 days in 2013, respectively.

Microbiological Source Characterization

Microbial analyses of surface water

Surface water samples were collected monthly from 2010 to 2015. Samples were collected from one point at the Danube (DSW5), and from points LSW 1 and 3 along the backwater channel (**Figure 1**). The sampling location LSW1 is situated in a lateral branch outside the flood-protected area delineated by the dam and therefore represents waterbodies with high connectivity to the Danube River. The location LSW3 represents waterbodies along the main backwater channel, with an average water depth of ca. 170 cm. The MST markers were quantified in 500–600 mL water samples using quantitative PCR. The human marker HF183/BacR287 (Green et al., 2014), the ruminant marker BacR (Reischer et al., 2006), the pig marker Pig2Bac (Mieszkin et al., 2009), and the bird marker DuckBac (Kobayashi et al., 2013) were selected and applied as described

previously (Kirschner et al., 2017). As a robust approximation for the SLOD (sample limit of detection), 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 L (ME/L) to account for potential extraction losses (Reischer et al., 2007, 2008). The TOD covers sampling and sample processing information and also the efficiency of qPCR analysis. The mean TODs during the calibration and validation periods were 564 and 382 ME/L for the human, 419 and 327 ME/L for the ruminant, 490 and 337 ME/L for the pig, and 419 and 327 ME/L for the bird MST marker. The samples were analyzed for *E. coli* according to ISO 16649-1 (ISO, 2001) with a limit of detection (LOD) of 1 CFU/100 mL. Additional surface water samples were collected monthly at the Danube 23 km upstream of DSW 5 from June 2018 to August 2020 and analyzed for *Giardia* and *Cryptosporidium* (oo)cysts. *Giardia* spp. and *Cryptosporidium* spp. (oo)cysts were isolated from 10-L water samples, using an adaptation of the flat membrane method described in ISO (2006). Parasites were recovered from the filters and further analyzed as described in Demeter et al. (2021) using 50 mL of 1M glycine pH 5.5 solution and centrifuged at 1,550 × g for 15 min. Pellets were resuspended in 2 mL of ultrapure water. One mL of the suspension was used for the immunomagnetic separation of the parasites using the Dynabeads GC Combo kit (Thermo Fisher, United Kingdom). Concentrates were stained with the EasyStain kit (BTF Pty. Ltd., Biomerieux, Australia) and quantified as described by Stevenson et al. (2015). The LOD of *Giardia* and *Cryptosporidium* in surface waters was 0.4 (oo)cysts/L.

Microbial concentrations in animal feces

Samples of fecal matter of ruminants (hunted herbivores), wild boar, avian fecal matter from great cormorant (*Phalacrocorax carbo*), wild duck (*Anas platyrhynchos*) and other *Anatidae*, common tern (*Sterna hirundo*), and *Charadriiformes* were previously collected and analyzed for MST markers and *E. coli* in the study area (Vierheilig et al., 2013; Farnleitner et al., 2014; Frick et al., 2018). Marker concentrations associated with human (n = 19), ruminant (n = 20), porcine (n = 18), and bird fecal pollution (n = 11) from these samples were determined via qPCR (Farnleitner et al., 2014; **Table 1**). For *Cryptosporidium* and *Giardia*, reported values were used (**Table 1**).

Data analysis

The fecal indicator and pathogen concentrations in the Danube, C_r, used in Equation 1, were described by selected statistical distributions (**Table 3**). The parameters of the distributions were obtained from fits to the observed dataset at point DSW 5 (**Figure 1**). We performed Kruskal–Wallis tests for the selection of the distribution types ($p > 0.05$, **Table 3**). During all model simulations, random values were drawn from the distributions for each time step and Monte Carlo run. A substantial fraction of the measured microbial concentrations were left-censored values (i.e., 20, 60, 76, 30% for the human, ruminant, pig, and bird MST markers, and, 32 and 39 % for *Giardia* and *Cryptosporidium*), meaning that the concentration was known only to be lower than the LOD (*Giardia* and *Cryptosporidium*) or the TOD (MST markers). Non-detects (ND) were replaced by half of the TOD in case of the MST markers and by half of the LOD in case of *Cryptosporidium* and *Giardia*. This continues to be the most common procedure within the disciplines of environmental sciences to deal with non-detects (Helsel, 2006). Concerning the MST data, we used these in the calibration process. Since we treated both simulated and observed values the same way (i.e., observed non-detects and simulated values < TOD were both replaced by TOD/2), the chosen performance metrics are not affected, leading to the best calibration possible. Concerning the *Giardia* and *Cryptosporidium* data, we compared different levels for the substitution, i.e., substitution by zero, LOD/2 and LOD. It was shown that the choice of the level did not affect the results of the Kruskal–Wallis tests ($p > 0.05$), so the method was justifiable in our case. For modeling the concentrations of *Giardia* and *Cryptosporidium* in the backwater branch and the QMRA, we merely used the data in the Danube as boundary condition. We did not use the data in the backwater river for this purpose, where the concentrations would be lower than the LOD in most cases. For the data analysis, Python 3.7 and Scipy package 1.3.1 were used.

Quantitative Microbial Risk Assessment Module

Exposure to the pathogens is given as the dose D [L/d], the number of ingested pathogens per person per day. For calculating D, the Monte Carlo samples of pathogen concentrations in the backwater branch (C_{bw} [particles/L]), recovery (R, [-]), pathogen treatment reduction (log reduction value, LRV), and consumption data (V, [L]) are multiplied according to Equation 12. Data on times of consumption and consumed volumes of unboiled drinking water per person (V_i in equation 12) during a day were available from the Dutch National Food Consumption Survey 2007–2010 (DNFCS) (Van Rossum et al., 2011). The cumulative dose per person per day is:

$$D = \sum_{i=1}^{d} \left(C_{bw,i} \times \frac{1}{R} \times 10^{LRV} \times V_i \right) \qquad (12)$$

where i denotes the hourly time step. Recovery rates were determined in the laboratory, resulting in mean values of 0.65 for *Giardia* (standard deviation: 0.28, n = 17) and 0.53 for *Cryptosporidium* (standard deviation: 0.27, n = 8). Beta distributions were fitted to the recovery data (α: 0.87, β: 0.53 for *Cryptosporidium* and α: 1.03, β: 0.44 for *Giardia*). Daily probabilities of infection for *Cryptosporidium* can be estimated

TABLE 3 | Observed values and descriptive statistics for the microbial concentrations of the Danube from 2010 to 2015 (C_r in Equation 1) and the Kruskal–Wallis test results showing that the simulated and observed values were not significantly different ($p \geq 0.05$).

Parameter	Observed dataset detected/n	Observed median (minimum, maximum) [particles/L]	Distribution	Descriptive statistical parameter values gamma distribution: shape/location/scale; normal distribution: mean/standard deviation	Kruskal–Wallis *p*	Comment
Human MST marker	50/62	5.39×10^3 (60, 1.41×10^6)	Gamma	0.23 / 58 / 80,000	0.05	After model optimization based on OF (Equation 14)
Ruminant MST marker	21/60	250 (60, 4.54×10^4)	Gamma	0.23 / 58.5 / 10,766	0.14	
Pig MST marker	14/60	220 (60, 1.58×10^5)	Gamma	0.25 / 58.5 / 3,700	0.11	
Bird MST marker	16/23	1.21×10^4 (60, 2.19×10^5)	Gamma	0.45 / 58 / 20,000	0.05	
E. coli	64/64	570 (20, 4.9×10^4)	Gamma	0.19 / 100 / 27,000	0.33	
Giardia	21/31	0.8 (0.2, 4.4)	Normal	1.39 / 1.17	0.45	stats fit function in Python 3.7
Cryptosporidium	19/31	0.8 (0.2, 6.44)	Normal	1.29 / 1.42	0.40	

Non-detects were replaced by half of the TOD (MST markers) or half of the LOD (E. coli, Giardia, and Cryptosporidium).

using a hypergeometric dose–response relation (Teunis and Havelaar, 2000):

$$P_{inf} = 1 - {}_1F_1(\alpha, \alpha + \beta, D) \quad (13)$$

where α and β are infectivity parameters that are pathogen-specific and ${}_1F_1$ is the confluent hypergeometric function. The dose-response model parameters for *Cryptosporidium* and *Giardia* were taken from the literature (**Table 1**). As daily health based target (hbt), $1 \cdot 10^{-6}$ infections/person/d was adopted in this study (Signor and Ashbolt, 2009). LRV was estimated iteratively until the criterion $P_{inf} \leq$ hbt according to Equation (13) was fulfilled for both the mean and 95^{th} percentile values of P_{inf}.

Validation of the Microbial Fate and Transport Model

To prove that the model captured the most relevant fecal sources and transport processes, we evaluated the model performance based on monthly measured concentrations of the human and animal MST markers and *E. coli* during 2010–2015. We selected the observation dates, when Danube discharged into the wetland area, or when rainfall occurred. The mean absolute error (MAE) was used as a performance metric (Willmott and Matsuura, 2005; Demeter et al., 2021). Log_{10} transformed concentrations were used in the MAE computations because microorganisms typically follow a lognormal distribution and the use of logarithms minimizes the influence of outliers present in the data (Hong et al., 2018; Demeter et al., 2021). The Kruskal–Wallis test was used for the distribution comparisons of the simulated and observed datasets in the backwater channel and the p-value of the Kruskal–Wallis statistic was a metric of model performance. In order to ensure an optimum model performance, the optimization parameters were adjusted to minimize the objective function (OF) (Demeter et al., 2021).

$$OF = MAE + (1 - p) \quad (14)$$

Non-detects (ND) in the observed dataset and simulated values below that level were set to the half of the threshold of detection (for the MST markers) in the calculations. The optimization parameters were the distribution parameters describing the microbial concentrations in the Danube and the microorganism-specific decay rate parameters (C_r in Equation 1, a_0 and a_1 in Equation 2). The former were adjusted while ensuring that the Krukal-Wallis p-value was greater than 0.05 (**Table 3**). Decay rates of the MST markers were collected from a literature survey and summarized in **Supplementary Table 4**. An ordinary least square method was used to fit the time-to-first-log (TFL) as a function of water temperature (dashed lines in **Figure 4**), using the Python 3.7 package statsmodels (0.10.1). During the adjustment of intercept a_0 (used in Equation 2, solid lines in **Figure 4**), it was ensured that the decay as function of temperature obtained lay within the prediction interval of the ordinary least square regressions (**Figure 4**, shaded area). The Bradford-Schijven release parameter a and β were kept the same for all animal sources, as their effects on the simulated concentrations was small in comparison with the optimization parameters (**Table 1**). We performed a stepwise, source-targeted model optimization of QMRAcatch:

- The model was validated, considering individual, presumably important fecal sources using measured concentrations of the respective MST markers. We simulated concentrations of the human MST marker in the backwater channel and compared them with the measured dataset on days when the Danube discharged into the backwater channel, i.e., during floods. We selected the data during days when the Danube discharged into the backwater channel (Section "Study Area," **Figure 1**). The distribution parameters describing the human-associated MST marker concentrations in the Danube and the decay rate coefficient a_0 were adjusted to minimize OF (Equation 14). The same procedure was applied consecutively for the ruminant, pig, and bird associated MST markers, except

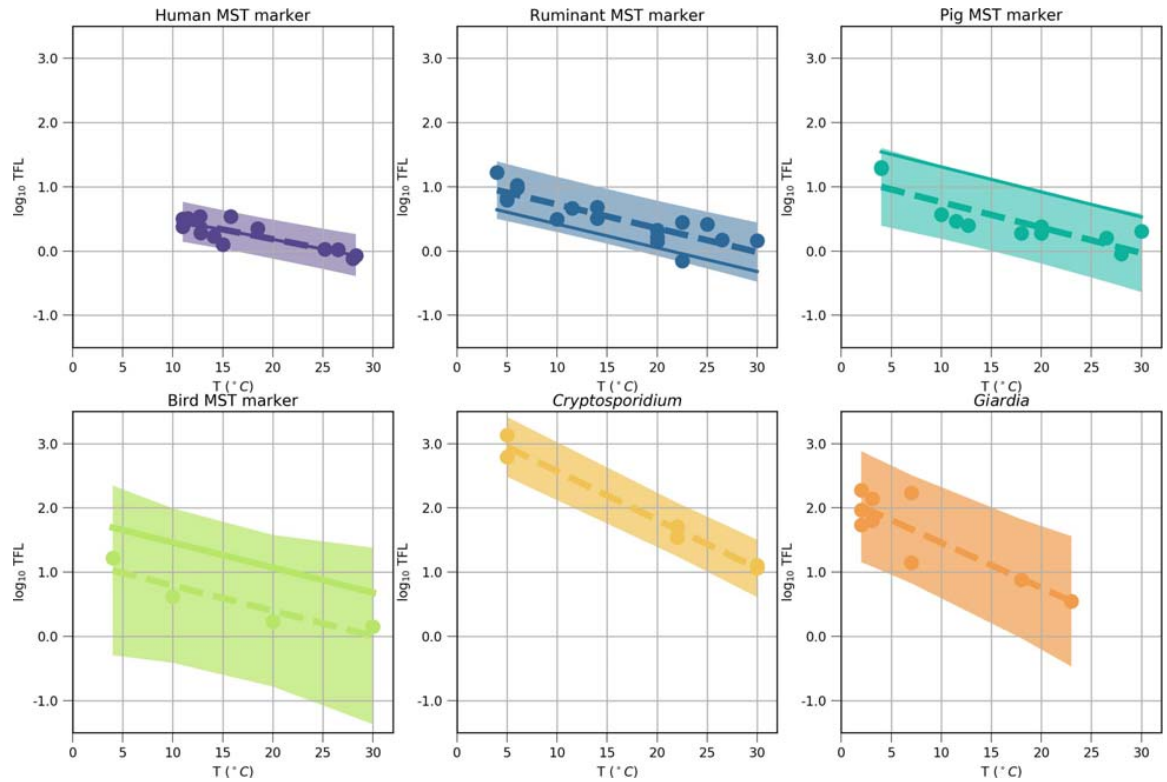


FIGURE 4 | Inactivation of the MST markers, *Cryptosporidium*, and *Giardia* after model optimization (solid lines) and as reported in experimental studies (dots, **Supplementary Table 4**), plotted as time to first $\log_{10}$ reduction (TFL, days) values $\log_{10}$-transformed ($\log_{10}$ (TFL) in function of the temperature. Ordinary-least-square regressions (dashed lines) were fitted to the literature values, shown with their 95% prediction intervals (shaded). The intercept and the slope of the solid lines were the result of the model optimization. These values were used as model input parameters a_0 and a_1 in Equation 2 (**Table 1**).

that animal fecal deposits were additionally considered as microbiological sources (**Table 1**). As for the human MST marker, we used the data collected during floods. In addition, we used data collected on days, when the backwater area was partially inundated or when it was raining (Section "Study Area," **Figure 1**). The observation sites were selected based on the findings of Frick et al. (2020) who conducted a comprehensive analysis of the spatial distribution of human and animal fecal pollution in the study area. To validate the model during floods, we selected the site, which was influenced by floods (LSW 1). To validate the model during days of rainfall or inundation of the area, we used the site, which was impacted by wildlife (LSW 3, **Figure 1**).

- In the second step, the model was validated using measured concentrations of *E. coli*. The same procedure was applied as for the MST markers, except that we considered all fecal sources combined and the decay rate coefficients were not adjusted but taken from the literature (**Table 1**).

Scenario Load and Infection Risk Assessment

We defined the following event-driven scenarios for quantifying the effects of fecal sources on the microbiological quality of the backwater channel considering safe drinking water:

- As allochthonous source, we considered *Cryptosporidium* and *Giardia* transport via Danube discharges into the backwater channel (scenario FLOODS).
- As autochthonous sources, we considered the resuspension of *Cryptosporidium* and *Giardia* from fecal deposits in inundated areas (scenario RESUSP), and the rainfall-release and runoff of *Cryptosporidium* and *Giardia* from fecal deposits (scenario RAIN).
- All of the above scenarios were considered simultaneously (scenario all combined).

Using QMRAcatch, we simulated the concentrations and loads of *Cryptosporidium* and *Giardia* in the backwater channel, and the drinking water infection risks relative to a health based target. We considered different hydrological conditions and fecal sources in the scenarios, as indicated in **Table 4**. To simulate no connection of Danube and backwater (**Table 4**), the Danube discharge was set to the mean flow rate, consequently there was no discharge into the backwater channel. All other parameter settings were taken from **Tables 1–3** and **Supplementary Material**.

TABLE 4 | Fecal sources and hydrological conditions in the event-driven scenarios investigating (i) the wastewater-impacted river water entering the backwater during floods (FLOODS), (ii) the resuspension of pathogens from fecal deposits in inundated areas (RESUSP), and (iii) the pathogen release and runoff from fecal deposits (RAIN), and (iv) all combined.

	Wastewater impacted river water (FLOODS)	Resuspension of pathogens from fecal deposits in inundated areas (RESUSP)	Pathogen release and runoff from fecal deposits (RAIN)	All combined
Hydrological conditions				
Rain	–	–	+	+
Connection between Danube and backwater	+	+	–	+
Fecal pollution sources				
River	+	–	–	+
Ruminants	–	+	+	+
Wild boar	–	+	+	+
Birds	–	+	+	+

+ taken into account, – not applied.

RESULTS

In order to test if we considered the most relevant fecal sources and transport pathways, we tested the applicability of the human and selected animal MST markers (Section "Applicability of the MST Marker Specificity for Modeling"), and validated the model based on measured concentrations of these MST markers and *E. coli* in the backwater channel (Section "Performance of the Microbial Fate and Transport Model"). We then simulated the *Giardia* and *Cryptosporidium* concentrations, loads and drinking water infection risks relative to a health-based benchmark for the defined fecal pollution scenarios and reference pathogens (Section "Contribution of Fecal Sources to the Reference Pathogen Impact on the Backwater Resource Considering Safe Drinking Water Production).

Applicability of the MST Marker Specificity for Modeling

The selected human, ruminant, pig and bird MST marker assays (Section "Microbiological Source Characterization") are primarily associated with their respective target sources. However, low numbers may also occur in the non-target pollution sources. According to the analysis of fecal samples, the reported MST marker concentrations in the non-target pollution sources were more than five orders of magnitude lower than those in the target pollution sources (**Table 1**). Nevertheless, the impact on false-positive MST marker detection rates in the backwater area may become significant, when a large non-target animal population is the source. To evaluate the applicability of the MST markers, we investigated the impact on the simulated concentrations from non-target animal sources in the floodplain river with QMRAcatch. The concentrations of each MST marker in the backwater channel were described by a gamma distribution based on the reported mean and 95^{th} percentile fecal source concentrations according to **Table 1**, and considering (i) both target (i.e., correct positive detections) and non-target host groups (i.e., false positive detections) and (ii) only the target group. The parameter settings were used according to **Tables 1–3** in the simulations.

For each measured MST marker, the simulated mean and 95^{th} percentile concentrations in the backwater branch during the simulation period were compared for the two cases. For all MST markers, the simulated concentrations considering both target and non-target groups differed by 0 - 5 % from the simulated concentrations considering only the target group. This means that at least 95 % of the simulated concentrations in the floodplain river were associated with the target pollution sources in the catchment. The animal and human associated qPCR assays and the measured concentrations at our study site were thus considered to be useful for a source–targeted evaluation of the microbial fate and transport model.

TABLE 5 | Model performance based on the observed microbial concentrations in 2010–2015 during days when the Danube discharged into the backwater branch and when rainfall occurred.

Parameter	Observed dataset	Observed median (minimum, maximum)	OF (Equation 14)	Mean absolute error	Kruskal–Wallis test
	Detected/n	[particles/L]		[$\log_{10}$ particles/L]	p
Human MST marker	9/10	4.1×10^3 (640, 9.3×10^4)	1.34	0.67	0.33
Ruminant MST marker	10/16	708 (56, 1.64×10^4)	0.70	0.61	0.91
Pig MST marker	6/15	231 (65, 1.29×10^4)	1.17	0.50	0.33
Bird MST marker	14/16	7.04×10^3 (89, 8.37×10^4)	1.53	0.69	0.16
E. coli	16/16	220 (10, 7.0×10^3)	1.19	0.62	0.44

Objective function values (OF), mean absolute error and Kruskal–Wallis p-values after comparison of the simulated and observed microbial concentrations in the backwater branch. Non-detects were replaced by half of the TOD (MST markers) or LOD (E. coli). Simulated values below the half of the LOD or the lowest TOD were set to the half of the LOD or lowest TOD.

Performance of the Microbial Fate and Transport Model

We validated the model based on measured concentrations of human-, ruminant-, pig-, bird-associated MST marker and *E. coli* in the backwater branch. The model validation resulted in mean absolute errors ranging from 0.5 to 0.7 $\log_{10}$ for the MST markers and *E. coli* (**Table 5**). The objective function values ranged from 0.7 to 1.5 (OF in Equation 14). The cumulative distribution plot of the simulated and observed concentrations confirmed the general good agreement (**Figure 5**). The majority (70–90 %) of the simulated concentrations of all fecal indicators resulted in errors ranging from −1.0 to 1.0 $\log_{10}$ particles/L after model optimization (**Figure 6**).

Contribution of Fecal Sources to the Reference Pathogen Impact on the Backwater Resource Considering Safe Drinking Water Production

For the scenarios, we evaluated the simulated concentrations of *Cryptosporidium* and *Giardia* during time steps when

- The Danube discharged into the backwater branch (for the FLOODS scenario),
- Part of the backwater area was inundated (for the RESUSP scenario),
- Rainfall occurred (for the RAIN scenario), and
- All of the above combined.

A more detailed definition of how we defined these events is given in the **Supplementary Information Section** "Detailed Definition of the Events for the Scenarios." The FLOODS and RAIN scenarios and all scenarios combined resulted in similar ranges of concentrations of *Cryptosporidium* and *Giardia* in the backwater branch (0.4–1.2 particles/L for the mean and 1.8–5.1 particles/L for the 95th percentiles, **Figure 7**). The concentrations were more than one $\log_{10}$ lower for the RESUSP scenario. The concentrations were the same for *Giardia* and *Cryptosporidium* for the FLOODS scenario, while they were 70–90 % smaller for *Giardia* than for *Cryptosporidium* for the RAIN and RESUSP scenario due the higher inactivation.

We further conducted a source apportionment by calculating the mean pathogen loads of *Cryptosporidium* and *Giardia* in

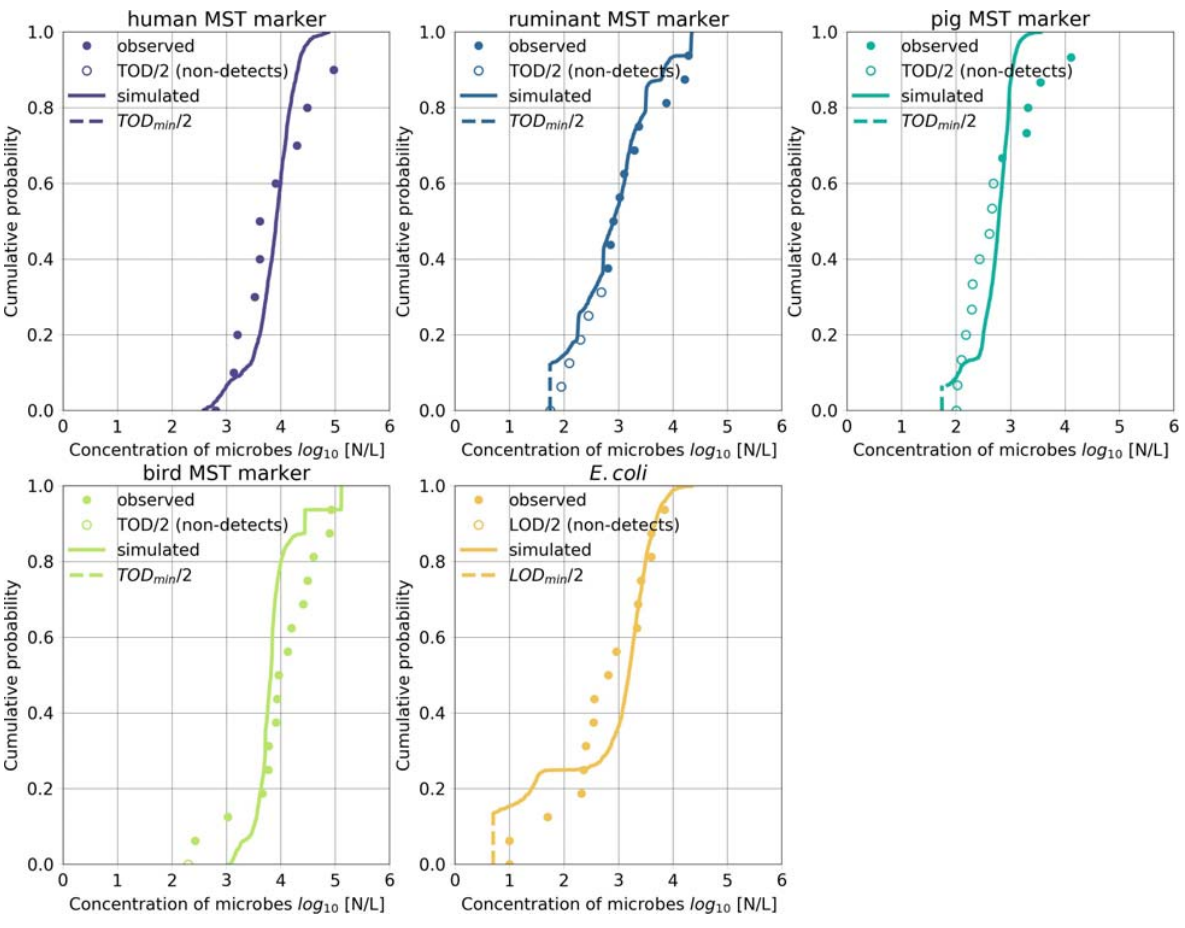


FIGURE 5 | Microbial fate and transport model performance. Simulated and observed concentrations of the host-associated MST markers and of *E. coli* during days when Danube water discharged into the backwater branch or on rainy days. Non-detects were replaced by half of the TOD (MST markers) or LOD (*E. coli*). Simulated values below the LOD or the lowest TOD (TOD_{min}) were set to half of the LOD or the TOD_{min}.

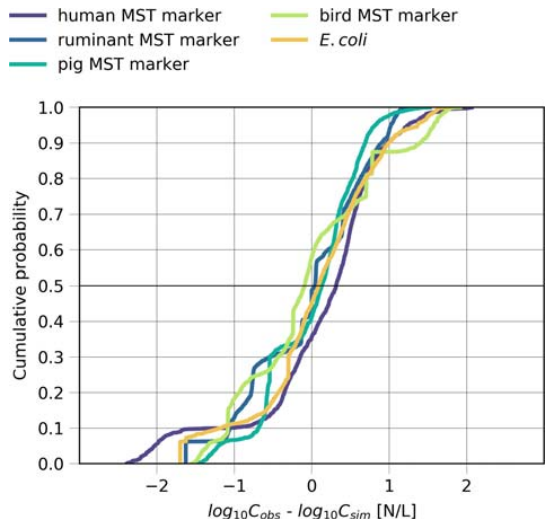


FIGURE 6 | Model performance for the source-targeted microbial fate and transport. Cumulative distribution plot of the difference between the simulated and measured concentrations (log_{10}-transformed) of the human, ruminant, pig and bird-associated MST markers and *E. coli* during days when Danube water discharged into the backwater branch or on rainy days.

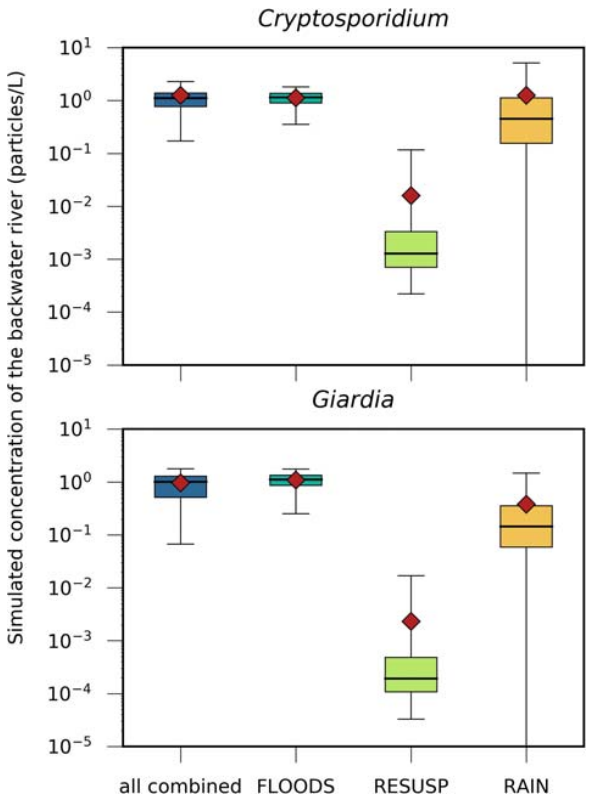


FIGURE 7 | Simulated concentrations of *Cryptosporidium* and *Giardia*, transported via (i) the discharge of Danube water (FLOODS), (ii) the resuspension of pathogens from fecal deposits in inundated areas (RESUSP), (iii) the rainfall-release and runoff from fecal deposits (RAIN), and all scenarios combined. Black horizontal lines, red diamonds, and whiskers mark the median, mean, and 95th percentile values, respectively.

the backwater branch. For that, we multiplied the simulated hourly concentrations by the hourly discharges and evaluated the mean loads for the selected time steps (Section "Scenario Load and Infection Risk Assessment"). The simulated mean loads of *Cryptosporidium* and *Giardia* were again in a similar range for the FLOODS and RAIN scenarios and all scenarios combined (3–13 $\times$ 10^9 particles/h), and were at least one log_{10} lower for the RESUSP scenario. The FLOODS scenario occurred during 20 % of the 6-year time period (**Figure 8**). The RAIN scenario, which occurred only during 7 % of the time, resulted in higher standard deviations and peaks of loads than the FLOODS scenario in case of *Cryptosporidium*. The RESUSP scenario resulted in the smallest source attribution in comparison, occurring during 8 % of the time.

The drinking water infection risks relative to a health based target of $\leq 1 \cdot 10^{-6}$ infections/person/d were estimated assuming a value of 6.2 and 6.0 as treatment reduction of *Cryptosporidium* and *Giardia* from backwater river water (LRV, Equation 13). The mean drinking water infection risks for *Cryptosporidium* and *Giardia* resulted in values one log_{10} below to close to the health based target for the FLOODS and RAIN scenarios, and all scenarios combined. For the RESUSP scenarios, the mean drinking water infection risks were $>$ 3 log_{10} below the health based target (**Figure 9**).

DISCUSSION

Strengths and Limitations of the Integrative Approach

In this study, we presented a new integrative modeling approach for evaluating the impact of fecal sources and transport pathways on the microbiological quality of a riverine wetland considering safe drinking water. By integrating measured concentrations of human and the most relevant animal MST markers, the approach allowed for the first time quantifying the relative drinking water infection risks from external (allochthonous, i.e., river water inflows) and internal fecal sources (autochthonous, i.e., wild boar, ruminants, birds in the backwater study area). This would not have been possible based on FIO data alone, which are sum indicators in contrast to MST markers (Zhang et al., 2019). The approach also allowed assessing if a given MST marker is appropriate in the study area, given its fecal specificity and fecal sensitivity. These performance characteristics of MST markers can be highly regional- and site-specific (Reischer et al., 2013). Furthermore the required MST performance criteria depend on the relative abundance of the specific fecal sources to be detected amongst the sum of total fecal pollution occurring at the investigation site, e.g., % fraction of human fecal pollution in relation to the sum of human and animal fecal pollution (Reischer et al., 2011). For example, if the animal numbers were different to our study site, the resulting non-target concentrations of the MST markers could render the selected MST marker assays inapplicable. In this case, other

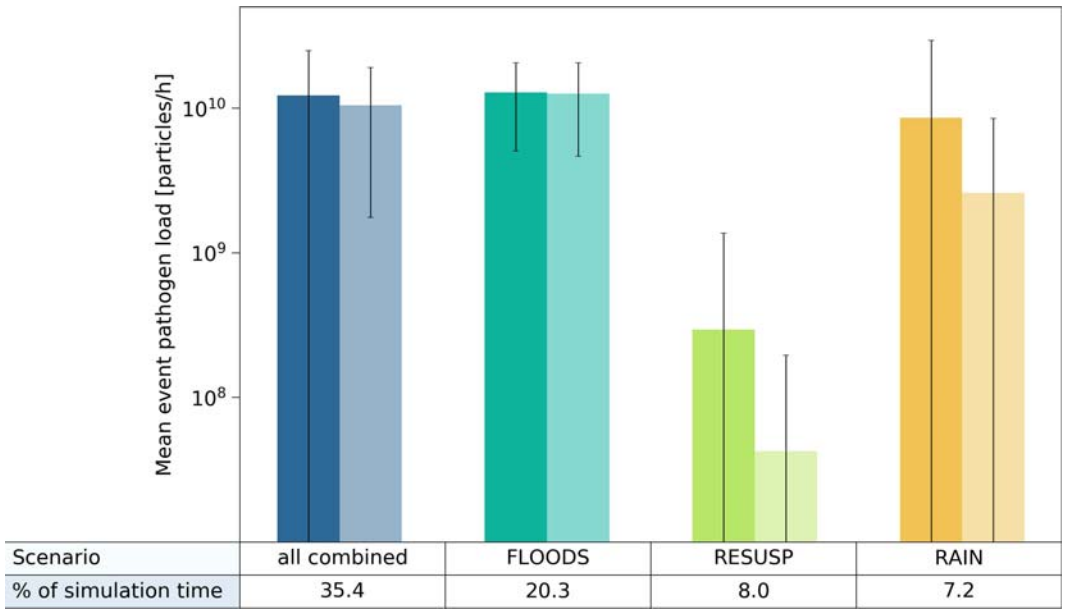


FIGURE 8 | Mean load attribution of *Cryptosporidium* (left columns) and *Giardia* (right columns) via (i) the discharge of Danube water (FLOODS), (ii) the resuspension from fecal deposits in inundated areas (RESUSP), (iii) the rainfall-release and runoff from fecal deposits (RAIN), and all scenarios combined from 2010 to 2015. Whiskers indicate the standard deviations.

markers with appropriate performance characteristics for the specific situation and question have to be chosen. A trade-off between fecal source sensitivity and specificity for MST qPCR assays often exists (Layton et al., 2013; Raith et al., 2013; Mayer et al., 2016). To illustrate this relationship, for recent fecal pollution detection bacterial MST qPCR assays often show high sensitivity but limited specificity as in contrast to many viral qPCR MST assay often showing high source specificity but limited sensitivity (Mayer et al., 2016). As an exemplary test case for the selected human MST marker assay at our study site, we assumed a theoretical increase of the non-target population number of animals by 10 fold, i.e., of ruminants, boar and birds, while leaving the human fecal sources unchanged (Section "Applicability of the MST Marker Specificity for Modeling"). We then simulated the resulting concentration signal of the human MST marker in the backwater river (i.e., the sum of the total qPCR signal from the correct positive (humans) and false negative (animals) DNA targets). The scenarios showed that the selected human MST markers would still be applicable at our study site and for the calibration of the model, even if the animal population number increased drastically by 10-fold (error $< 5\%$, results not shown).

The model was validated in two steps over a 6-year time period, considering (i) individual, presumably important fecal sources using measured concentrations of the respective MST markers, and (ii) all fecal sources combined using measured concentrations of *E. coli*. This was to ascertain that we accounted for the most relevant fecal sources and transport pathways. Interestingly, the measured and simulated MST marker and *E. coli* concentrations were similar, even though other sources of FIO may have potentially contributed (**Figure 5**). Frick et al. (2018) identified poikilothermic animals (earthworms, gastropods, frogs, and fish) as further potential autochthonous reservoirs of bacterial fecal indicators in our study area. To validate the transient concentration changes during floods and rainfall, it would be advantageous to collect microbial data at high temporal resolution. Current advances in online monitoring techniques may provide this opportunity in the near future (Stadler et al., 2019).

In this study, we modified and extended the microbial fate, transport and infection risk model QMRAcatch (v 1.1 python backwater) to simulate rainfall - runoff and mixing with released microbial particles from animal fecal deposits as functions of transient soil moisture processes according to Bradford and Schijven (2002) and Blöschl et al. (2008). The QMRA framework was fine-tuned for making use of the simulated exposure concentrations of *Cryptosporidium* and *Giardia* at hourly time steps based on human drinking water consumption data (Van Rossum et al., 2011). The discharge rates, volumes and surface water areas simulated by a validated hydrodynamic model allowed accounting for the spatiotemporal changes of these hydrological variables by means of polynomial regression. This was an essential input information for accurately predicting the microbial fate and transport in the alluvial wetland, as pointed out by Sanders et al. (2005) and Liu et al. (2015). Integrating a probabilistic Monte Carlo framework into the model analysis allowed accounting for the uncertainty of the source and transport variables and conducting a microbial infection risk assessment (Liao et al., 2016). One limitation of our model was that it did not account for the microbial particle interaction with the riverbed sediments. While Sanders et al. (2005) showed that the sediment erodibility parameters,

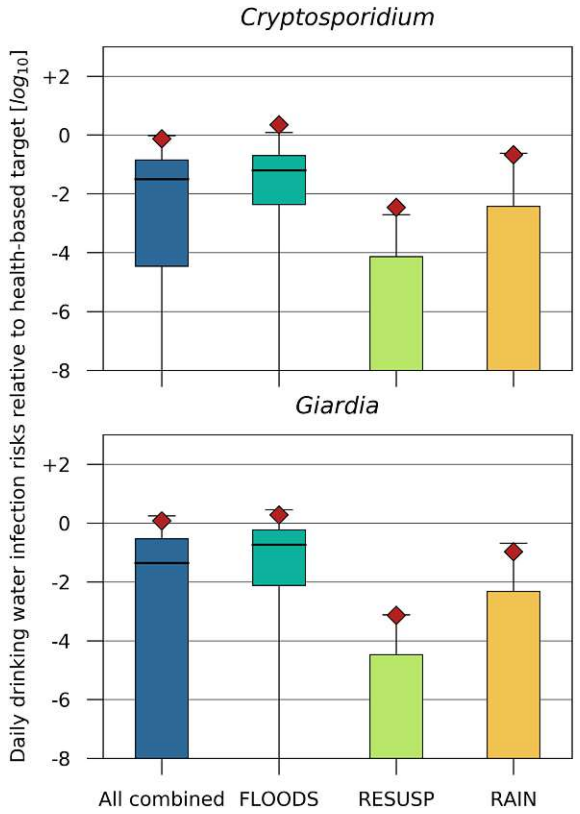


FIGURE 9 | *Cryptosporidium* and *Giardia* daily drinking water infection risks relative to a health-based benchmark of –6 $\log_{10}$/person/d (Signor and Ashbolt, 2009) for the scenarios (i) discharge of Danube water (FLOODS), (ii) resuspension from fecal deposits in inundated areas (RESUSP), (iii) rainfall-release and runoff from fecal deposits (RAIN), and all scenarios combined during 2010–2015. The treatment reduction of *Cryptosporidium* and *Giardia* from backwater river water (LRV, Equation 13) was 6.2 and 6.0 $\log_{10}$.

and sediment concentrations were important for FIO transport in a coastal wetland, sediment erosion was presumably of minor importance at our study site. Our model predicted 70–80 % of the observed concentrations within acceptable error limits (± 1 $\log_{10}$ particles/L), and the simulated and observed cumulative concentrations were not significantly different (Kruskal–Wallis $p > 0.05$). The settling of microbial particles and sediment transport simulations may be included for future applications. To estimate pathogen source loads from animal fecal deposits, we assumed that they were evenly distributed in the backwater area. This simplifying assumption was justifiable in our 14 km^2 sized model area. In larger wetlands, the spatial distribution of animals may need to be accounted for (Kay et al., 2007).

Impact of Event-Driven Fecal Pollution Sources and Pathways

The new integrative modeling approach allowed determining the transfer rates of pathogens from diverse fecal sources into wetlands during storm events and floods. Such weather extremes are of increasing concern due to climate change in many parts of the world. Several studies identified links of severe rainfall and flood events to elevated concentrations of pathogens such as *Giardia* and *Cryptosporidium* in rivers (Atherholt et al., 1998), the associated drinking water infection risks (Tolouei et al., 2019), or, to the number of outbreaks and sporadic cases of waterborne illness (Galway et al., 2015; Chhetri et al., 2019). For quantifying the impact of such events on the microbiological water quality of wetlands, modeling frameworks were developed either for pathogen transport via the rainfall-induced release and runoff (Guber et al., 2013), or via floods and resuspension (Sanders et al., 2005; Daniels et al., 2014; Liu et al., 2015). Our integrated modeling approach was developed to quantitatively compare these transfer pathways in a probabilistic framework. Our study showed that rainfall-induced pathogen release from animal fecal deposits, and floods can result in similar ranges of concentrations and loads of *Cryptosporidium* and *Giardia* and required reductions to achieve safe drinking water. This implies for water safety planning, that the autochthonous, homeothermic animal sources, such as ruminants, wild boar and birds, can be similarly important fecal pollution sources as the allochthonous human wastewater. This also implies that additional treatment may be required for drinking water production in wetlands inhabiting abundant wildlife, even in the absence of human wastewater discharges from upstream. According to our estimates, a 5–6 $\log_{10}$ reduction of *Cryptosporidium* and *Giardia* is required to achieve safe drinking water during floods and rainfall events. Demeter et al. (2021) considered only human wastewater sources to calculate the required reductions of *Cryptosporidium* to achieve safe drinking water at the Danube study site. Our estimation during floods is 0.5 $\log_{10}$ higher due to the additional contribution of diffuse animal sources in the Danube catchment.

For the estimation of infection risks, we used a mixture of beta distributions for the prevalence of the reference pathogens *Cryptosporidium* and *Giardia* in animal waste. We conducted a comprehensive literature survey, and selected values from the most recent, data-intensive studies conducted in temperate, high-income regions as our study area. The human infection risks from the animal fecal sources, however, may still be an overestimate, as we assumed the same dose-response models as for the human wastewater sources. To date, there are no reports about dose-response studies including different genotypes of *Cryptosporidium* or *Giardia* in the scientific literature. However, as long as this information is missing, it seems acceptable for risk assessment to choose this conservative risk assessment approach. Besides the reference pathogens *Cryptosporidium* and *Giardia*, other zoonotic pathogens such as EHEC and *Salmonella* spp. could be included in future analysis. These bacteria are also important reference pathogens occurring both in human and animal sources (Stalder et al., 2011), and their effects will depend on region and microorganism-specific source concentrations, prevalence and decay.

The modeling approach is transferrable to other riverine wetlands worldwide, even though the results of our study are

site-specific. To support water safety planning, it is important to integrate site-specific data into the modeling analysis and to validate the different transfer pathways of pathogens. In contrast to our local-scale approach, larger scale modeling studies previously identified hot spots of fecal pollution or evaluated the impact of system changes on the microbiological water quality (Medema and Schijven, 2001; Vermeulen et al., 2015; Sterk et al., 2016). These studies commonly made generalizing assumptions about the pathogen source and transport parameters as well as the hydrological and environmental boundary conditions and were not validated on real-world data.

CONCLUSION

- This study presents a new integrative modeling approach for determining the transfer rates of pathogens from diverse fecal sources into alluvial wetlands during storm events and floods considering safe drinking water supply.
- The modified and extended QMRAcatch (v1.1 Python backwater) combines microbial source tracking (MST) with 2-D hydrodynamic flow, rainfall-runoff, microbial fate and transport, and QMRA.
- The modeling approach allowed assessing the applicability of the chosen MST markers for the targeted fecal pollution source in relation to the total sum of all fecal pollution sources, considering fecal sensitivity and fecal specificity. They were found fully applicable for the modeling requirements and the research question in this study. The model captured the most relevant fecal sources and transport pathways, as proven by the model validation based on MST markers and *E. coli*.
- Allochthonous and autochthonous fecal sources during floods and rainfall events contributed similar ranges of concentrations and loads of *Cryptosporidium* and *Giardia* in the backwater branch, and drinking water infection risks relative to a health-based target.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

JD extended the computer code of QMRAcatch, did the computational and model analyses, and wrote the manuscript. KD contributed to the design of the study. KD and RL organized the microbial source tracking database. RL, SC-A, and KD did the molecular and microbiological analyses, supported by RS and JW. GL took the water samples in the field. GS conducted the literature survey on pathogen prevalence and source concentrations. JS gave support with QMRA modeling and provided data on the cumulative doses to calculate daily drinking water infection risks. JK did the hydrodynamic model simulations and provided support regarding the hydrological model. SC-A, RS, JW, AK, AB, and AF contributed to conception and in the acquisition of funding support. SC-A and KD wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.668778/full#supplementary-material

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