

RELATIONS BETWEEN CLIMATE SENSITIVE FACTORS AND FECALLY DERIVED MICROORGANISMS IN RIVERS

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SUMMARY

Microbial pollution of surface waters poses substantial risks for public health, amongst others during recreational use. Microbial pollution was studied at selected sampling sites in rivers Rhine, Moselle and Lahn (Germany) on the basis of commonly used fecal indicator organisms (FIO) indicating bacterial (*Escherichia coli*, intestinal enterococci) and viral (somatic coliphages) fecal contamination. In addition, *bla*_{CTX-M} antibiotic resistance genes (ARG) were quantified at two sites in river Lahn and were used as markers for tracking the spread of antibiotic resistance in the aquatic environment. The impact of changes in climate-related parameters on FIO was examined by studying monitoring results of contrasting flow conditions at rivers Rhine and Moselle. Analyses at all studied river sites clearly indicate that high discharge and precipitation enhance the influx of FIO, ARG and thus potentially (antibiotic resistant) pathogens into rivers. In contrast, a decrease in hygienic microbial pollution was observed under high solar irradiation and increasing water temperatures. Based on identified contributing key factors, multiple linear regression (MLR) models for five sites at a stretch of river Lahn were established that allow a timely assessment of fecal indicator abundances. An interaction between abiotic and biotic factors (i.e. enhanced grazing pressure) considerably contributed to the formation of seasonal patterns among FIO abundances. This was enhanced during extraordinary low flow conditions in rivers with pronounced trophic interactions, clearly hampering a transfer of model approaches between rivers of different biological and hydrological characteristics. Bacterial indicators were stronger influenced by grazing pressure than phages. Hence, bacterial indicators alone do not sufficiently describe viral pollution in rivers. *Bla*_{CTX-M} genes were omnipresent in Lahn River water and corresponded to distribution patterns of FIO, indicating fecal sources. Agriculture and waste water treatment plant effluents contributed to ARG loads and participants in non-bathing water sports were found to be at risk of ingesting antibiotic resistant bacteria (ARB) including ARG, bearing the risk of infection or colonization. Results of the present study highlight the need to be aware of such risks not only in designated bathing waters. ARG abundance at both riverine sampling sites could largely be explained by *E. coli* abundance and may thus also be incorporated into multiple regression models using *E. coli* specific environmental predictors. It can be expected that the frequency of short-term microbial pollution events will increase over the next decades due to climate change. Several challenges were identified with regard to the implementation of early warning systems to protect the public from exposure to pathogens in rivers. Most importantly, the concept of the Bathing Water Directive (Directive 2006/7/EC) itself as well as the lack of harmonization in the regulatory framework at European Union (EU) level are major drawbacks and require future adjustments to reliably manage health risks related to microbial water pollution in waters used in multifunctional ways.

ZUSAMMENFASSUNG

Da die Belastung von Gewässern mit Mikroorganismen ein Gesundheitsrisiko für den Menschen unter anderem bei dessen Freizeitnutzung birgt, wurde die mikrobielle Belastung der Flüsse Rhein, Mosel und Lahn anhand von Indikatoren für fäkalbürtige bakterielle (*Escherichia coli*, intestinale Enterokokken) und virale Belastungen (somatische Coliphagen) untersucht. Des Weiteren wurden *bla*_{CTX-M} Gene als Marker für die Verbreitung von Antibiotikaresistenzen in der Lahn quantifiziert. Durch vergleichende Analysen von Monitoringdaten gegensätzlicher Abflussgeschehen in Rhein und Mosel wurde ein Einfluss von Veränderungen klimarelevanter Parameter auf die Verbreitung der fäkalen Indikatororganismen (FIO) geprüft. Die Analysen an Rhein, Mosel und Lahn zeigen deutlich, dass hohe Abflüsse und Niederschläge den Eintrag von FIO und Antibiotikaresistenzgenen (ARG) und damit potentiell antibiotikaresistenter Bakterien in Gewässer begünstigten, während unter dem Einfluss hoher Globalstrahlung und steigenden Wassertemperaturen ein Rückgang zu beobachten war. Basierend auf als relevant identifizierten Einflussparametern wurden multiple lineare Regressionsmodelle (MLR Modelle) entwickelt, die eine zeitnahe Vorhersage von FIO Konzentrationen an fünf Stellen eines Lahnabschnitts ermöglichen. Wechselwirkungen zwischen abiotischen und biotischen (Fraßdruck) Faktoren tragen zur Entstehung saisonaler Muster in den FIO Konzentrationen bei. Diese sind unter Niedrigwasserverhältnissen und besonders in Flüssen mit ausgeprägten trophischen Interaktionen besonders deutlich ausgeprägt und beeinflussen eine Übertragbarkeit von Modellansätzen zwischen Flüssen unterschiedlicher biologischer und hydrologischer Charakteristika. Daraus, dass Bakterien davon stärker betroffen sind als Coliphagen, lässt sich schließen, dass bakterielle Indikatoren virale Belastungen nicht ausreichend abbilden. *Bla*_{CTX-M} Gene waren in der Lahn parallel zu den FIO ubiquitär nachzuweisen, was auf einen fäkalen Ursprung hinweist. Landwirtschaft und kommunale Kläranlageneinleiter trugen zur Belastung mit FIO bei. Es zeigte sich, dass auch nicht badende Wassersportler mit eingeschränktem Wasserkontakt nachweislich dem Risiko ausgesetzt waren, antibiotikaresistente Bakterien (ARB) samt ihren Genen zu verschlucken, was das Risiko einer Infektion oder Kolonisierung mit den entsprechenden Bakterien birgt. Da ARG zu großen Teilen durch *E. coli* erklärt werden konnten, können ARG gegebenenfalls in MLR Modelle mit *E. coli* spezifischen Vorhersagevariablen integriert werden. Kurzzeitige und kurzfristig auftretende Verschmutzungsereignisse werden in Zukunft als eine Folge des Klimawandels in ihrer Häufigkeit weiter zunehmen. Identifizierte Herausforderungen hinsichtlich der Implementierung von Frühwarnsystemen zur Bewertung der mikrobiellen Wasserqualität in Flüssen liegen in den Anforderungen der Badegewässerrichtlinie (Richtlinie 2006/7/EC) und in einer unzureichenden Harmonisierung des Rechtsrahmens der Europäischen Union (EU) begründet, um Gesundheitsrisiken aufgrund mikrobieller Belastung in multifunktional genutzten Gewässern angemessen zu begegnen.

CHAPTER 1

1 General Introduction

1.1 Microbial water quality in rivers and public health protection during recreational use

Rivers and streams are important renewable freshwater resources that are intensively used by humans for a wide range of purposes, which include:

- ▶ drinking water supply,
- ▶ agricultural uses (irrigation, livestock, aquaculture),
- ▶ industrial and municipal water supplies,
- ▶ industrial and municipal waste disposal,
- ▶ navigation,
- ▶ energy generation and cooling,
- ▶ recreation and
- ▶ aesthetic value (Chapman, 1996).

In 2015, most of the water used by different economic sectors across Europe originated from rivers (64.4 %). Most of it (40 %) was accounted for by the agricultural sector, followed by 28 % for cooling in the energy sector and 18 % for manufacturing and mining, while public water supplies accounted for 14 % of the water abstracted (EEA, 2018). In 2016, 69.1 % of the raw water abstracted for drinking water purification originated from groundwater and 15.6 % from surface water. 8.2 % originated from bank filtration and 7.0 % from artificial groundwater recharge. 0.1 % was made available from other sources (UBA, 2018a). Therefore, rivers substantially contribute to drinking water supply. Besides these purposes, rivers are also used for recreation and serve as tourist attractions due to their aesthetic value. Waterborne tourism is becoming

increasingly popular in many regions of Germany with more than 6 million people engaging in water sports such as sailing, motor boating, canoeing, water skiing as well as surfing and diving (BMVI, 2019). Thus, there are efforts ongoing to implement a waterborne tourism strategy to make minor waterways more attractive to tourists (BMVI, 2016). Rivers such as Rhine, Moselle, and Lahn are already highly frequented by tourists in terms of waterborne recreation (BMW, 2016). Taking the “Living Lahn” project (LiLa, project number IP-LIFE14/DE/022) funded by the European Union (EU) LIFE IP program as an example, one of its objectives is the promotion of sustainable tourism at the Lahn River e.g., by sustainable offers in the field of canoeing or rowing (HMUKLV, 2019). More than 2000 bathing waters were officially registered in Germany in the bathing season of 2018. The majority of designated German bathing waters is located at inland lakes (i.e., 2289, 83 %) and only 366 (16 %) are of the coastal type, located on the coasts of the North and Baltic Sea. Rivers represent the minority of bathing waters in Germany with 33 sites (1.5 %) (EEA, 2019; UBA, 2019). Good water quality of surface waters was shown to enhance the recreational demand (Breen et al., 2018). Thus, tourism benefits from good environmental water quality. Water tourism at federal waterways is an important economic factor in Germany, generating approximately 4 billion Euros in gross sales revenue, corresponding to a calculated income equivalent of approximately 66,500 people (BMW, 2016). However, as river water is used in many ways for different purposes, it can also serve as a medium for the effective transmission of infectious agents to a large number of people (Moe, 2007). Therefore, the contamination of water bodies with harmful microorganisms represents a substantial health threat and diminishes their touristic value.

1.1.1 Microbial contaminants of public health concern transmitted via water

Water-related diseases can be transmitted by ingestion, inhalation and aspiration of aerosols or direct contact, which can occur not only as a result of drinking water and participating in recreational activities, but also when contaminated water is used for crop irrigation and food processing. This can

cause foodborne outbreaks that affect a large number of people in wide geographic areas (Moe, 2007; WHO, 2003; WHO, 2008). Foodborne outbreaks occurred in Germany with enterohemorrhagic *Escherichia coli* (EHEC) (Buchholz et al., 2011) and *Norovirus* (Bernard et al., 2014), for example.

Routes of exposure to infectious agents in water during recreational activities will vary depending on the degree of water contact, which may be classified as

- ▶ no contact (or where water contact is incidental e.g., fishing from shore, sunbathing on a beach);
- ▶ incidental contact (partial contact which may include swallowing water, e. g. boating, fishing, wading) as well as
- ▶ whole-body contact (whole body or face and trunk frequently immersed or wetted by spray, swallowing water is likely e.g., swimming, diving, canoeing) (WHO, 2003).

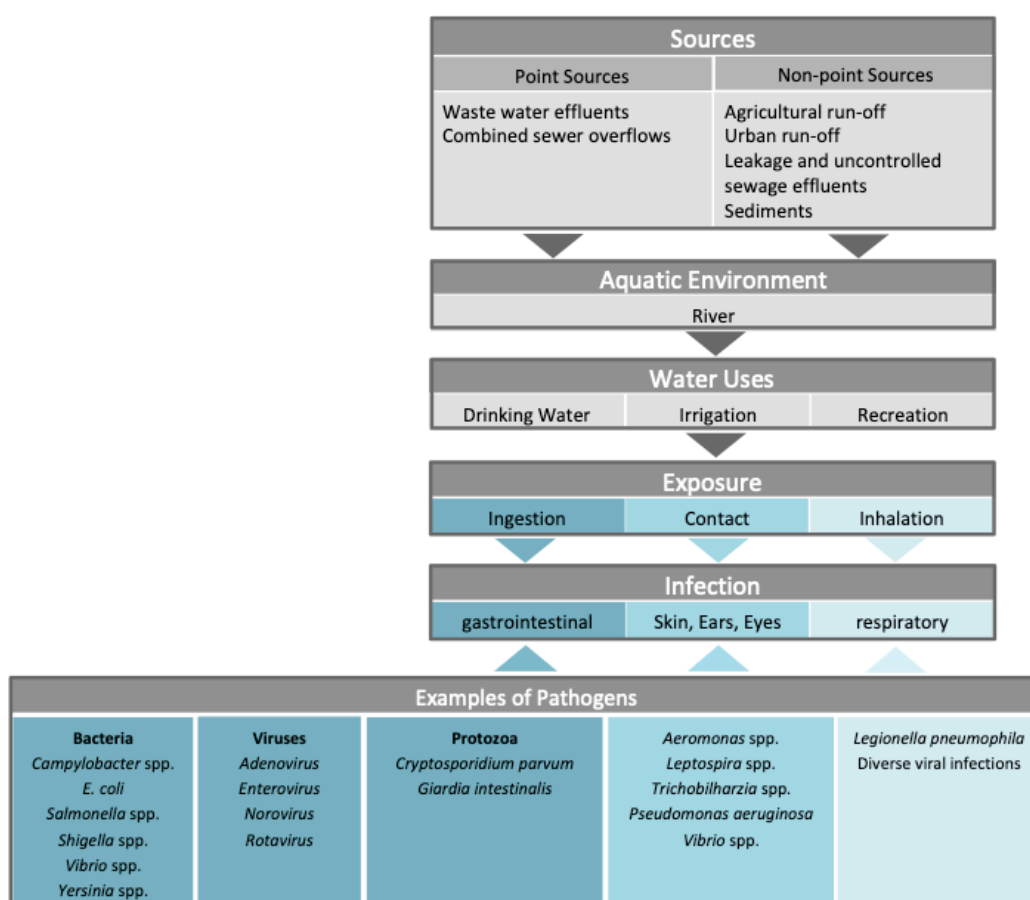


Figure 1.1: Transmission pathways and examples of relevant water-related pathogens

Infections transmitted via water can be caused by a variety of pathogens including viruses, protozoa and helminths as well as bacteria causing gastrointestinal symptoms, skin complaints including mucous membranes, wounds, ears and eyes, as well as respiratory illness (Prüss, 1998; Fig. 1.1). It was estimated that worldwide there are annually more than 120 million cases of gastrointestinal diseases and 50 million cases of respiratory diseases caused by swimming and bathing in coastal waters polluted with waste water (Shuval, 2003). Outbreaks linked to swimming or bathing in freshwater rivers or lakes were reported in Finland in the summer of 2014 (Kauppinen et al., 2017), the Netherlands in the summer of 2012 (Schets et al., 2018) and in Germany in 2006 (Brockmann et al., 2010), for example. While some waterborne pathogens are indigenous microorganisms naturally occurring in aquatic environments (e.g. *Vibrio* spp., *Aeromonas hydrophila*, *Legionella pneumophila*, and *Pseudomonas aeruginosa*), the majority of them is fecally derived and originates from the gastrointestinal tract of humans and warm-blooded animals (Moe, 2007). Diseases most frequently associated with exposure to fecally contaminated water are illnesses of the gastrointestinal type, which are mostly caused by enteric viruses (de Wit et al., 2001; Lopman et al., 2003; Prüss, 1998). They include *Rotavirus*, *Astrovirus*, *Adenovirus*, *Sapovirus* and *Norovirus*, but studies have found *Norovirus* to be responsible for most gastroenteritis infections (de Wit et al., 2001, Lopman et al., 2003, Sinclair et al., 2009). In terms of bathing at a German river site, it was shown that the highest risk of acquiring gastrointestinal disease was posed by *Norovirus* and *Rotavirus* (Timm et al., 2016).

In the group of parasitic protozoa, several species may be transmitted to humans through water, but *Giardia* spp. and *Cryptosporidium* spp. causing gastrointestinal diseases account for the majority of outbreaks worldwide (Efstratiou et al., 2017). Most of the human cryptosporidium cases in Europe are caused by *C. parvum* and *C. hominis* (Cacciò and Chalmers, 2016). Outbreaks have been reported, among others, in bathers and water sports players (ECDC, 2012). Starting in August 2012, the United Kingdom (UK), the Netherlands and Germany simultaneously registered an unusual increase in cryptosporidiosis notifications (ECDC, 2012,

Fournet et al., 2013), which could not be traced back to an identifiable single source (Fournet et al., 2013). The largest outbreak of cryptosporidiosis occurred in Halle, Germany in 2013 following a flood (Gertler et al., 2015). *Trichobilharzia* spp. are trematodes of the family *Schistosomatidae*, which require waterfowl and aquatic snails as final and intermediate hosts. Its free-swimming larvae (cercariae) can cause an inflammatory skin disease also known as “swimmer’s itch”. Exposure to water contaminated with cercariae emitted from snails can result in an accidental infection of humans (e.g. Selbach et al., 2016; Soldánová et al., 2013). A case study from Lake Baldeney, River Ruhr, Germany, revealed a potential risk of *Trichobilharzia* spp. infections while bathing (Selbach et al., 2016). Even if the disease is considered relatively harmless, outbreaks can have considerable negative economic impacts on local tourism in affected regions (Leighton et al., 2000). Leptospirosis caused by spirochaetes of the genus *Leptospira* is a zoonotic disease of global significance. As transmission to humans occurs due to contact with urine of infected animals, and more often via contaminated soil or water (Bharti et al., 2003), participants in water sports are frequently affected (Farr, 1995; Haake et al., 2002). In Germany, several cases of leptospirosis occurred in athletes after a triathlon held in the Neckar River (Brockmann et al., 2010).

Bacterial waterborne pathogens of fecal origin with high human health significance include members of the *Enterobacteriaceae* family such as *E. coli*, *Campylobacter*, *Salmonella*, *Shigella* and *Yersinia* species (Rosner et al., 2010; WHO, 2003; WHO, 2008). Enteric bacteria mostly cause gastrointestinal infections, including salmonellosis (*Salmonella* spp.), dysentery (*Shigella* spp.) and yersiniosis (*Yersinia* spp.) (Rosner et al., 2010; Santamaría and Toranzos, 2003). The most common causative bacterial agents of gastrointestinal disease in Europe are *Campylobacter* (ECDC, 2019). Following campylobacteriosis and salmonellosis, yersiniosis is the third most common bacterial zoonose in Germany (Rosner et al., 2010). Water recreation is a risk factor for infections with *E. coli*, *Campylobacter*, *Salmonella* or *Shigella* species and exposures like water recreation were shown to be as relevant as food-related exposures with regard to the proportion of studied

cases (Denno et al., 2009). Even if most *E. coli* strains are non-pathogenic, there are intestinal pathogenic *E. coli* (IPEC) and extraintestinal pathogenic *E. coli* (ExPEC) of clinical importance (Sarowska et al., 2019). IPEC, also known as diarrheagenic *E. coli* (DEC) cause severe intestinal illnesses (Nataro and Kaper, 1998). DEC, classified as enteropathogenic *E. coli* (EPEC), enterohemorrhagic (Shiga toxin-producing) *E. coli* (EHEC/STEC), enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC), and enteroinvasive *E. coli* (EIEC) have been responsible for multiple waterborne gastroenteritis outbreaks worldwide (Gomes et al., 2016). ExPEC are facultative pathogens comprising uropathogenic *E. coli* (UPEC), neonatal meningitis *E. coli* (NMEC) and sepsis-associated *E. coli* (SEPEC), which are part of the normal human intestinal flora (Sarowska et al., 2019) and thus comprise enteric pathogens causing extraintestinal illnesses. Besides enteric illnesses, water has also been recognized as transmission medium for extraintestinal infections caused by natural bacterial inhabitants of the aquatic system (Pitlik et al., 1987). Aeromonads are Gram-negative bacteria that occur naturally in aquatic environments. They include opportunistic pathogens of serious public health concern as they can cause a variety of human diseases, which, besides gastroenteritis, include soft-tissue and skin infections as well as septicemia (Igbinosa et al., 2012). *Pseudomonas aeruginosa* is also a Gram-negative and ubiquitous environmental bacterium and opportunistic pathogen known to cause a wide range of human diseases (Moradali et al., 2017). It is responsible for 10 % of nosocomial infections in Germany (Ott et al., 2013). They are of great public health concern as they often bear intrinsic and obtained resistances against a wide range of antibiotics, including beta-lactam antibiotics, due to the production of extended spectrum beta-lactamases, in particular of the OXA, TEM, PER and SHV type (Jenny and Kingsbury, 2018; Moradali et al., 2017). In nature, it is commonly found in aquatic environments and thus a frequent cause of water related infections, such as otitis externa (Mena and Gerba, 2009; van Asperen et al., 1995), also known as „swimmer’s ear“ (e.g. Strauss and Dierker, 1987).

Concern regarding the treatment of infectious bacterial diseases with common antibiotics is growing, since antibiotic resistance is increasingly noticed in clinically relevant species (e.g. McKenna, 2013; Ventola, 2015; WHO, 2018). Antibiotic resistance accounted for 67000 infections resulting in 33000 deaths in Europe in 2015 (Cassini et al., 2019), and a further global increase is projected (O'Neill, 2016). In members of the *Enterobacteriaceae* family such as *E. coli* a dramatic increase of resistance to 3rd and 4th generation cephalosporins is subject of growing concern as well as resistance to carbapenems, which are often claimed as "the last line of antibiotic defense" (Bassetti et al., 2009; Hawkey and Jones, 2009; McKenna, 2013; Paterson, 2006). Cephalosporin resistant *E. coli* and carbapenem resistant *P. aeruginosa* belong to the antibiotic resistant bacteria (ARB) with the largest human health impact (Cassini et al., 2019). Resistance to cephalosporins, carbapenems and also penicillins in *Enterobacteriaceae* is mainly mediated by beta-lactam hydrolyzing enzymes (extended spectrum beta-lactamases, ESBL), of which CTX-M-type enzymes, encoded by *bla*_{CTX-M} genes, are the most common ESBL in Germany (Pfeifer et al., 2013). *E. coli* represents the dominant host of *bla*_{CTX-M} genes (Carattoli, 2009; Livermore et al., 2006; Robin et al., 2017). Antibiotic resistance genes (ARG) located on plasmids, like *bla*_{CTX-M}, can be mobilized between bacteria, enabling an exchange of ARG between environmental bacteria, human and animal commensals and pathogens (Forsberg et al., 2012; Nordmann, 2014; von Wintersdorff et al., 2016; Wellington et al., 2013).

1.1.2 Indicators of fecally derived microbial pollution

The assessment of microbial water quality relies on the quantification of surrogate microorganisms for pathogens, which are usually associated with fecal origin (fecal indicator organisms, FIO), but are generally not human pathogens themselves (Fewtrell and Bartram, 2001). Ideally, a fecal indicator should

- ▶ not be able to multiply in the environment
- ▶ have similar survival and transport properties than pathogens
- ▶ correlate with pathogenic microorganisms of fecal origin and

- ▶ be affordable to analyze (Ferguson and Signoretto, 2011).

The abundance of these indicators is thus supposed to provide an indication of

- ▶ fecal pollution,
- ▶ the presence of domestic sewage,
- ▶ the presence of pathogens,
- ▶ the efficiency of waste water treatment processes and
- ▶ the environmental fate and transport of a pathogen (Saxena et al., 2015).

Recreational water quality standards vary by country, but they generally relate indicator counts to defined thresholds. Widely used microbial indicators comprise coliforms (total coliforms), fecal or thermotolerant coliforms, *E. coli*, enterococci (fecal streptococci or intestinal enterococci) and bacteriophages (Fewtrell and Bartram, 2001; Saxena et al., 2015). As intestinal enterococci are able to persist under a wide range of environmental conditions it is assumed that they may be a more stable indicator for pathogens compared to *E. coli* (Jin et al., 2004). Bacteriophages that infect *E. coli*, particularly F-specific (F+ phage) and somatic coliphages, have been suggested to more reliably indicate the presence of pathogenic viruses than fecal indicator bacteria (FIB) (Gerba, 2009; Grabow, 2004; Skrabber et al., 2004). For the assessment of antibiotic resistances in environmental settings, *E. coli* and fecal enterococci were proposed as possible bacterial indicators (Berendonk et al., 2015; Gekenidis et al., 2018). Because of their wide distribution and frequent detection in the environment (e.g. *bla*_{CTX-M} abundance in enteric bacteria in some UK bathing waters is assumed to be as high as 0.1 % (Finley et al., 2013)), *bla*_{CTX-M} genes have been suggested as an adequate surrogate to indicate and trace the dissemination of resistance in natural watercourses (Berendonk et al., 2015, Tacão et al., 2012).

1.1.3 Sources of fecally derived microbial pollution in rivers

Many of the pathogens found in surface water originate from feces of humans and warm-blooded animals (Pandey et al., 2014). The sources of fecal pollution can be divided in point and non-point sources. Point sources are defined as fixed

locations or facilities from which pollutants are discharged. Effluents from waste water treatment plants (WWTP) commonly discharged into rivers represent important point sources impacting river water quality (Pandey et al., 2014; Servais et al., 2007). Combined sewer overflows (CSO) have been seen as major point sources of microbial contamination in river water. Many urban centers are drained by combined sewer systems, which collect surface runoff and sewage water in a shared system connected to a WWTP. During rainfall events or snowmelt, the systems are designed to overflow when collection capacity is exceeded, resulting in a CSO that discharges a mixture of untreated waste water and surface runoff directly into the river. Although limited in time, CSO discharges can considerably impact microbiological water quality (Eleria and Vogel, 2005; Olds et al., 2018; Passerat et al., 2011).

Non-point source pollution of rivers originates from surface runoff and soil leaching mainly driven by rain events (George et al., 2004; Ouattara et al., 2011). Agriculture is a major cause of diffuse pollution entering surface waters (WHO, 2008). Fecal material is washed off from livestock feces and manure (Crane et al., 1983), but microbial water quality is also threatened by runoff and direct deposition of feces of wildlife such as waterfowl (Fleming et al., 2001). Poorly maintained sanitation systems or misconnections to the rainwater collection system can also result in untreated waste water directly entering streams in urban areas, as for example examined in the case of the city of Berlin. There, a high degree of false pipe connections or leaks was shown to considerably increase FIO concentrations in the receiving waters during rainfall as opposed to areas without (Raber et al., 2019). Treated and untreated waste water from households, hospitals, industry as well as animal agriculture is further known to also contain elevated levels of ARB, ARG and antibiotics which are released into the environment (e. g. Berendonk et al., 2015; Bueno et al., 2018). The rise of antibiotic resistance is linked with the widespread use of antibiotics in human and veterinary medicine (e.g. Berendonk et al., 2015; O'Neill, 2016). The aquatic environment then acts as a collecting vessel of ARB, offers exposure routes to humans and animals and facilitates the spread of ARB and resistance markers

through surface water or groundwater. Aquatic environments further represent a reservoir for ARG and enable their exchange between bacterial species (e.g. von Wintersdorff et al., 2016). Since sediments of aquatic environments constitute a reservoir for microorganisms, resuspension of sediments can considerably increase pathogen levels (e.g. Cho et al., 2010; Droppo et al., 2009). Sediments thus represent a further non-point source of pathogen contamination. It has also been reported that maintenance dredging can lead to a release of bacteria from river bottom sediments, resulting in significantly increased fecal coliform levels in the vicinity of dredging operations (Grimes, 1975). Also bathers themselves may be a non-point source, by distributing microorganisms from their bodies or by resuspending contaminated sediments (Fewtrell and Kay, 2015). Rainfall events are considered to be an important driver of fecal-microbial water deterioration. Many studies have confirmed the link between precipitation and increased microbial pollution in different aquatic environments (e.g. Eleria and Vogel, 2005; Pandey et al., 2014; Passerat et al., 2011). Once in the river, the survival of microorganisms is impacted by a variety of environmental factors including predation (Boenigk and Arndt, 2002; Menon et al., 2003), inactivation due to sunlight exposure (Sinton et al., 2002), or mortality due to undesirable physicochemical conditions (Hipsey et al., 2008).

1.1.4 Microbiological aspects in EU water-related legislation

Over the last decades, the EU has adopted several legislations aiming at the protection of waters and public health (Fig. 1.2). Their implementation basically occurred in three waves (Bernasconi et al., 2003; European Commission, 1997; Kaika, 2003).

The first wave starting in the 1970s focused on protecting and setting standards for particular waters including fish waters (Freshwater Fish Directive; Directive 78/659/EEC; Council of the European Union, 1978), shellfish waters (Shellfish Water Directive; Directive 79/923/EEC; Council of the European Union, 1979a), groundwater (Groundwater Protection Directive; Directive 80/68/EEC; Council of the European Union, 1980a), drinking water (Drinking Water

Directive (DWD); Directive 80/778/EEC; Council of the European Union, 1980b) and surface waters intended for drinking (Surface Water Directive; Directive 75/440/EEC; Council of the European Union, 1975) as well as bathing waters (Bathing Water Directive (BWD); Directive 76/160/EEC; Council of the European Union, 1976a). The Dangerous Substances Directive (Directive 76/464/EEC; Council of the European Union, 1976b) and its Daughter Directives (Directive 82/176/EEC (Council of the European Union, 1982); Directive 83/513/EEC (Council of the European Union, 1983); Directive 84/491/EEC (Council of the European Union, 1984a); Directive 84/156/EEC (Council of the European Union, 1984b); Directive 86/280/EEC (Council of the European Union, 1986)) were adopted to set emission limit values for certain dangerous substances discharged into the aquatic environment. The information exchange decision (Decision 77/795/EEC; Council of the European Union, 1977) aimed at setting up a common system for monitoring and exchange of information of water quality in the European Community. Within Directive 79/869/EEC (Council of the European Union, 1979b), methods of measurement and frequencies of sampling and analysis of surface water intended for the abstraction of drinking water were recommended (Bernasconi et al., 2003; European Commission, 1997).

The second wave included the Urban Waste Water Treatment Directive (UWWTD; Directive 91/271/EEC; Council of the European Union, 1991a) and the Nitrates Directive (NiD; Directive 91/676/EEC; Council of the European Union, 1991b). Other elements were revisions of the DWD of 1980, resulting in a new DWD (Directive 98/83/EC; Council of the European Union, 1998). For industrial installations, the IPPC Directive (Directive 96/61/EC; Council of the European Union, 1996) set out a legislative framework dealing with integrated pollution prevention and control, which also covered water pollution (Bernasconi et al., 2003; European Commission, 1997).

During the third wave, EU water policy was substantially reformed with the implementation of the Water Framework Directive (WFD, Directive 2000/60/EC; European Parliament and Council of the European Union, 2000),

which combined many of the previous directives. Thus, the directives concerning dangerous substances, freshwater fish, shellfish, groundwater protection, information exchange, surface water abstraction and methods of sampling were repealed by the WFD in 2007 and 2013, respectively, whereas the UWWTD, NiD, DWD and BWD remained as stand-alone directives besides the WFD (Fig. 1.2; Bernasconi et al., 2003; European Commission, 1997).

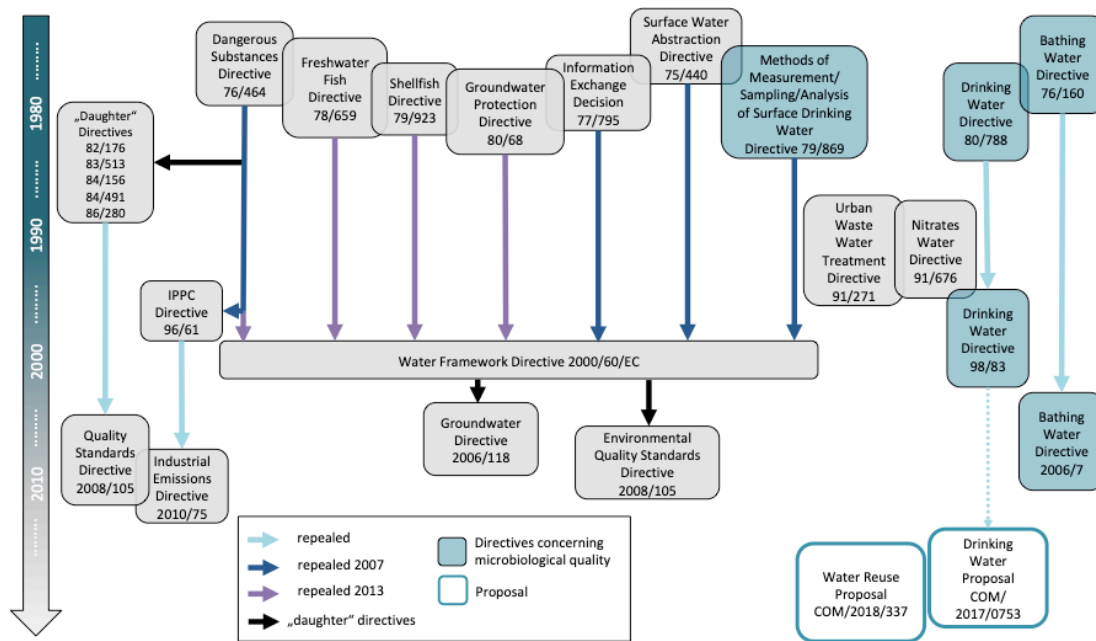


Figure 1.2: Overview of water-related legislation (adapted from Bernasconi et al., 2003).

The 1976 BWD was revised resulting in a new BWD in 2006 (Directive 2006/7/EC; European Parliament and Council of the European Union, 2006). The implementation of the UWWTD, BWD and DWD by EU Member States, which are sometimes summarized as “the water industry directives”, is regarded as a minimum requirement by the WFD (European Commission, 2008; EEA, 2016). Monitoring of key parameters of water quality and management measures have to be conducted by the Member States under these directives at different compliance points. While UWWTD quality standards have to be met at the WWTP outlet (EEA, 2016), the BWD compliance point is the water body, where the majority of bathers or the highest pollution is expected. The DWD compliance point is at the point of human consumption (EEA, 2016).

Microbiological aspects are addressed in the DWD and BWD, while monitoring of specific bacteria is not directly required in the UWWTD (EEA, 2016). While there have been microbiological requirements for surface water used for drinking water abstraction in the past, the WFD, which is currently applied instead, does not stipulate any microbiological requirements for surface water bodies (Exner and Schwartz, 2015; Fig. 1.2). The WFD complements the DWD by the formation of safeguard zones for areas of the abstraction of surface water intended for human consumption. The compliance point under the WFD for drinking water protection is thus the water body at source where it is abstracted before being processed and delivered at the tap (European Commission, 2008). While the microbiological quality of drinking water has to be tested for a broad variety of microbial determinants in an end point control, the microbiological status of a water body is not regarded as relevant, until it is designated as bathing water. In 2018 the European Commission proposed new rules to stimulate and facilitate water reuse in the EU (Proposal COM/2018/337 final; European Commission and Directorate-General for Environment, 2018a). The proposals objective is to regulate the use of reclaimed municipal waste water for agricultural irrigation. The proposal is intended to supplement the WFD, the Groundwater Directive, the UWWTD, and the NiD (UBA, 2018b). Another proposal (Proposal COM/2017/0753 final; European Commission and Directorate-General for Environment, 2018b) aims at improving drinking water quality among others by tightening up maximum limits for certain pollutants including harmful bacteria.

The Bathing Water Directive

To ensure the safety of bathing waters the BWD obliges national authorities to monitor and report on bathing water quality. In Germany, the responsibility of its enforcement is up to the federal states (Länder), which have implemented the demands of the BWD in national law. The BWD applies „to any element of surface water where the Competent Authority expects a large number of people to bathe and has not imposed a permanent bathing prohibition, or issued permanent advice against bathing“ (European Parliament and Council of the European Union, 2006). These waters have to be designated as EU bathing

waters. At these sites, local authorities have to collect water samples throughout the bathing season according to a prescheduled monitoring plan. The collected samples have to be analyzed for *E. coli* and intestinal enterococci according to ISO 7899-1 (ISO, 1998a) or ISO 7899-2 (ISO, 2000a) (intestinal enterococci) and ISO 9308-3 (ISO, 1998b) or 9308-1 (ISO, 2014) (*E. coli*). Depending on the microbial monitoring results, a bathing water can be classified as „poor“, „sufficient“, „good“, and „excellent“. At bathing water sites with sufficient or poor water quality rating, management measures must be implemented to upgrade bathing water quality. Collection of one pre-season sample shortly before the start of the bathing season and a minimum of four samples per season is required by regulation, generally resulting in at least four samples per bathing season and 16 samples in the four-year reference period.

The BWD differentiates between coastal waters and inland freshwater but no difference is made between surface standing waters such as lakes and flowing waters such as rivers. Classification for inland bathing waters is based on a 95- and 90-percentile calculation according to the criteria given in Tab. 1.1. That means that 95 % or 90 % of the sample measurements taken during this four-year reference period must not exceed a defined threshold value (Tab. 1.1) in order to meet the bathing water quality standard.

Table 1.1: Inland bathing water classification criteria according to BWD

Parameter	Excellent	Good	Sufficient	Reference Methods
Intestinal enterococci (CFU/100 mL)	200*	400*	330**	ISO 7899-1 or ISO 7899-2
<i>E. coli</i> (CFU/100 mL)	500*	1000*	900**	ISO 9308-3 or ISO 9308-1
*Based upon a 95 th percentile evaluation; **Based upon a 90 th percentile evaluation				

Bathing water quality results have to be made available to the public. Furthermore, the responsible authorities have to establish and regularly update bathing water profiles, which provide additional information on cyanobacteria occurrences and pollution sources and notify the public if the bathing water is subject to short-term pollution. To limit exposure risks of bathers to short-term pollution of bathing water sites, the BWD sets out specific demands and calls for

adequate management measures and early warning systems (BWD Article 12 (2) (c)).

1.1.5 Current progress of research

In view of the implementation of WFD and BWD, (predictive) modeling has become more and more important. To support water resource managers in implementing adequate water management measures, researchers have been putting much effort over the last couple of years in providing model-based tools, for example in the fields of bathing water or river basin management. With regard to microbial aspects models are generally used for a variety of applications:

- ▶ to explore the dominant processes within an aquatic system
- ▶ to guide the design of targeted monitoring programs
- ▶ to quantify differences between species
- ▶ to quantify the impact of management scenarios
- ▶ to support real-time decision-making (Hipsey and Brookes, 2013)

Real-time sensors for example for temperature, conductivity, discharge, rainfall, turbidity and chlorophyll-*a* are widely used at meteorological and hydrological monitoring stations and provide high temporal resolution data, which are immediately available in many cases. Regression modeling for the prediction of FIB densities using (near) real-time information such as hydro-meteorological and physical parameters is frequently used to produce a timely alternative to cultivation-based microbiological monitoring at coastal and inland bathing waters (Boehm et al., 2007; de Brauwere et al., 2014; Olyphant and Whitman, 2004). For example the Scottish Environment Protection Agency (SEPA) runs real-time bathing water quality predictions at bathing sites throughout Scotland (McPhail and Stidson, 2009). Ideally, bathing water quality prediction should serve both purposes: on the one hand, it should prevent the exposure of users to poor water quality and on the other hand it should help to prevent unnecessary bathing site closings.

Despite the increasing recreational demand (BMWi, 2016) riverine bathing waters in Germany as well as tools for a reliable water quality prediction for riverine bathing waters are scarce as they cause specific challenges in terms of microbial risk assessment for multiple reasons. Treated waste water is commonly discharged into rivers. This results in elevated effluent contributions in the river depending on its flow regime (Karakurt et al., 2019), altering microbiological water quality especially downstream of WWTP. Furthermore, water quality in rivers is strongly impacted by changing meteorological conditions such as heavy rainfall that can cause sudden inputs of fecal pollution by CSO or surface runoff from urban and agricultural areas (e.g. Passerat et al., 2011, Servais et al., 2007). Enhanced river discharge especially after rainfall events may also lead to resuspension of contaminated sediments, enhancing microbial pollution (e.g. Cho et al., 2010; Droppo et al., 2009). Thus, flowing surface waters are subject to rapid changes in microbial water quality with strong variations even within a day. Since culture techniques for FIB (as proposed by the BWD) require up to 48 hours until results are available, the microbial water quality may have changed considerably during this time period and near real-time decisions regarding bathing water quality classifications can hardly be made. Thus alternative methods allowing a timely prediction of microbial water quality are needed. However, predictive models are known to be often system and organism specific, making an implementation difficult (Hipsey et al., 2008).

ARB along with ESBL-producing members of the *Enterobacteriaceae* family are increasingly detected in various surface waters including rivers (e. g. Blaak et al., 2015; Leonard et al., 2015; Zurfluh et al., 2014). Furthermore surface waters have been recognized as a setting where (re)transmission of ARB to humans may occur (Ashbolt et al., 2013; Iversen et al., 2004; Laurens et al., 2018; Leonard et al., 2018), posing further potential health risks for water users. A better understanding of how resistance spreads via water in the environment is important for the development of management tools, measures, policies and regulations to tackle health risks due to antimicrobial resistance. As the popularity of water borne tourism is increasing (BMWi, 2016), it is of urgent need to

examine the occurrence of antibiotic resistance along the river to assess potential risks and prevent human exposure in rivers that are used in multifunctional ways. Current knowledge gaps include the relative contributions of different sources of antibiotics and ARB into the environment as well as health impacts caused by exposure to environmental antibiotic resistant bacteria (Larsson et al., 2018). Viruses and bacteria may exhibit other persistence and distribution properties in flowing surface waters due to their divergent biology, and bacteria may thus not represent the many viral pathogens adequately, despite their high health significance (e.g. Rodrigues and Cunha, 2017; Schets et al., 2018). Yet, the selection of appropriate determinants to indicate human health risk from recreational water exposure is still a challenge in the field of water quality management.

1.2 Project goals and outline

Implications of the BWD and WFD and emerging risks such as the increase of antibiotic resistances in the aquatic environment elicited the need to better understand the processes driving microbial dynamics in river catchments. A better understanding will be mandatory to generate a sound knowledge base for the development of reliable management measures, tools, policies and regulations to address the health risks due to microbial water pollution.

Thus, the main overarching goals of the study were to contribute to the aforementioned knowledge base by (i) exploring the influence of environmental factors on input and dynamics of fecally derived microbial pollution in rivers and (ii) establishing tools for a timely prediction of microbial water quality (Fig. 1.3).

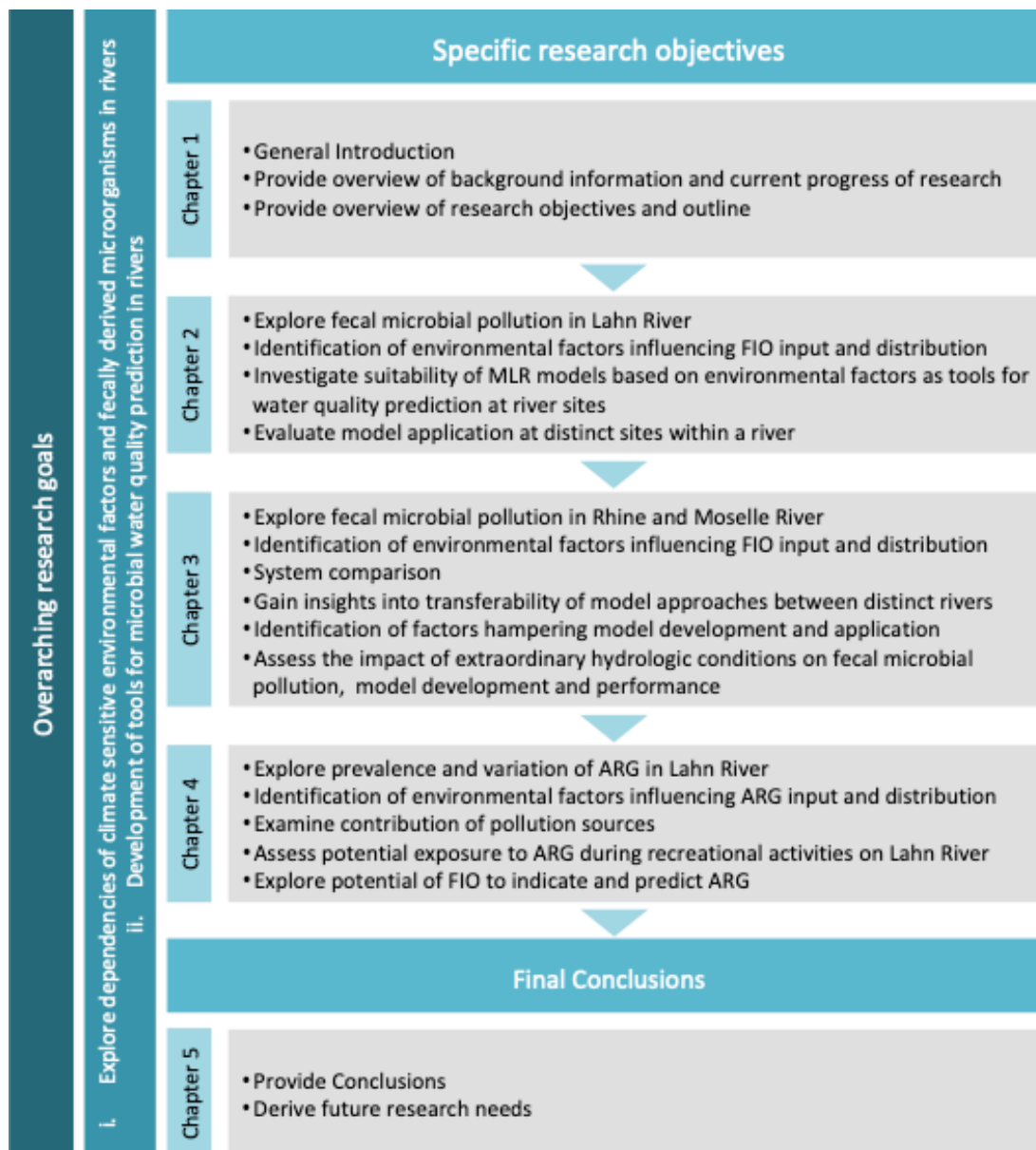


Figure 1.3: Schematic illustration of thesis outline and research objectives

Therefore, the hygienic condition of three distinct rivers (Fig. 1.4) was determined over > 12 months each at selected sampling sites by measuring general hydrological, physicochemical and microbiological water quality parameters including fecal indicators to estimate fecal microbial pollution and *bla*_{CTX-M} ARG to trace the dissemination of antibiotic resistance in the riverine environment. All of the rivers are under particular interest for recreational use (BMW_i, 2016) but differ considerably in their hydrodynamic characteristics. Data were analyzed with respect to specific research objectives as outlined in Fig. 1.3 and Fig. 1.4.

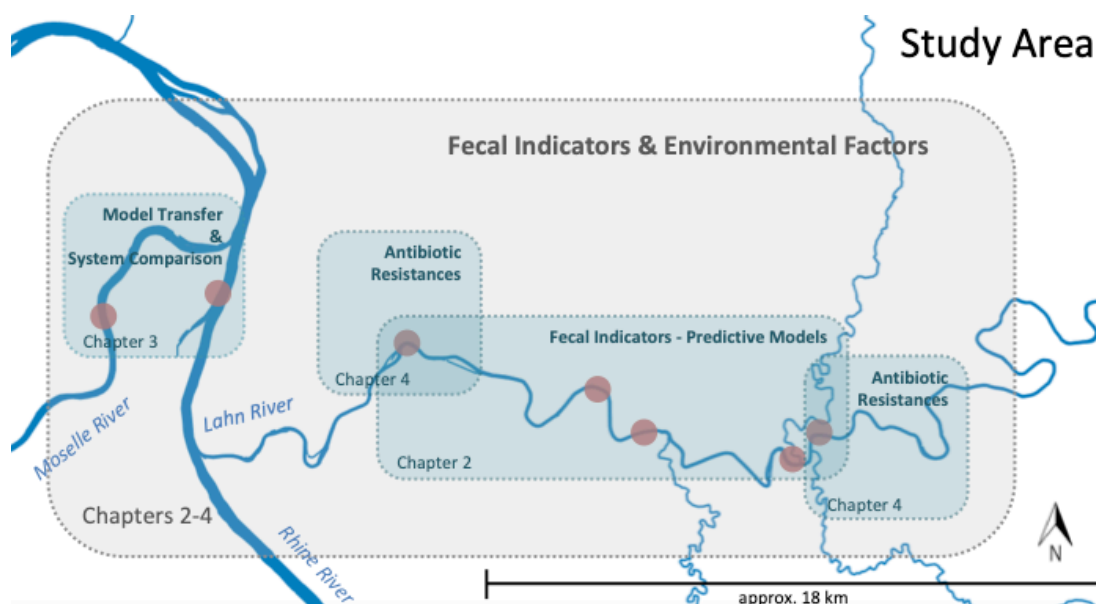


Figure 1.4: Spatial scope of the study with sampling sites indicated as red circles (Map modified from: <https://geoportal-wasser.rlp-umwelt.de/servlet/is/2025/>)

Following a general introduction in [Chapter 1](#), [Chapter 2](#) describes research conducted at lower Lahn River, which aimed at achieving an understanding of the spatial dissemination of viral and bacterial FIO in river surface waters and the identification of key factors influencing their distribution. A main focus was the identification of explanatory variables suitable for the prediction of FIO using multiple linear regression (MLR) model approaches and to gain insights into MLR model applicability at several sites within a river stretch.

In [Chapter 3](#) prevalence and seasonal variation of fecal microbial pollution in Rhine and Moselle River was explored. Due to their spatial proximity, sampling sites were subject to similar meteorological conditions. Comparative analyses were conducted to also allow conclusions on the influence of autochthonous riverine factors influencing FIO distribution. By transferring previously established model approaches (Seis et al., 2018) to Rhine and Moselle data, the transferability of predictive model approaches between distinct rivers with different characteristics was assessed and factors hampering model development and transferability were identified. Considering that rivers underlie strong and rapid changes in water quality driven by various climate sensitive environmental factors, changes in climatic conditions may impact river water quality and thus its uses. Therefore, the impact of contrasting hydro-meteorological conditions was

examined in order to draw conclusions on potential impacts of climate change on fecal pollution levels and model validity under extraordinary conditions.

In **Chapter 4** seasonal and spatial distribution patterns of *bla*_{CTX-M} resistance gene concentrations were investigated in relation to environmental factors at two of the sampling sites of lower Lahn River and compared with FIO abundances. ARG, specifically those most frequently associated with enteric bacteria, such as *bla*_{CTX-M} genes, are proposed as key indicators of water quality suitable to trace the dissemination of antibiotic resistances in riverine environments (Berendonk et al., 2015; Tacão et al., 2012). The results allow drawing conclusions on ARG sources, fate and potential human exposure as well as on the ability of fecal indicators to indicate the presence and distribution of ARG in flowing surface waters. Because Lahn River is very popular for waterborne recreation, especially boating and canoeing, a potential exposure of participants to antibiotic resistances in aquatic recreational activities was assessed. Enhanced knowledge of the prevalence of ARB and ARG in surface waters and environmental factors driving their input and dissemination may allow establishing model approaches for estimation of antibiotic resistance abundances in multifunctional flowing waters as well. To indicate risks of exposure the possibility of using 'classic' indicator bacteria to monitor water quality with respect to antibiotic resistance would be extremely convenient as FIB concentrations are widely measured in routine monitoring schemes according to standardized protocols. Thus, the ability of FIO to depict the dissemination of ARG was examined by linear regression analyses. Conclusions and future research needs are discussed in **Chapter 5** with a focus on climate change, challenges of the implementation of water quality assessment strategies and shortcomings of current water quality legislation with an emphasis on microbiological aspects.

CHAPTER 2

2 Multiple linear regression models as predictive tools for fecal indicator concentrations

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Herrig, I., Böer, S., Brennholt, N., Manz, W., 2015. Development of multiple linear regression models as predictive tools for fecal indicator concentrations in a stretch of the lower Lahn River, Germany. *Water Res.* 85, 148–157.
DOI: 10.1016/j.watres.2015.08.006
.....

Abstract

Rivers are typically subject to rapid changes in microbiological water quality. In contrast to traditional water quality assessment based on the time-consuming cultivation of fecal indicator organisms (FIO), predictive models facilitate a near-real-time assessment of the actual hygienic status of a particular water body. In our study, we developed multiple linear regression (MLR) models in order to predict the abundance of the FIO *Escherichia coli* (EC), intestinal enterococci (IE) and somatic coliphages (SC) in the Lahn River, Germany. The models were developed on the basis of an extensive set of environmental parameters collected during a 12-months monitoring period. Two models were developed for each type of indicator:

- An extensive model including the maximum number of variables significantly explaining variations in indicator abundance and

- A simplified model reduced to the three most influential explanatory variables, thus obtaining a model, which is less resource-intensive with regard to required data.

Both approaches have the ability to model multiple sites within one river stretch. $\text{NH}_4\text{-N}$, turbidity and global solar irradiance turned out to be the three most important predictive variables for the fecal bacterial indicators. In contrast, somatic coliphages were predicted comparably well by chlorophyll-*a* content, discharge and $\text{NH}_4\text{-N}$. Depending on indicator type, the models employing the maximum number of explanatory variables (extensive mode models) additionally include the variables rainfall, O_2 content, pH and chlorophyll-*a*. The extensive mode models could explain 69 % (EC), 74 % (IE) and 72 % (SC) of the observed variance in FIO concentrations. The models based on the three best predictive variables (optimized models) explained the observed variance in FIO concentrations to 65 % (EC), 70 % (IE) and 68 % (SC). Station-specific efficiencies ranged up to 82 % (EC) and 81 % (IE, SC). Our results suggest that MLR models are generally promising tools for a timely water quality assessment in the Lahn area.

CHAPTER 3

3 Prediction of fecal indicator organism concentrations in rivers: The shifting role of environmental factors under varying flow conditions

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DOI: 10.1186/s12302-019-0250-9
.....

Abstract

Background: Fecal indicator organisms (FIO) like *Escherichia coli*, enterococci and coliphages are important to assess, monitor and predict microbial water quality in natural freshwater ecosystems. To improve predictive modeling of fecal indicators in surface waters, it is vital to assess the influence of autochthonous and allochthonous environmental factors on microbial water quality in riverine systems. To better understand how environmental conditions influence the fate of fecal indicators under varying weather conditions the interdependency of environmental parameters and concentrations of *E. coli*, intestinal enterococci, and somatic coliphages was studied at two rivers (Rhine and Moselle in Rhineland-Palatinate, Germany) over a period of two years that exhibited contrasting hydrological conditions. Both riverine sampling sites were subject to similar meteorological conditions based on spatial proximity, but differed in

hydrodynamics and hydrochemistry, thus providing further insight on the role of river-specific determinants on fecal indicator concentrations. Furthermore, a Bayesian multiple linear regression (MLR) approach that complies with the European Bathing Water Directive (BWD) was applied to both rivers' datasets to test model transferability and the validity of microbial water quality predictions in riverine systems under varying flow regimes.

Results: According to multivariate statistical analyses, rainfall events and high water discharge favored the input and dissemination of fecal indicators in both rivers. As expected, concentrations declined with rising global solar irradiance, water temperature, and pH. While variations in coliphages concentrations were predominantly driven by hydro-meteorological factors, bacterial indicator concentrations were strongly influenced by autochthonous biotic factors related to primary production. This was more pronounced under low flow conditions accompanied by strong phytoplankton blooms. Strong seasonal variations pointed towards bacterial indicator losses due to grazing activities. The Bayesian linear regression approach provided appropriate water quality predictions at the Rhine sampling site based on discharge, global solar irradiance and rainfall as fecal indicator distributions were predominantly driven by hydro-meteorological factors.

Conclusions: Assessment of microbial water quality predictions implied that rivers characterized by strong hydrodynamics qualify for MLR models using readily measurable hydro-meteorological parameters. In rivers where trophic interactions exceed hydrodynamic influences, such as the Moselle, viral indicators may pose a more reliable response variable in statistical models.

CHAPTER 4

4 Prevalence and seasonal dynamics of *bla*_{CTX-M} antibiotic resistance genes and fecal indicator organisms in the lower Lahn River, Germany

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Manuscript¹ authored by:

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Abstract

Antibiotic-resistant bacteria (ARB) represent an emerging global health problem and are frequently detected in riverine environments. Thus, it is of public interest to analyze the occurrence of respective antibiotic resistance genes (ARG) in rivers, as it will take a step towards understanding origin and dissemination of these emerging contaminants via water, which is critical for devising and evaluating strategies to mitigate the spread of ARG in the environment.

Concentrations of *bla*_{CTX-M} ARG were quantified weekly over a 12-month period in Lahn River surface water at two sampling sites using quantitative real-time PCR (qPCR). ARG were analyzed in relation to previously determined concentrations of fecal indicator organisms (FIO) *Escherichia coli*, intestinal enterococci and somatic coliphages, as well as environmental factors influencing their distribution.

¹This is an author's version of a work that was published in PLOS ONE. Changes made to this version prior or during submission and publishing process are not reflected in this manuscript. A definitive version was subsequently published as Herrig, I., Fleischmann, S., Regnery, J., Wesp, J., Reifferscheid, G., Manz, W., 2020. Prevalence and seasonal dynamics of *bla*_{CTX-M} antibiotic resistance genes and fecal indicator organisms in the lower Lahn River, Germany. PLOS ONE 15(4): e0232289. DOI: 10.1371/journal.pone.0232289.

Similar seasonal patterns and strong correlations between FIO and ARG concentrations indicated identical sources of these pollutants at both sampling sites. The environmental factor rainfall proved to be more influential at the upstream site, hinting at a prominent role of surface runoff there. Notably, the level of agricultural impact on land use increased from downstream to upstream, linking increasing ARG concentrations after rainfall events to the degree of agricultural land use.

Exposure assessment revealed that even participants in non-swimming recreational activities such as boating, canoeing or fishing were at risk of incidentally ingesting potential ARB and ARG. Linear regression analyses showed that *bla*_{CTX-M} concentrations could largely be explained by fecal pollution indicated by FIO. *E. coli* provided the best estimates (75 % explained variance) of ARG concentrations at site 2, where proportions of ARG in relation to FIO were highest. Results suggest, that *E. coli* might serve as a suitable estimate for the presence of respective ARB strains. This information will be helpful in the field of water quality management.

Finally, considering that *bla*_{CTX-M} genes are ubiquitous in Lahn River and participants in bathing and non-bathing water sports are at risk of exposure, results highlight the importance and necessity of microbial water quality monitoring with an emphasis on ARB and ARG not only in designated bathing waters.

4.1 Introduction

Antibiotic-resistant bacteria (ARB) represent an emerging global health problem, which accounted for 67000 infections resulting in 33000 deaths in Europe in 2015 (Cassini et al., 2019), and a further global increase is projected for the future (O'Neill, 2016). Increasing resistance to 3rd and 4th generation cephalosporins in *Enterobacteriaceae* such as *E. coli* is subject of growing concern (Basseti et al., 2009; Hawkey and Jones, 2009; McKenna, 2013; Paterson, 2006). Cephalosporin resistant *E. coli* belong to the antibiotic resistant bacteria (ARB) with the largest human health impact (Cassini et al., 2019). Resistance to cephalosporins,

carbapenems and also penicillins in *Enterobacteriaceae* is mainly mediated by beta-lactam hydrolyzing enzymes (extended spectrum beta-lactamases, ESBL), of which CTX-M-type enzymes, encoded by *bla*_{CTX-M}, are the most common ESBL in Germany (Pfeifer et al., 2013). *E. coli* represents the dominant host of *bla*_{CTX-M} genes (Carattoli, 2009; Livermore et al., 2006; Robin et al., 2017).

ARB and their respective ARG including *bla*_{CTX-M} are increasingly detected outside clinical settings in various surface waters including rivers (e. g., Blaak et al., 2015; Jørgensen et al., 2017; Leonard et al., 2015; Zhang et al., 2009; Zurfluh et al., 2014). They are released into aquatic environments from human and animal sources (Baquero et al., 2008). Studies emphasized that rivers comprise long distance transport and dispersal routes for ARB and ARG (Okeke and Edelman, 2001; Rath and Patra, 2018) and pose transmission pathways to humans considering their manifold use for recreational activities, drinking water abstraction, and crop irrigation. An increased risk of (re)transfer of ARB to humans through contact with surface water or waste water was shown in previous studies (e. g. Iversen et al., 2004; Laurens et al., 2018). As ARG proliferate among bacteria through horizontal gene transfer, human pathogens can acquire antibiotic resistance from non-pathogenic microorganisms and vice versa in natural environments under favorable conditions (e.g. Baquero et al., 2009; von Wintersdorff et al., 2016). Although being recognized as a collecting vessel of antimicrobial resistance, knowledge about the factors governing the spread of ARB and ARG in the aquatic environment is still incomplete (Berendonk et al., 2015; Larsson et al., 2018). Relative contributions of different sources and human health impacts caused by exposure to environmental resistant bacteria have also been identified as areas urgently needing research (Larsson et al., 2018).

Yet, ARG are important markers for tracking the spread of antibiotic resistance in the environment and characterizing associated human health risks. In particular, *bla*_{CTX-M} genes that are frequently identified in *E. coli* as well as *E. coli* itself might serve as meaningful indicators to trace antibiotic resistance in the environment (Berendonk et al., 2015; Tacão et al., 2012).

To gain more information on ARG prevalence and dynamics in effluent receiving surface waters and the contribution of fecal pollution sources, the relationship of *bla*_{CTX-M} gene and FIO concentration (i.e., *E. coli*, intestinal enterococci, and somatic coliphages) and their mutual dependence on environmental driving factors were investigated at two riverine sampling sites of Lahn River, Germany, characterized by different degrees of waste water impact. Corresponding data of FIO and environmental parameters were obtained from a previous study by (Herrig et al., 2015).

Because Lahn River is very popular for waterborne recreation especially boating and canoeing (BMW, 2016), a potential exposure of recreational water users was assessed using water ingestion rates published in literature (Dorevitch et al., 2011; Dufour et al., 2006; Rijal et al., 2011). To indicate risks of exposure, the possibility of using 'classic' fecal indicator bacteria (FIB) to monitor water quality with respect to antibiotic resistance would be extremely convenient as FIB concentrations are widely measured in routine monitoring schemes according to standardized protocols. Thus, the ability of FIB to depict the dissemination of ARG was examined.

4.2 Materials and Methods

4.2.1 Study site

The study area is located at the lower stretch of Lahn River, a tributary of the Rhine River (Herrig et al., 2015). Lahn River with its source in the low mountain range Rothaar Mountains, North Rhine-Westphalia (Germany) has a total length of 245 km, a catchment area of approximately 5900 km² and flows into the Rhine south of Koblenz. The river is impounded by multiple weirs and locks affecting the river's discharge regime. Its mean annual discharge is approximately 46.6 m³/s (BfG, 2019). The study area is mostly surrounded by forested slopes, as well as narrow strips of meadows and pastures at the valley bottom. Agricultural areas are concentrated on the heights surrounding the valley; they are less prevalent in the river valley. Within the studied area, the degree of agricultural impact increases upriver, i.e., from West to East (<https://geoportal-wasser.rlp->

umwelt.de/servlet/is/8262/; accessed 04 September 2019). Although the lower Lahn valley is considered a rural environment, the proportion of municipal waste water effluent at the studied river stretch is in the range of 10-20 % during average flow conditions and greatly exceeds 50 % under low flow conditions (Drewes et al., 2018). The river is predominantly used by smaller motor yachts, as well as paddle- and rowboats. Other recreational activities along this river stretch include fishing, canoeing, or water skiing (BMW, 2016).

Sampling site 1 near the small town of Nievern (population of ~1000) is located approximately 1 km downstream of a municipal waste water treatment plant (WWTP) outfall in the town of Bad Ems (population of ~9300) and thus more directly effluent-impacted compared to site 2. The WWTP with a treatment capacity of 33,000 person equivalents applies conventional treatment (i.e., tertiary treatment) and receives raw waste water corresponding to 27,828 person equivalents from the surrounding municipalities (<https://geoportal-wasser.rlp-umwelt.de/servlet/is/2025/>; accessed 04 September 2019). Sampling site 2 is located 18 km upstream of sampling site 1 in the tiny town of Obernhof (population of ~375). Upstream of sampling site 2, no immediate municipal effluent outfalls or tributaries discharge into Lahn River over a stretch of approximately 9 km.

4.2.2 Collection of samples for molecular analyses

The collection of surface water samples for molecular analyses on a weekly basis between October 2011 and December 2012 was part of a broader monitoring campaign (Herrig et al., 2015) at Lahn River. Grab samples at sampling sites 1 ($n = 46$) and 2 ($n = 51$) were collected into sterile glass bottles from a depth of approximately 0.3 m below water surface and 1 m off the shore using a telescopic stick. Samples were transported to the laboratory in an ice chest and were immediately processed upon arrival. Sample volumes of 100 mL to 250 mL (depending on the amount of suspended particles) were filtered through cellulose acetate membrane filters with 0.2 μm pore size to retain bacteria for ARG

analysis. The membrane filters with retained material were immediately placed in individual Cryo tubes and were frozen at -80 °C for further molecular analyses.

4.2.3 DNA extraction and quantitative real-time-PCR

Genomic DNA was extracted from the cellulose acetate filters using the DNeasy PowerWater DNA extraction Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Extracted DNA was kept at -20 °C until further analysis (i.e., less than 3 months). Previous research demonstrated that freezing and extended storage of samples at -80 °C does not alter ARG profiles compared to respective fresh samples (Li et al., 2018).

SYBR Green qPCR was used to quantify *bla*_{CTX-M} genes encoding resistance to beta-lactam antibiotics and performed similar to (Marti et al., 2013). All qPCR assays were conducted on an Mx3005P system (Agilent Technologies, Santa Clara, USA). Each reaction was carried out in triplicate in a total volume of 30 µl, containing 20 µL of Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix (Agilent Technologies), 3 µl of template DNA and forward and reverse primers (biomers, Ulm, Germany) in end concentrations of 300 nM for each primer. The final volume of 30 µl was completed with the respective amount of ultrapure water. Primers used (forward primer: CTATGGCACCACCAACGATA, reverse primer: ACGGCTTTCTGCCTTAGGTT) were originally published by Kim et al. (2005) and modified by Marti et al. (2013). Cycling conditions consisted of one cycle at 95 °C for 3 min followed by 45 cycles at 95 °C for 15 s and 20 s at 60 °C. To verify the specificity of PCR products, melting curve analyses were performed immediately after amplification in the range of 60 °C to 95 °C. Specificity and quality of products was additionally checked on 2.5 % agarose gels (data not shown).

DNA of *E. coli* IMT 14355, which is known to harbor *bla*_{CTX-M-3} (Büchter, 2011; Coque et al., 2002), was obtained from the Institute of Microbiology and Epizootics (Freie Universität Berlin) and was analyzed as reference material.

Standard curves comprised 10-fold serial dilutions of *E. coli* IMT 14355 DNA in the range of 10 to 100,000 copies per reaction. The required dilutions to obtain the desired copy numbers had been calculated according to the manufacturer's protocol (Applied Biosystems, 2003) based on photometrically (Implen Nanophotometer P 330) determined reference DNA concentration (ng/μL). Thereby, calculation of the mass per genome was based on the *E. coli* median total genome length of 5.142 Mb (NCBI, 2019).

The number of target gene copies per reaction was derived from the standard curves using the MxPro™ QPCR Software (Agilent Technologies). Gene copy number per 100 mL of sample volume was calculated as follows:

$$\text{copies per 100 mL} = \left(\frac{\text{copies per reaction}}{\text{volume DNA per reaction}} \right) \times \left(\frac{\text{volume of extracted DNA}}{\text{water sample volume}} \right) \times \text{reference volume}$$

Negative and positive controls were included in each run. Negative controls contained all the ingredients of the reaction mixture while template DNA was replaced by ultrapure water. Positive controls included DNA of *E. coli* IMT 14355.

4.2.4 Fecal indicator organism data and environmental parameters

Corresponding spatiotemporal data of FIO abundances and general surface water characteristics for both sampling sites were retrieved from a previous investigation (Herrig et al., 2015). In brief, microbiological analyses had been conducted according to standard methods as described in (Herrig et al., 2015). *E. coli* were enumerated following ISO 9308-3 (ISO, 1998b). Intestinal enterococci were analyzed by membrane filtration methods according to ISO 7899-2 (ISO, 2000a). Procedures for somatic coliphage enumeration were carried out by means of a plaque assay as described in ISO 10705-2 (ISO, 2000b). FIO counts were expressed as MPN/100 mL (*E. coli*), CFU/100 mL (enterococci) and PFU/100 mL (coliphage).

General water characteristics including water temperature, specific conductivity, pH, turbidity, dissolved oxygen (O₂) and chlorophyll-*a* had been

measured in situ with a YSI 6600 V2 multiparameter sensor (YSI, USA) throughout the broader monitoring campaign (Herrig et al., 2015). Spectrophotometric measurements of nutrient concentrations (Xion 500, Hach-Lange, Germany) relied on ready-to-use cuvette tests (Hach-Lange, Germany). Global solar irradiance and precipitation data of nearby weather stations reported as daily totals and daily mean water discharge at gauge Kalkofen originated from Rhineland-Palatinate rural area service centers and the Federal Institute of Hydrology, respectively (Herrig et al., 2015).

4.2.5 Statistical analyses

All statistical analyses were performed using the statistical software R (R Core Team, 2018). Individual Spearman's rank correlations as well as principle component analyses (PCA) were run to identify relationships between gene and indicator concentrations and environmental parameters as well as for identification of seasonal patterns. Assignment of seasons (spring, summer, fall, winter) followed the astronomical beginning of seasons for the Central European Time Zone (UTC+1). Samples containing ARG concentrations below the limit of quantification (LOQ) of 10 copies per reaction were excluded from the statistical analyses. PCA and Spearman's rank correlations were performed on z-standardized data. FIO concentrations and ARG concentrations were log₁₀ transformed and linear regression was performed on the whole dataset including concentration data of both sites using the *lm()* function in R. ARG concentrations in surface water were predicted on the basis of *E. coli*, intestinal enterococci and somatic coliphages by the linear model using the function *predict()* in R.

4.2.6 Exposure and risk assessment

Assuming that 32 % to 48 % (Blaak et al., 2011) of *E. coli* detected in freshwater are antibiotic resistant, theoretical minimum, average and maximum concentrations of resistant *E. coli* in Lahn River were calculated, based on minimum, average and maximum *E. coli* concentrations measured in Lahn during the study period (Herrig et al., 2015). Similar calculations were performed to

estimate minimum, average and maximum theoretical concentrations of ESBL-producing *E. coli* in Lahn River, assuming, that ESBL-producing *E. coli* represent 0.05 % (Blaak et al., 2014) to 1.7 % (Haberecht et al., 2019) of total *E. coli* in freshwater. Taking into account that 8.5 % of all ESBL-producing *E. coli* in surface water can be suspected gastrointestinal pathogens (Franz et al., 2015), theoretical minimum, average and maximum numbers of ESBL-producing diarrheagenic *E. coli* in Lahn River were calculated. Subsequently, human exposure was estimated for total *E. coli*, antibiotic-resistant *E. coli*, ESBL-producing *E. coli*, diarrheagenic ESBL-producing *E. coli* as well as for *bla*_{CTX-M} genes using ingestion rates from literature (Dorevitch et al., 2011; Dufour et al., 2006; Rijal et al., 2011). Ingestion rates of bacteria and genes per hour as well as bacteria and genes per water sport session were calculated, using data about average times people spend with a particular water sport. Exposure assessment was conducted for non-swimming water sports including boating, fishing, rowing, canoeing, kayaking (Dufour et al., 2006; Rijal et al., 2011), and for swimming (Dorevitch et al., 2011). Dufour et al. (2006) reported mean water ingestion rates during active swimming in a swimming pool of 16 mL/45 min (49.33 mL/h) for adults and 37 mL/45 min (21.33 mL/h) for children. Rates were determined by measuring cyanuric acid as a tracer of pool water in swimming pools and human urine over the next 24 hours after 45 minutes of swimming. It was suggested that these volumes may also apply to swimming in fresh water. As the study did not evaluate the time people spend on average with swimming in freshwater environments, these data were obtained from (Schets et al., 2011). Schets et al. (2011) found out by questionnaires that visits at freshwater sites lasted up to 79 minutes (1.32 h). The extent to which participants in boating, fishing and canoeing may be exposed via incidental ingestion of water was estimated based on median ingestion rates (50th percentile) published by (Rijal et al., 2011), who used a probability density function for water consumption to calculate ingestion rates. They assumed durations of 4 h for boating and fishing and 2.6 h for canoeing. Dorevitch et al. (2011) reported ingestion rates for boating, canoeing, fishing, rowing and kayaking of 3.7 mL, 3.9 mL, 3.6 mL, 3.5 mL, and 3.8 mL, respectively based on questionnaires. For reasons of

comparability, ingestion rates for boating, canoeing and fishing by Dorevitch et al. (2011) were used together with the respective durations specified by (Rijal et al. (2011) (i.e. 4 h for boating and fishing, 2.6 h for canoeing).

Risk assessment was conducted using established beta-Poisson dose–response models, as shown in equation (1) (Haas et al., 1999), to calculate the probability of infection after exposure to presumptive ESBL-carrying diarrheagenic *E. coli*.

$$P_{(response)} = 1 - \left[1 + dose \frac{\left(\frac{1}{2\alpha-1} \right)}{N_{50}} \right]^{-\alpha} \quad (\text{eq. 1})$$

$P_{(response)}$ is the probability of infection, $dose = c*V$ (where c is the assumed concentration of hazards in the water and V is the volume of water ingested). Median infectious doses (N_{50}) and slope parameters (α) were obtained from Haas et al. (1999) and DuPont et al. (1971). Haas et al. (1999) fitted a beta-Poisson model to several pooled datasets describing the disease response from non-enterohaemorrhagic strains including enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), and enteroinvasive *E. coli* (EIEC) with diarrhea as the response. Model parameters obtained from DuPont et al. (1971) describe a positive isolation in stool as the response to EIEC.

4.3 Results and Discussion

4.3.1 Prevalence of *bla*_{CTX-M} genes and fecal indicators

In accordance with their spatial proximity, both sampling sites at Lahn River were very similar in terms of their hydrological, hydrochemical, and meteorological characteristics throughout the study period in 2011 and 2012 (Tab. 4.1).

Table 4.1: Overview of parameters measured during the sampling period between October 2011 and December 2012 at both sampling sites (adapted from Herrig et al. 2015).

parameter	unit	site 1						site 2					
		median	min	max	average	SD	n (n below LOQ)	median	min	max	average	SD	n (n below LOQ)
<i>bla</i> _{CTX-M}	[copies/100 mL]	1630	349	12320	2905	3097	33 (13)	1454	412	20083	3453	4640	45 (6)
<i>E. coli</i>	[MPN/100 mL]	1579	212	27730	3571	5296	63	596	15	23670	2745	5105	64
enterococci	[CFU/ 100 mL]	252	75	10150	665	1417	60	112	3	11450	621	1622	61
coliphages	[PFU/100 mL]	1010	120	6760	1492	1539	61	615	60	8550	1531	1986	62
discharge	[m ³ /s]	19	10	248	34	45	65	19	10	248	34	45	65
water temperature	[°C]	10.2	1.1	22.6	11.3	5.9	61	9.9	0.2	22.4	11.1	5.9	61
conductivity	[µS/cm]	452	231	607	447	96	61	450	232	608	447	92	61
pH	[-]	8.2	7.0	8.7	8.1	0.3	61	8.1	7.2	8.9	8.1	0.3	61
turbidity	[NTU]	3.5	0.9	69.4	8.5	12.5	61	3.2	1.0	75.9	7.6	13.9	61
chlorophyll- <i>a</i>	[µg/L]	5.1	1.2	69.2	10.3	13.5	61	4.3	0.7	68.7	9.7	13.4	61
oxygen	[mg/L]	11.1	8.2	14.3	11.0	1.7	61	10.8	6.7	14.7	10.9	2.2	61
rainfall	[mm]	0.2	0.0	15.0	1.9	3.3	65	0.2	0.0	15.0	1.9	3.3	65
rainfall _(4d-sum)	[mm]	5.8	0.0	36.4	7.8	7.6	64	5.8	0.0	36.4	7.8	7.6	64
NO ₂ -N	[mg/L]	0.03	0.01	0.07	0.03	0.01	53	0.03	0.01	0.10	0.03	0.02	54
NO ₃ -N	[mg/L]	2.72	1.85	5.46	2.75	0.59	53	2.75	1.89	5.69	2.82	0.63	53
NH ₄ -N	[mg/L]	0.06	0.01	0.43	0.08	0.07	53	0.05	0.01	0.51	0.09	0.09	53
PO ₄ -P	[mg/L]	0.23	0.02	0.55	0.23	0.07	51	0.24	0.12	0.55	0.24	0.07	50
TN _b	[mg/L]	3.28	1.62	7.44	3.36	0.78	50	3.41	2.61	6.39	3.46	0.67	50
global solar irradiance	[Wh/m ²]	1925	177	7940	2692	2207	65	1925	177	7940	2692	2207	65
global solar irradiance _(3d-sum)	[Wh/m ²]	6655	593	22395	8322	5982	65	6655	593	22395	8322	5982	65

The river's daily mean discharge ranged between a minimum of 9 m³/s in September 2012 (low flow conditions) and a maximum of 381 m³/s in January 2012 (high flow conditions) (Fig. 4.1). Sampling events covered daily mean discharge conditions that varied between 10 and 248 m³/s, respectively (Tab. 4.1).

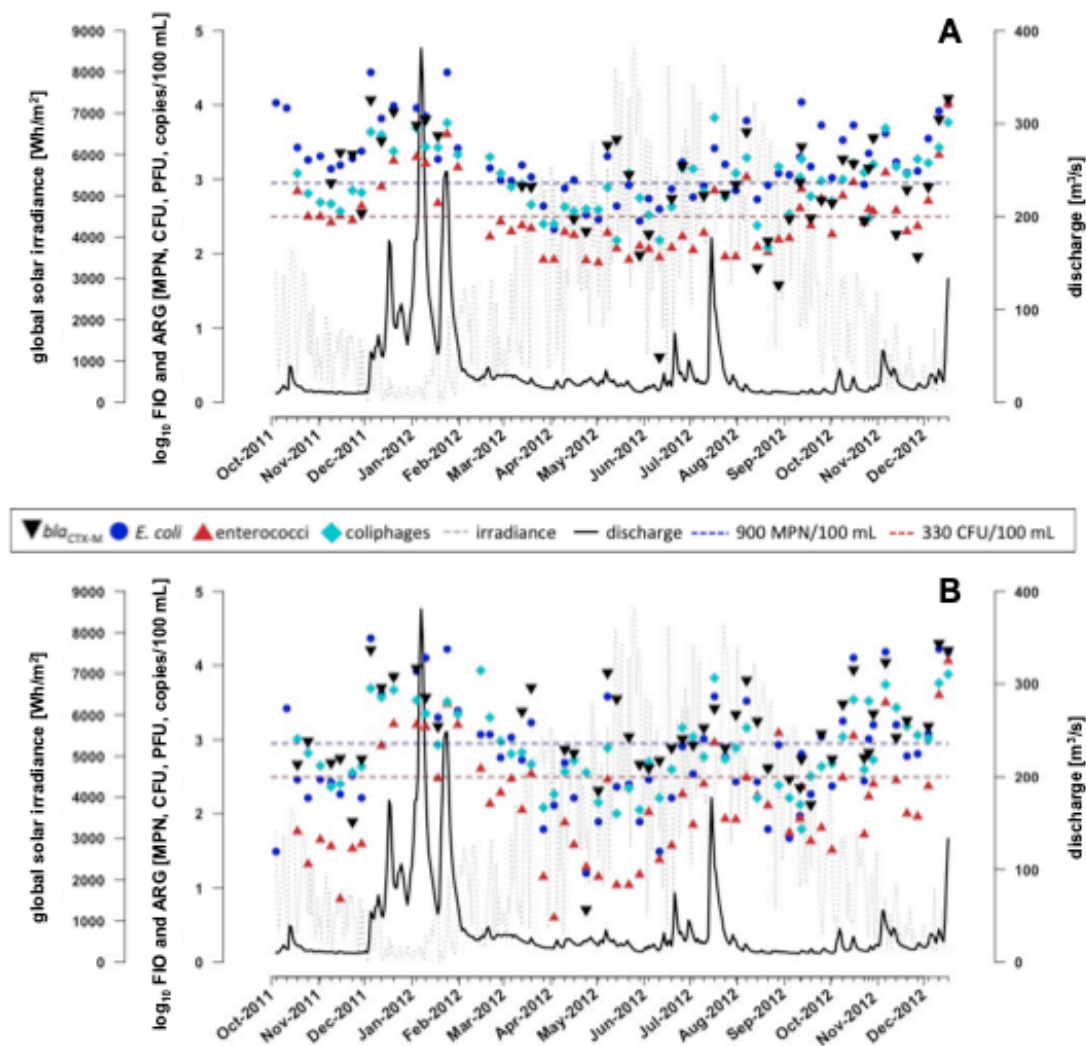


Figure 4.1: \log_{10} -transformed concentrations of FIO *E. coli*, intestinal enterococci, somatic coliphages and *bla*_{CTX-M} genes in relation to global solar irradiance and discharge throughout the study period from October 2011 until December 2012 at site 1 (A) and 2 (B), respectively.

FIO (Herrig et al., 2015) and ARG *bla*_{CTX-M} were detected in all water samples at both sites throughout the year. Highest average FIO concentrations were detected for *E. coli*, while enterococci average concentrations were lowest. Measured *bla*_{CTX-M} concentrations ranged between 3.49×10^2 and 2.01×10^4 copies/100 mL (Tab. 4.1).

The coefficients of determination of standard curves in qPCR experiments ranged from $R^2 = 0.994$ to 0.999 and efficiencies between 90.3 % and 92.3 % were obtained over at least 5 orders of magnitude in all qPCR assays (Appendix:

Fig. A.1), confirming the validity of the assay. At sampling site 1 in Nievern, 13 out of 46 observations of *bla*_{CTX-M} genes were below the LOQ of 10 copies per reaction. At sampling site 2 in Obernhof, only 6 out of 51 samples analyzed were below LOQ. In total, annual *bla*_{CTX-M} concentrations did not differ significantly between both sites (Fig. 4.2 A). At site 2, relative proportions of ARG expressed as the ratio of ARG and individual FIO were elevated compared to site 1, in particular for *E. coli* and enterococci (Fig. 4.2 B).

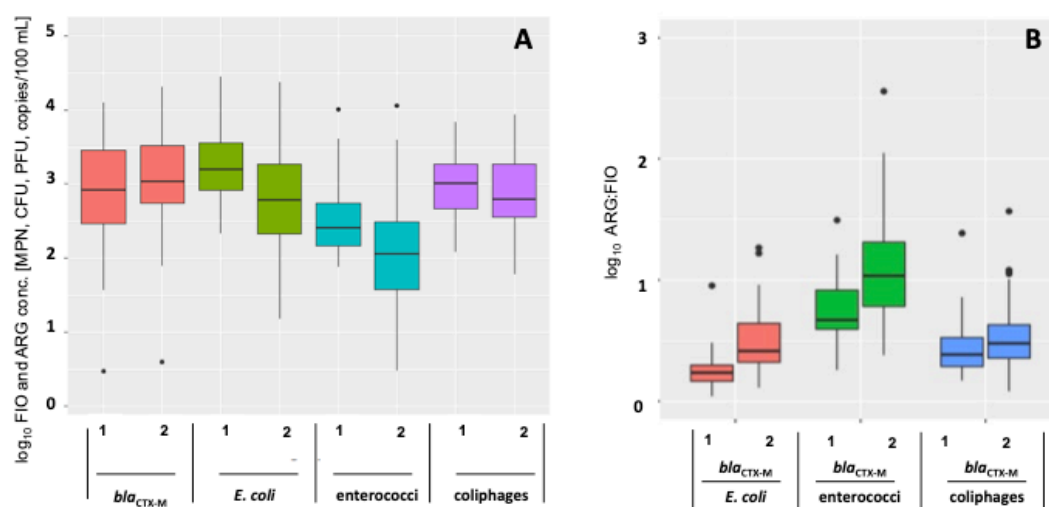


Figure 4.2: Concentrations of FIO *E. coli*, intestinal enterococci, somatic coliphages and *bla*_{CTX-M} ARG (A) measured in Lahn River surface water samples throughout the sampling period at sites 1 and 2 and ARG normalized to FIO concentrations (B).

4.3.2 Relations between ARG, fecal indicators and environmental parameters

PCA and individual Spearman's rank correlations revealed relations between ARG, FIO and environmental data, as well as seasonal patterns. FIO were strongly correlated with ARG and among each other (Tab. 4.2) and generally exhibited similar variation patterns over the year (Fig. 4.1). This is an indication that ARG and FIO originate from the same pollution sources and are distributed within the river in similar ways. Interestingly, *bla*_{CTX-M} genes correlated strongest with bacteria concentrations at site 2 (Tab. 4.2). Among the bacterial indicators, strongest correlations were observed between *bla*_{CTX-M} and *E. coli* at both sampling sites, corroborating current findings of other studies (Gekenidis et al.,

2018; McConnell et al., 2018) and reflecting that *E. coli* is the dominant host of *bla*_{CTX-M} genes (Carattoli, 2009; Robin et al., 2017).

Table 4.2: Individual Spearman's rank correlations between the fecal indicator and ARG concentrations and environmental parameters (color gradient indicates strength of correlation with positive correlations in blue, negative correlations in purple, significant ($p < 0.05$) correlations in bold).

	site 1 (n=25)				site 2 (n=35)			
	<i>bla</i> _{CTX-M}	<i>E. coli</i>	enterococci	coliphages	<i>bla</i> _{CTX-M}	<i>E. coli</i>	enterococci	coliphages
<i>bla</i> _{CTX-M}		0.76	0.71	0.78		0.88	0.81	0.73
<i>E. coli</i>	0.76		0.91	0.75	0.88		0.93	0.79
enterococci	0.71	0.91		0.71	0.81	0.93		0.78
coliphages	0.78	0.75	0.71		0.73	0.79	0.78	
discharge	0.55	0.29	0.29	0.50	0.69	0.65	0.66	0.72
water temperature	-0.43	-0.47	-0.56	-0.43	-0.58	-0.60	-0.53	-0.54
conductivity	-0.17	0.11	0.11	-0.12	-0.42	-0.30	-0.30	-0.49
pH	-0.22	-0.19	-0.31	-0.09	-0.40	-0.41	-0.41	-0.43
turbidity	0.58	0.38	0.32	0.65	0.30	0.29	0.33	0.44
chlorophyll- <i>a</i>	0.05	-0.24	-0.25	-0.09	-0.19	-0.34	-0.26	-0.32
dissolved oxygen	0.48	0.36	0.44	0.43	0.53	0.52	0.47	0.46
rainfall	0.13	0.33	0.28	0.03	-0.01	0.14	0.12	0.03
rainfall _(4d-sum)	0.69	0.56	0.58	0.54	0.56	0.65	0.64	0.52
NO ₂ -N	0.54	0.36	0.35	0.55	0.42	0.45	0.42	0.53
NO ₃ -N	0.35	0.50	0.64	0.34	0.33	0.41	0.42	0.35
NH ₄ -N	0.69	0.73	0.73	0.62	0.61	0.74	0.69	0.64
PO ₄ -P	0.07	0.08	0.02	-0.05	0.01	0.07	0.03	-0.03
TN _b	0.17	0.42	0.55	0.26	0.44	0.50	0.47	0.43
global solar irradiance	-0.43	-0.65	-0.77	-0.51	-0.44	-0.55	-0.51	-0.48
global solar irradiance _(3d-sum)	-0.51	-0.68	-0.78	-0.57	-0.58	-0.72	-0.66	-0.62

All measured environmental parameters, except PO₄-P correlated with at least one FIO or ARG and can thus be considered as potentially relevant for their fate and transport. Levels of FIO and ARG increased with discharge, turbidity, dissolved oxygen, rainfall, and nutrient concentrations, while water temperature, conductivity, pH, chlorophyll-*a*, and global solar irradiance were associated with a decline in FIO and ARG concentrations (Tab. 4.2).

Accordingly, *bla*_{CTX-M} concentrations showed strong seasonal alterations and varied over nearly 2 orders of magnitude (Tab. 4.1, Fig. 4.1). High concentrations of FIO and ARG were measured predominantly during fall and winter which were characterized by high discharge following rainfall events, elevated oxygen levels due to cold water temperatures, elevated turbidity due to resuspension and runoff, and rising $\text{NH}_4\text{-N}$ contents, indicating an influence of waste water. During spring and summer characterized by increasing global solar irradiance, accompanied by rising water temperature and chlorophyll-*a* levels, FIO and ARG concentrations declined (Fig. 4.1, Fig. 4.3). Water temperature, global solar irradiance (3d-sum), turbidity, $\text{NH}_4\text{-N}$ and discharge were environmental parameters that contributed most to explained variance in PCA (Fig. 4.3). Similar relationships had been demonstrated for FIO concentrations at rivers Rhine and Moselle and dependencies between environmental parameters and FIO were extensively discussed in previous studies by Herrig et al. (2015) and Herrig et al. (2019).

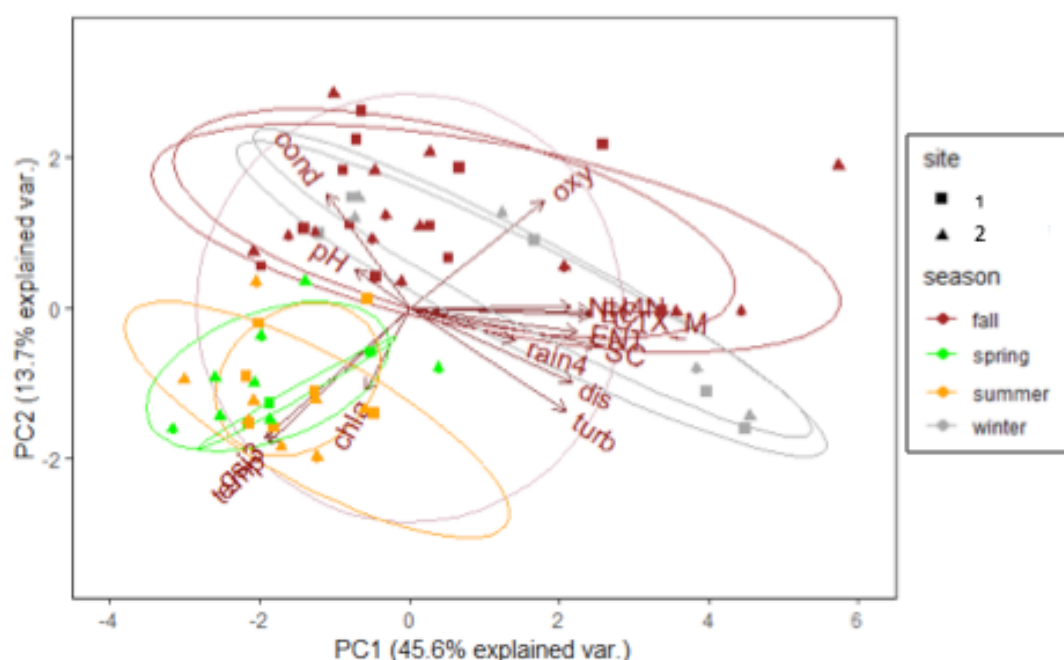


Figure 4.3: Principle component analyses of ARG, FIO and environmental parameters of site 1 and 2 with correlation circle grouped by season (ellipses).

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A comparison of PCA results between sites 1 and 2 showed similar contributions and comparable proportions of explained variance at both sites (Fig. 4.4). However, it is notable that rainfall, pH, and conductivity contributed to a greater extent to the explained variance of site 2 data (Fig. 4.4).

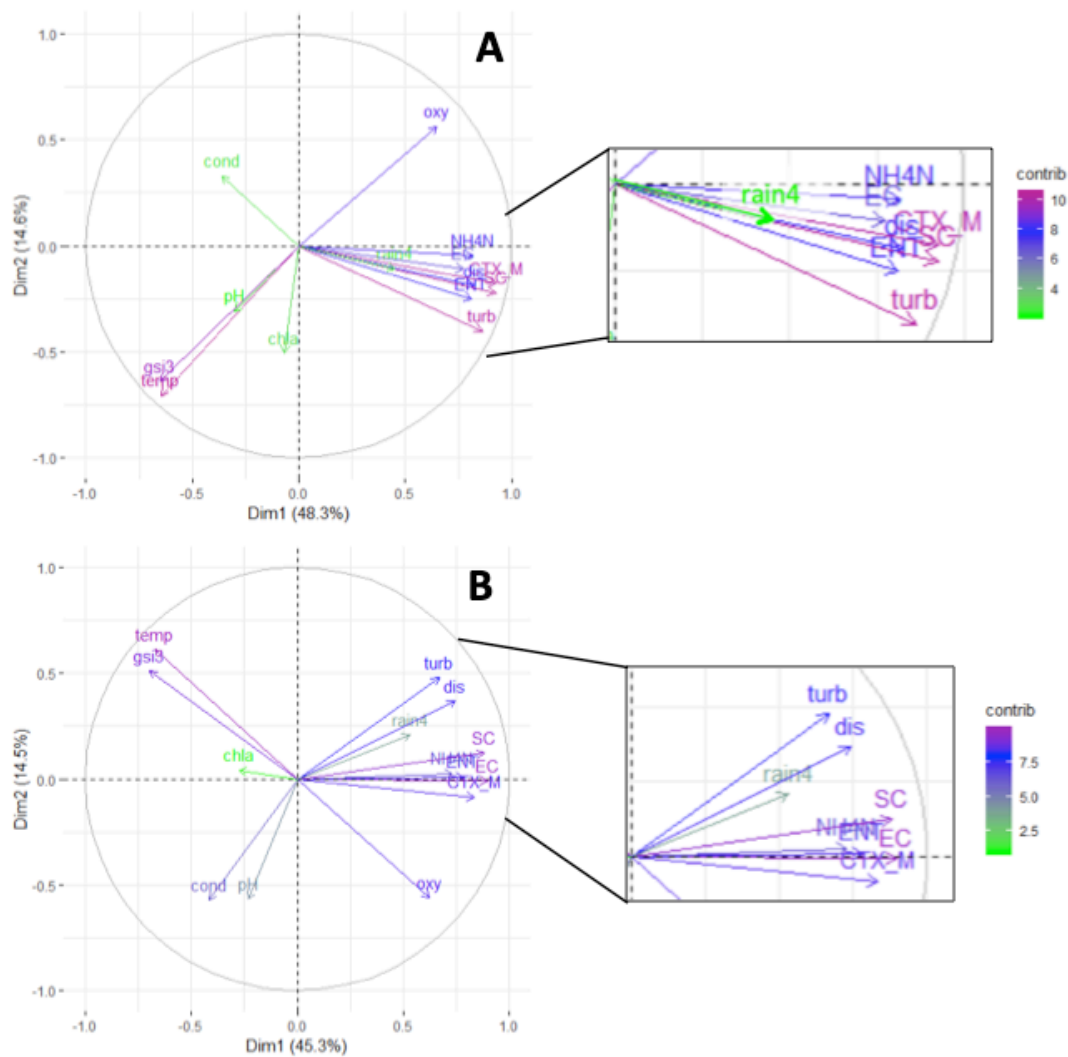


Figure 4.4: Principle component analyses of ARG, FIO and environmental parameters with correlation circle and contributions indicated by color gradient individually for site 1 (A) and site 2 (B). rain4: 4d-sum of rainfall; temp: water temperature; cond: conductivity; gsi3: 3d-sum of global solar irradiance; chl_a: chlorophyll-*a*; dis: discharge; CTX_M: bla_{CTX-M} genes; EC: *E. coli*; ENT: intestinal enterococci; SC: somatic coliphages; turb: turbidity; oxy: oxygen content; NH₄N: ammonium-N; contrib: contribution.

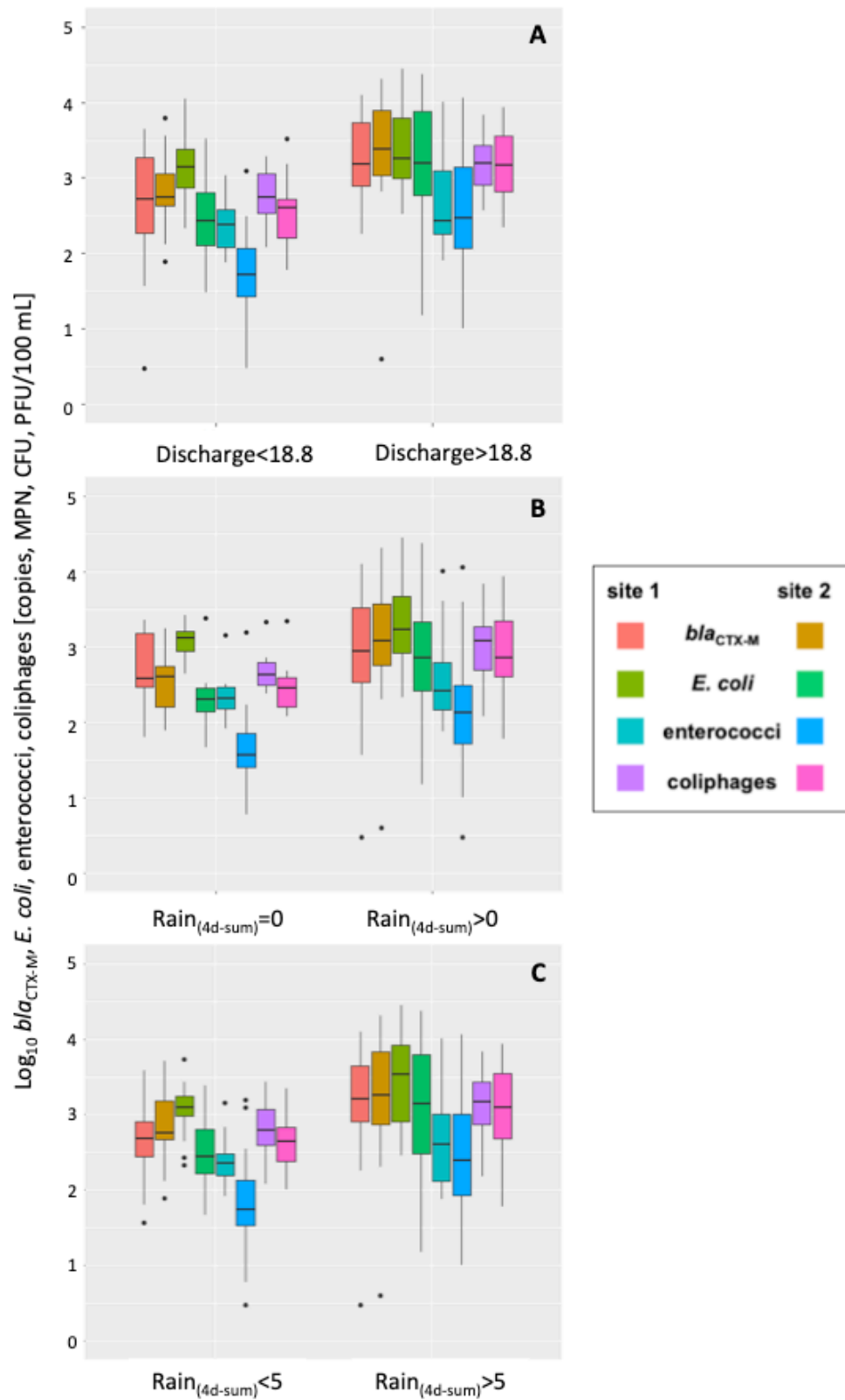


Figure 4.5: Boxplots of fecal indicator and ARG concentrations in (A) low flow periods (discharge < median of 18.8 m³/s) vs. high flow periods (discharge > median of 18.8 m³/s) (B) in dry periods (4d-sum of rainfall = 0 mm) vs. wet periods (4d-sum of rainfall > 0 mm) and (C) in periods with low rainfall (4d-sum of rainfall < 5mm) vs. high rainfall (4d-sum of rainfall > 5 mm).

A strong impact of rainfall and related discharge especially at site 2 is also shown in Fig. 4.5. During high flow periods, annual FIO and *bla*_{CTX-M} levels were comparable between both sampling sites (Fig. 4.5 A). During periods without rainfall 4 days prior sampling, FIO and *bla*_{CTX-M} concentrations tended to be higher at site 1 (Fig. 4.5 B). If precipitation was higher than 5 mm over the 4 days period prior sampling, *bla*_{CTX-M} concentrations at site 2 exceeded those measured at site 1 (Fig. 4.5 C), indicating a more prominent role of surface runoff at site 2.

4.3.3 Impact of point and non-point sources

It is well known that ARB and ARG are released into the environment from various sources including waste water discharges and agriculture (e.g. Gothwal and Shashidhar, 2015) and studies found WWTPs to significantly enhance ARG levels in rivers (Sabri et al., 2018) including *bla*_{CTX-M} (Amos et al., 2014, Marti et al., 2013). A strong correlation with NH₄-N indicates an influence of waste water on elevated levels of FIO and ARGs. But despite the close proximity of site 1 to the WWTP in Bad Ems, no direct influence of the WWTP on elevated annual total ARG levels could be observed (Fig. 4.2 A). Yet, an influence of the WWTP is illustrated by decreased relative percentages of ARG at site 1 (Fig. 4.2 B). Similar observations were made by Haberecht et al. (2019), where percentages of *E. coli* harboring ESBL resistance were lower in WWTP effluent (0.28 %) compared to surface water (1.7 %). To depict the impact of individual waste water inputs with regard to ARG levels in rivers dry-season sampling proved to be useful (Knapp et al., 2012). Are dry- and wet-weather data regarded separately, the impact of WWTP discharges becomes apparent at site 1 during periods without rainfall events and low discharge (Fig. 4.1, Fig. 4.5). Without input of pollutants by rainfall, the measured microbiological determinants substantially declined at site 2 during low flow periods between May and June, whereas their concentrations remained elevated at site 1 (Fig. 4.1, Fig. 4.5 B). Clearly, the WWTP provided a constant input of microbial pollutants at sampling site 1. However, other inputs of fecal pollution exceeding that of the municipal WWTP mask the constant effluent-related background pollution during high flow conditions.

Rainfall events likely promote a flush of (suspended) particulate matter in runoff from the agricultural areas surrounding the valley at site 2 into the river, explaining the higher impact of rainfall at this site. Trends of an increase in ARG pollution with agricultural impact were also described for other riverine environments (Chen et al., 2013). Interestingly, rainfall at site 2 increasingly impacted the *bla*_{CTX-M} genes but not FIO (Fig. 4.5 C). This may be due to different detection rates, persistence and transport properties of DNA and living cells. As ARG quantified by qPCR may comprise extracellular DNA (eDNA) (which may be retained on the cellulose acetate filters, when attached to particles) and intracellular DNA (iDNA), ARG are thus likely to be detected in higher concentrations than living cells of FIO quantified by cultivation dependent methods.

eDNA is known to adsorb to soil components including clay, sand, silt and humic substances (Pietramellara et al., 2009). In this way it is protected against degradation, which can considerably prolong its persistence in soil and sediments, which may even facilitate ARG propagation (Mao et al., 2014; Nielsen et al., 2007; Pietramellara et al., 2009). Thus, while there may be losses of viable cells during transport from source to point of detection, DNA may be detectable for a longer time. It may be transported over longer distances compared to living cells, due to its different persistence properties and adsorption to particles. Transport of DNA including ARG through soil is known to occur (Agnelli et al., 2004; Ceccherini et al., 2007; Poté et al., 2003). Even an on-site selection due to antibiotic residues in the environment, shifting ARG proportions cannot be excluded. However, to answer these uncertainties, future research is needed. Microbial source tracking could reliably identify the sources and measuring antibiotic residues in the water could reveal potential pressures triggering on-site selection.

4.3.4 Exposure and risk assessment

Nevertheless, the presence of resistances in surface water bears the risk of transfer to water users, for example by ingestion of water during water-related

recreational activities (e.g. Leonard et al., 2015). Although Lahn River is no designated bathing water, canoeing and boating in particular became very popular there in recent years (Biedenkapp and Stührmann, 2004; BMWi, 2016).

Theoretical prevalence of antibiotic-resistant *E. coli*, ESBL-producing *E. coli* and diarrheagenic ESBL-producing *E. coli* was calculated based on prevalence values obtained from the literature (Blaak et al., 2011; Franz et al., 2015; Haberecht et al., 2019; Herrig et al., 2015). Based on these results amounts of bacteria and ARG potentially ingested during various recreational activities including swimming and non-swimming activities were calculated using water ingestion rates published by Dorevitch et al. (2011), Dufour et al. (2006) and Rijal et al. (2011) (Tab. 4.3, Appendix: Tab. A.1). Reported proportions of antibiotic resistant *E. coli* isolates in rivers range between 32 and 48 % (Blaak et al. 2011). In the Meuse, 48 % of *E. coli* were resistant to at least one tested antibiotic, compared to 32 % of *E. coli* in the Rhine and 34 % of *E. coli* in the New Meuse (Blaak et al., 2011). Values are similar to antibiotic resistance in *E. coli* isolates from the Seine River in France, which was as high as 42 % (Servais and Passerat, 2009). *bla*_{CTX-M} abundance in enteric bacteria in some United Kingdom bathing waters was assumed is 0.1 % (Finley et al., 2013). This is within the range reported elsewhere in the literature. Leonard et al. (2015) found a prevalence of 3rd generation cephalosporin-resistance in *E. coli* in coastal surface waters in England and Wales of 0.12 %. In some Dutch recreational waters (including North Sea, a river and freshwater lakes), ESBL-producing *E. coli* represented 0.05–1 % of the total *E. coli* population (Blaak et al., 2014). Higher values were reported by (Haberecht et al., 2019), who found 1.7 % of *E. coli* harboring ESBL resistance in surface water of Cache La Poudre River, USA. 8.5 % of all ESBL-producing *E. coli* isolates from surface waters (including rivers, canals, lakes, North Sea) in the Netherlands were suspected diarrheagenic variants (Franz et al., 2015).

Table 4.3: Minimum, average and maximum prevalence values and numbers of total *E. coli*, antibiotic-resistant *E. coli*, ESBL-producing *E. coli* and diarrheagenic ESBL-producing *E. coli* theoretically ingested during non-swimming and swimming activities in Lahn River during the study period; (a detailed version of this table is provided in Appendix: Tab. A.1).

	activity	range	(1) total <i>E. coli</i>	(2) resistant <i>E. coli</i>	(3) resistant <i>E. coli</i>	(4) ESBL <i>E. coli</i>	(5) ESBL <i>E. coli</i>	(6) diarrheagenic ESBL <i>E. coli</i>	(7) diarrheagenic ESBL <i>E. coli</i>
prevalence [MPN/100 mL]		MIN	15	5	7	0	0	0	0
		AVG	3158	1011	1516	2	54	0	5
		MAX	27730	8874	13310	14	471	1	40
ingested per hour [MPN/h]	non-swimming ^a	MIN	0	0	0	0	0	0	0
		AVG	125	40	60	0	2	0	0
		MAX	2085	667	1001	1	35	0	3
	swimming (children) ^b	MIN	7	2	4	0	0	0	0
		AVG	1558	499	748	1	26	0	2
		MAX	13679	4377	6566	7	233	1	20
	swimming (adults) ^b	MIN	3	1	2	0	0	0	0
		AVG	674	216	323	0	11	0	1
		MAX	5915	1893	2839	3	101	0	9
ingested per session [MPN/session]	non-swimming ^c	MIN	1	0	1	0	0	0	0
		AVG	430	138	206	0	7	0	1
		MAX	5422	1735	2602	3	92	0	8
	swimming (children) ^d	MIN	10	3	5	0	0	0	0
		AVG	2057	658	987	1	35	0	3
		MAX	18057	5778	8667	9	307	1	26
	swimming (adults) ^d	MIN	4	1	2	0	0	0	0
		AVG	889	285	427	0	15	0	1
		MAX	7808	2498	3748	4	133	0	11

a) based on ingestion rates by Rijal et al. 2011, Dorevitch et al. 2011, Dufour et al. 2011, including boating, canoeing, fishing, kayaking and rowing

b) based on ingestion rates by Dufour et al. 2011

c) based on ingestion rates by Rijal et al.2011, Dorevitch et al. 2011, Dufour et al. 2011, durations by Rijal et al. 2011, including boating, canoeing, fishing

d) based on ingestion rates by Dufour et al. 2011 and average duration by Schets et al. 2011

(1) based on Herrig et al. 2015

(2) based on (1) and Blaak et al. 2011 (32 % of *E. coli* AB-resistant *E. coli*)

(3) based on (1) and Blaak et al. 2011 (48 % of *E. coli* AB-resistant)

(4) based on (1) and Blaak et al. 2014 (0.05 % of *E. coli* producing ESBL)

(5) based on (1) and Haberecht et al. 2019 (1.7 % of *E. coli* producing ESBL)

(6) based on (4) and Franz et al. 2015 (8.5 % of ESBL producing *E. coli* diarrheagenic)

(7) based on (5) and Franz et al. 2015 (8.5 % of ESBL producing *E. coli* diarrheagenic)

Results revealed that participants in swimming and non-swimming recreational activities theoretically might incidentally swallow considerable amounts of potentially resistant *E. coli* and ARG (Tab. 4.3, Tab. 4.6, Appendix: Tab. A.1, Tab. A.2). Amounts depend on degree of water contact and the level of water pollution. Not surprisingly, swimming activities pose a higher risk of exposure as non-swimming water sports, especially for children (Tab. 4.3, Tab. 4.6, Appendix: Tab. A.1, Tab. A.2).

Based on the assumption, that 32 % to 48 % (Blaak et al., 2011) of *E. coli* detected in rivers may be antibiotic-resistant, participants are theoretically at risk

of ingesting 0-1001 MPN of potentially antibiotic-resistant *E. coli* per hour and 0-2602 MPN of potentially antibiotic-resistant *E. coli* per session during non-swimming activities (Tab. 4.3). Considering, that 0.05 % (Blaak et al., 2014) to 1.7 % (Haberecht et al., 2019) of *E. coli* in freshwater may carry ESBL genes, 0-35 MPN of potentially ESBL-producing *E. coli* may be ingested per hour and 0-92 MPN of potentially ESBL-producing *E. coli* may be ingested per non-swimming water sports session (Tab. 4.3). Taking into account that 8.5 % of all ESBL-producing *E. coli* in surface water can be suspected to be potential gastrointestinal pathogens, including enteroaggregative *E. coli* (EAEC) and enterotoxigenic *E. coli* (ETEC) (Franz et al., 2015), participating in swimming can theoretically result in ingestion of 0-26 MPN of potentially diarrheagenic ESBL-producing *E. coli* per children's swimming session (Tab. 4.3, Appendix: Tab. A.1). The probability of infection due to ingesting the calculated maximum possible concentration of 26 MPN (Tab. 4.3, Tab. 4.4) was estimated based on beta-Poisson dose-response-models using models from the literature to describe dose-response-relations for several non-enterohaemorrhagic strains of *E. coli* (Haas et al. 1999) and EIEC (DuPont et al., 1971) (Tab. 4.5). Independent of model parameters used, results reveal that a risk of infection is actually very small (Tab. 4.5). However, Haas (1983) clearly emphasized that even a single microorganism has the potential to cause an infection.

Table 4.4: Exposure of children to presumptive diarrheagenic ESBL-producing *E. coli* during swimming in Lahn River during times of peak concentrations

Exposure	
Parameter	
Max. assumed number of infective determinants/liter ^a	400
Water ingestion rate (swimming, children) [mL/h] ^b	49.33
Average duration swimming session [h] ^c	1.32
Volume ingested per event [L]	0.065
Dose = contact rate (L/session) x concentration [number/L]	26
a) based on prevalence values by Herrig et al. 2015, Blaak et al. 2011, Haberecht et al. 2019, Franz et al. 2015; b) Dufour et al. 2006; c) Schets et al. 2011	

Table 4.5: Risk assessment for infection with presumptive diarrheagenic ESBL-producing *E. coli* for children during swimming in times of peak concentrations

Risk assessment		
Reference	Haas et al. 1999	DuPont et al. 1971
Host type	Human	Human
Pathogen type	Non-enterohaemorrhagic strains including ETEC, EPEC, EIEC	EIEC 1624
Response	Diarrhea	Positive stool isolation
Best Fit Model	Beta-Poisson	Beta-Poisson
alpha	1.78E-01	1.55E-01
ND ₅₀	8.60E+07	2.11E+06
P _(response)	2.59E-06	1.65E-04

Depending on the degree of water contact and the level of water pollution, up to 1510 copies of *bla*_{CTX-M} ARG may be ingested per hour of non-swimming water sports resulting in up to 3927 copies theoretically ingested per session (Tab. 4.6). During swimming, up to 13,077 copies may be swallowed by children per session (Tab. 4.6). However, results of this study do not reveal how many of the detected genes are actually carried by specific (potentially pathogenic) bacteria. Thus results cannot relate the amount of ARG potentially ingested to an actual risk of infection.

Table 4.6: Minimum (MIN), average (AVG) and maximum (MAX) copy numbers of *bla*_{CTX-M} genes theoretically ingested during non-swimming and swimming activities in Lahn River during the study period; (a detailed version of this table is provided in Appendix: Tab. A.2).

	activity	range	<i>bla</i> _{CTX-M} genes
prevalence [copies/100 mL]		MIN	349
		AVG	3179
		MAX	20083
ingested per hour [copies/h]	non-swimming ^a	MIN	7
		AVG	126
		MAX	1510
	swimming (children) ^b	MIN	172
		AVG	1568
		MAX	9907
	swimming (adults) ^b	MIN	74
		AVG	678
		MAX	4284
ingested per session [copies/session]	non-swimming ^c	MIN	27
		AVG	435
		MAX	3927
	swimming (children) ^d	MIN	227
		AVG	2070
		MAX	13077
	swimming (adults) ^d	MIN	98
		AVG	895
		MAX	5654

a) based on ingestion rates by Rijal et al. 2011, Dorevitch et al. 2011, Dufour et al. 2011, including boating, canoeing, fishing, kayaking and rowing

b) based on ingestion rates by Dufour et al. 2011

c) based on ingestion rates by Rijal et al.2011, Dorevitch et al. 2011, Dufour et al. 2011, durations by Rijal et al. 2011, including boating, canoeing, fishing

d) based on ingestion rates by Dufour et al. 2011 and average duration by Schets et al. 2011

Results concerning infection risk and exposure to ARB as well as ARG have to be interpreted with caution as they are based on numerous assumptions. People may become exposed to the calculated maximum numbers only during times of peak pollution. These, however, occurred predominantly during fall and winter and pronounced precipitation events, when less water sport participants have to be expected.

Furthermore, the risk assessment conducted focused solely on theoretically diarrheagenic *E. coli* carrying ESBL genes and thus risks posed by total potentially pathogenic ARB present in Lahn River are most likely considerably underestimated. Also the proportionally high prevalence of *bla*_{CTX-M} genes as opposed to *E. coli* measured in Lahn River on the one hand but low ESBL prevalence in *E. coli* on the other hand suggest that the actual level of resistance

in Lahn River due to *bla*_{CTX-M} might be considerably higher than estimated based on ESBL prevalence in *E. coli*.

Moreover, transmission of ARG from the environment to humans does not occur solely by pathogens, but in particular by vector bacteria carrying ARG. Risk assessment of the transmission of vector bacteria cannot rely on the same model used for pathogens, because most vectors are not pathogens themselves (Manaia, 2017). They may colonize a healthy host without causing diseases but infection can break out, if the host experiences some degree of debilitation (Manaia, 2017). Risk assessment must thus be conducted using vector bacteria instead of their resistance genes (Manaia, 2017). Therefore, this study cannot relate the concentrations of *bla*_{CTX-M} genes measured to any infection risk. Nevertheless the presence of ARG is associated with the risk of being transferred to the human bacterial flora even by nonpathogenic harmless bacteria. There is evidence that *E. coli* can act as a vector able to transfer ARG between environment and host as well as *in vivo* (Knudsen et al., 2018; Leonard et al., 2018). Swallowing water contaminated with *bla*_{CTX-M}-bearing *E. coli* was shown to be linked to gut colonization by these bacteria in surfers (Leonard et al., 2018). In addition transfer of plasmids carrying *bla*_{CTX-M} between *E. coli* within the human gut has been described (Knudsen et al., 2018).

As participants in water related recreation at Lahn River are at risk of swallowing considerable amounts of *bla*_{CTX-M} genes and also presumptive antibiotic-resistant *E. coli*, it might be possible that participants in water sports swallowing Lahn River water could become colonized by ARB.

Moreover, extracellular DNA in the environment is bioavailable for prokaryotic competent cells for transformation (Pietramellara et al., 2009) and extracellular DNA in rivers can constitute a reservoir of ARG that could facilitate the spread of antibiotic resistance (Mao et al., 2014). For these reasons, not only ARB but also ARG themselves can be considered as critical emerging risk factors for which strategies are needed to mitigate their dissemination.

Further research is needed to close the knowledge gaps between the presence of ARG, ARB, and infection risks. Thus, the qualitative and quantitative evaluation

of the occurrence of ARG in vector bacteria in Lahn River water that are able to colonize humans should be analyzed in future research studies. To further validate the assumptions made on prevalence and ingestion rates in this study, data on proportions of *bla*_{CTX-M} carrying *E. coli* and antibiotic-resistant *E. coli* should be assessed in Lahn River water samples.

4.3.5 *E. coli* as estimator for ARG concentrations

Even if the risk of acquiring gastrointestinal infections due to ESBL-carrying diarrheagenic *E. coli* is very low, results show that a transmission of ARG and antibiotic-resistant *E. coli* to humans via contact with Lahn River surface water during recreational activities is a realistic scenario. To prevent exposure or to identify times of enhanced risks of human exposure, predictive models allowing a timely assessment of microbial pollution with ARB or ARG could be used. It has been suggested that common FIB might serve as a suitable estimate for the presence of ARB strains (Berendonk et al., 2015; Gekenidis et al., 2018). FIB are already commonly used in water quality assessment it would be beneficial, if they provide also reliable estimates for ARG and ARB, respectively.

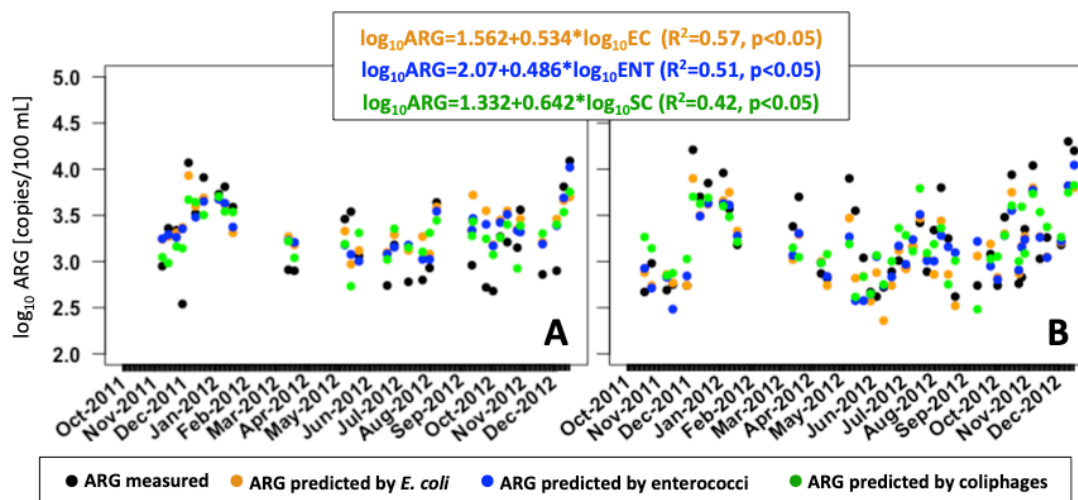


Figure 4.6: Linear model equations and measured and predicted \log_{10} ARG concentrations during the sampling period at site 1 (A) and site 2 (B).

In accordance with other studies (e.g. Karkman et al., 2019) correlation showed that the presence of ARG could largely be explained by fecal pollution (Tab. 4.2, Fig. 4.6, Fig. 4.7) indicated by FIO. Hence, the ability of FIO to explain \log_{10} ARG occurrence was investigated by simple linear regression analyses.

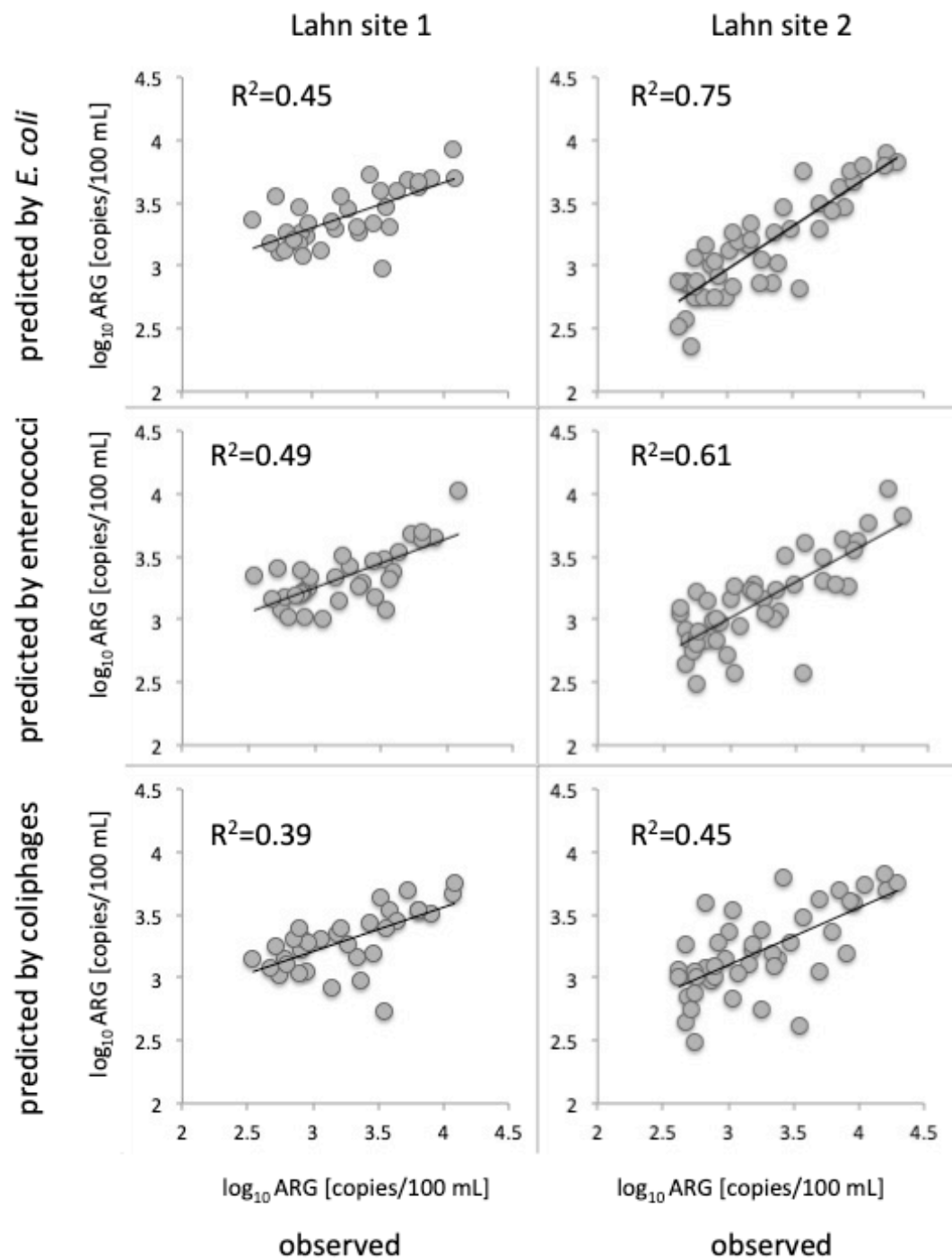


Figure 4.7: Predicted vs. observed concentrations of *bla*_{CTX-M} ARG for each individual sampling site.

Linear regression models based on FIO can explain *bla*_{CTX-M} concentrations with varying accuracy, depending on type of FIO and study site. Notably, ARG concentrations can be estimated by FIO with a considerably higher accuracy at site 2 (Fig. 4.7). With three-quarters (75 %) of variance explained, *E. coli* provided

the best estimates for ARG in Lahn River, at least at site 2. There, proportions of *bla*_{CTX-M} genes in relation to *E. coli* were highest (Fig. 4.2, Fig. 4.7), potentially explaining the better accuracy at this site.

ARG and FIO were shown to be similarly distributed and were influenced by environmental factors in a similar way. Thus, multiple linear regression models (MLR) as previously established for the prediction of FIO based on environmental factors (Herrig et al., 2015; Herrig et al., 2019; Seis et al., 2018) may also be suitable for the prediction of ARG. Future research should also focus on testing, if such model approaches can be combined with the findings of this study and be adapted to also cover ARG. Despite the limitation that the amount of ARG and FIO ingested cannot be linked to an actual risk of infection, results demonstrate a potential health risk associated with microbial water quality and water related recreational activities and highlight the relevance of microbial water quality assessment also with respect to antibiotic resistances, even when the water body is not designated as bathing water. Future research should thus focus on human risk assessment under these exposure scenarios and prevention of associated health risks. To close the knowledge gap between the prevalence of *bla*_{CTX-M} genes and the actual level of antibiotic resistance in Lahn River, a variety of bacterial isolates should be tested for antibiotic resistances and correlations between *bla*_{CTX-M} genes should be analyzed. Results could strengthen the assumptions made on the suitability of *bla*_{CTX-M} genes as proxy for the total level of antibiotic resistance in a river environment.

4.4 Conclusions

- ▶ *Bla*_{CTX-M} genes are omnipresent in Lahn River surface water.
- ▶ FIO and ARG follow a similar overall trend and vary according to temporal variations in hydro-meteorological factors.
- ▶ Influence of WWTP discharge is visible as elevated ARG levels under low flow conditions and decreased relative proportions of ARG as opposed to *E. coli*.

- ▶ Results revealed a trend of an increase in total concentrations of *bla*_{CTX-M} genes with the degree of agricultural impact after rainfall events.
- ▶ Participants in swimming and non-swimming recreational activities (i.e. boating, fishing, canoeing) are at risk of ingesting potentially resistant FIO and ARG.
- ▶ ARG abundances can largely be explained by fecal pollution, with *E. coli* providing the best estimates. This information will be helpful in the fields of risk assessment and water management.

Acknowledgements

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CHAPTER 5

5 Final conclusions

5.1 Influence of environmental factors and potential impact of climate change

The first overarching goal to gain insights into the influence of environmental factors on fecally derived microbial pollution including ARG in rivers was achieved by in depth examinations of extensive monitoring data of three different rivers (Fig. 5.1).

Microbial pollution was studied in rivers Rhine, Moselle and Lahn on the basis of commonly used fecal indicator organisms (FIO) indicating bacterial (*Escherichia coli*, intestinal enterococci) and viral (somatic coliphages) fecal contamination. As markers for tracking the spread of antibiotic resistances in the aquatic environment, *bla*_{CTX-M} genes were analyzed exemplarily at two sites of river Lahn. The impact of changes in climate-related parameters on seasonal distribution of fecal organisms was examined by studying monitoring results of contrasting flow conditions at rivers Rhine and Moselle.

Analyses at Rhine, Moselle and Lahn altogether clearly indicated that high discharge and precipitation enhance the influx of FIO, antibiotic resistance genes (ARG) and thus potentially (antibiotic-resistant) pathogens into waters. In contrast, a decrease in hygienic microbial pollution was observed under high solar irradiation and increasing water temperatures.



Figure 5.1: Schematic illustration of thesis outline with brief descriptions of the main findings and corresponding publications

Comparative analyses of FIO in relation to ARG allowed conclusions on similarities in their distribution patterns and on potential sources. *Bla_{CTX-M}* ARG, which were omnipresent in Lahn River water correspond to the distribution patterns of FIO, indicating fecal sources. Besides precipitation, agriculture and WWTP effluents contributed to increasing ARG loads in lower Lahn River. ARG distribution is influenced by environmental factors in a similar way as FIO distribution. Results highlight the need to be aware of the threat of antimicrobial resistance in rivers used in multifunctional ways and to apply best practice in

agriculture and waste water treatment in order to mitigate the spread of antibiotic resistance in the environment.

The interaction between abiotic and biotic factors (i.e. enhanced grazing pressure) was shown to considerably contribute to the formation of seasonal patterns of indicator concentrations, whereby bacteria were stronger affected than phages. This particularly applies to rivers with prolonged water residence time and thus less pronounced hydrodynamic impacts, which enhance phytoplankton blooms accompanied by elevated grazer densities (e.g. Moselle). Seasonal variations in FIO concentrations were further enhanced by low flow conditions.

It is expected that the frequency of heavy precipitation events and the magnitude of surface runoff across Europe will increase over the next decades (EASAC, 2013; Semenza and Menne, 2009). Furthermore, temperatures will continue to rise in the future causing more frequent periods of very warm weather (e.g. Patz and Kovats, 2002) resulting in periods of low water levels. As a consequence, changing discharge and precipitation patterns will influence microbial water quality of rivers. Floods caused by heavy rainfall events lead to elevated levels of FIO in the river sections examined. As shown by the results of rainfall event associated samples of Lahn River (Fig. 5.2), microbial pollution peaks can be expected before the actual flood peaks. Moreover, heavy precipitation, especially after longer dry periods, can cause a severe increase of FIO in the water. Known as the first flush effect, this phenomenon is based upon the assumption that a significant amount of pollution accumulated in the catchment is washed-off during the initial period of storm events (Cinque and Jayasuriya, 2010; McCarthy, 2009; Taebi and Droste, 2011). While these pollution peaks in rivers will be of short-term duration, they will occur more frequently. Short-term pollution events bear the risk of potential hazards, especially with regard to recreational water uses, as rapid changes in pollution levels are likely to be missed by regular bathing water quality monitoring according to Bathing Water Directive (BWD).

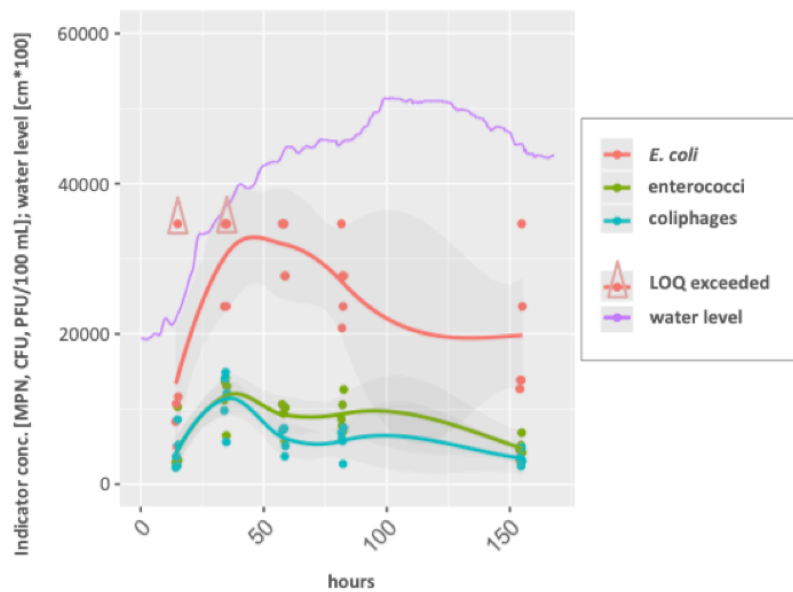


Figure 5.2: FIO concentrations (circles) measured at 5 sites in river Lahn during a rainfall event accompanied by rising water levels between 01-29-2013 and 02-04-2013. A locally weighted scatterplot smoothing (LOESS) function (lines) was used to discern trends in indicator concentrations. Triangles indicate *E. coli* concentrations higher than the limit of quantification (LOQ) of 34660 MPN/100 mL.

Furthermore, increasing water temperatures decrease the oxygen-carrying capacity of water and contribute to enhanced algal growth, which is expected to increase in the future (Poff et al., 2002). Enhanced algal growth, indicated by chlorophyll-*a* content in this study, was observed associated with low water levels in particular in Moselle and also in the Rhine during the low flow period in 2010/11. In summer 2018 it peaked in a widespread cyanobacteria bloom during extraordinary low water levels, especially in the Moselle (Belz et al., 2018). These conditions are likely to occur more frequently, resulting in more frequent bathing site closures or restrictions of water uses not only after rainfall events but also during dry periods.

Changes in environmental conditions indirectly also influence the reliability of fecal indicators. For the studied rivers it was shown that viral and bacterial indicators exhibit distinct behavioral patterns due to varying sensitivities primarily to grazing pressure and temperature in particular in spring and winter. Even differences between the bacterial indicators themselves were observed especially in river Moselle under low flow conditions. Hence, especially in slow flowing rivers with pronounced trophic interactions, abundances of viruses are

underestimated when relying on bacterial indicators alone. Thus, in accordance with other studies (e.g. Schets et al., 2018; Rodrigues and Cunha, 2017) it has to be concluded that bacteriological indicators do not always adequately reflect the risk of exposure to viruses in the water. Yet, the assumption that enterococci may better depict the distribution of viruses (Rodrigues and Cunha, 2017; Wade et al., 2006) was not confirmed in this study. Currently, fecal viral indicators are neglected under the BWD microbial water quality assessment.

According to the results of this study, rising water temperature during summer as one side effect of climate change is not expected to increase FIO levels in the studied rivers. In case of potential growth of FIO in the environment due to warming water temperatures (Rodrigues and Cunha, 2017), it is likely to be outnumbered by enhanced grazing activities in spring/summer and the negative impact of global solar irradiance. However, this finding cannot be unrestrictedly transferred to all pathogens in all aquatic environments, as fecally derived indicators do not represent the variety of pathogens of non-fecal origin, for example such as *Vibrio vulnificus*. *V. vulnificus* related incidences are expected to increase in the future because of predicted global warming in coastal regions of Northern Europe (Baker-Austin et al., 2012; Baker-Austin et al., 2017), posing serious challenges in terms of health protection and waterborne tourism in coastal areas.

5.2 Prediction of microbial water quality in rivers

5.2.1 Development and transfer of modeling approaches

The second overarching goal of this study to develop tools for a timely prediction of microbial water quality in rivers was achieved by the establishment of multiple linear regression (MLR) model approaches. Based on contributing key factors identified, MLR models for river Lahn were established that allow a timely assessment of fecal indicator concentrations, address bacterial and viral indicators, are applicable to several sites within a river stretch, are kept simple and as uniform as possible with respect to indicator types and explanatory variables and do thus not require time and resource intensive data collection.

While the models could be harmonized for indicator bacteria in terms of explanatory variables, different explanatory variables were needed for coliphages, emphasizing a different behavior of phages and bacteria in the water. Even if models generally tend to be site-specific, yet, a large-scale application at 5 sites of a river stretch was possible with only minor losses of accuracy. Thus, MLR model development for various sites requires a compromise between application scale and accuracy. However, it can be concluded that MLR models have potential for future applications as management tools for water quality prediction at river sites.

To gain insights into the transferability of predictive models between rivers, a Bayesian model approach for the prediction of *E. coli* established by Seis et al. (2018) was transferred to Rhine and Moselle data. Results revealed, that a transfer of model approaches between rivers with different characteristics is not unrestrictedly possible. While a decent model could be established for the Rhine site, no valid model could be built for river Moselle. Enhanced trophic interactions, which were not accounted for in the data, were identified as the main reason hampering model development and application. Accordingly, model accuracy declined when models were fitted with low flow condition data. For rivers like the Moselle, viral indicators may pose a more reliable response variable in MLR models because they are less affected by trophic interactions. For the Rhine site, where FIO concentrations are predominantly driven by hydro-meteorological factors, the Bayesian linear regression approach represents a valuable tool for water quality prediction.

Transmission of ARG carried by ARB to humans via contact with Lahn River surface water even during non-swimming recreational activities is a realistic scenario as shown by calculated theoretical ingestion rates during recreational activities at Lahn River. Further, studies have shown that DNA containing ARG in river environments represent a reservoir of ARG that could facilitate the spread of antibiotic resistance (Mao et al., 2014). Not only ARB but also ARG themselves represent critical emerging risk factors for which strategies are needed to mitigate their dissemination. Until now, reliable assessment tools to estimate the risk of ARB in aquatic environments are missing. Under these circumstances

it made sense to assess, if common fecal indicators are suitable estimators for the qualitative and quantitative presence of ARG in surface waters. The conclusion was reached that linear regression models based on *E. coli* can provide a good estimate of ARG concentrations at certain river sites with high relative proportions of ARG compared to *E. coli*. At sites, where linear models based on *E. coli* achieved imprecise estimates, it should be tested, if ARG can be integrated into MLR models based on environmental parameters.

5.2.2 Challenges of implementing the Bathing Water Directive

In perspective of the BWD to implement early warning systems, MLR models based on environmental variables proved to be promising tools for short-term bathing water quality assessment in rivers. Nevertheless, there are several challenges in implementing MLR models while still meeting the demands of the BWD. The BWD does neither make specific recommendations on how to implement early warning systems and nor does it provide single sample thresholds. Both model approaches are capable of predicting FIO concentrations and circumvented the time lag of 24-48 h needed to complete cultivation-dependent microbiological quantification methods. While conventional MLR models achieved promising results in predicting fecal indicator concentration in Lahn River, they are not in compliance with the percentile approach of the BWD. A solution is provided by Bayesian regression models as developed by Seis et al. (2018), which are capable of meeting the probabilistic character of the BWD.

As shown by a comparative analysis of Moselle and Rhine data, model fitting and accuracy is impeded by extraordinary years with high- or low flow conditions during dry or wet years, respectively. To meet these uncertainties, extensive data sets including varying weather conditions are needed for model development. However, data availability is often limited. Riverine microbiological water quality is not commonly included in harmonized regulatory monitoring programs and data obtained during bathing water monitoring alone generally cover solely the bathing season and only few samples per year.

Moreover, the percentile approach of the BWD has received much criticism. The calculation of percentiles proposed in the BWD (also known as parametric approach) relies on mean and standard deviation, which are suitable descriptors of data populations only if data are log normally distributed. However, log normal distribution in river bathing water quality data is barely achieved, thus violating the approach's statistical validity criteria (Martínez et al., 2010). Furthermore, the precision of the estimate of the 95th percentile is questionable when sample numbers are low. Chawla and Hunter (2005) even go as far as to claim that “in conclusion, the parametric method for calculating bathing water compliance is so severely and fatally flawed statistically that it should not form the basis of any legislation”.

Furthermore, the fecal indicators used in the BWD are not sufficient to describe viral pathogens or risks posed by the various pathogens of non-fecal origin, such as *V. vulnificus* for example. Although there were no indications of FIO proliferation in the environment in this study, regrowth cannot be fully excluded, exacerbating their use in risk assessment (e. g. Anderson et al., 2005; Whitman et al., 2014).

The BWD protects people swimming in designated bathing waters, but there are no formal microbiological EU standards applying to non-designated bathing waters, despite the fact that inland waters are extensively used for a variety of other recreational purposes. As there is evidence that water recreation activities others than swimming such as canoeing and boating also pose a potential health risk for participants, this is of particular concern, especially on the background of emerging risks such as antibiotic resistances or *V. vulnificus*, for example. Yet, it is not clear whether the current criteria for bathing waters are suitable for non-swimming recreational activities. The contamination of surface water with ARG is also of particular concern if the water is –besides recreation- used for livestock, irrigation, or drinking water abstraction, for example.

However, ARB and resistance markers are yet not commonly monitored and knowledge concerning their environmental release and spread is still kind of limited. Although guidelines including critical values for indicator bacteria exist

for bathing waters, up to now compulsory guidelines addressing ARB in surface waters serving multiple purposes are lacking. The high prevalence of ARG in surface waters highlight the need to establish monitoring guidelines for microbiologic hazards in the aquatic environment to identify health risks for users of multifunctional waters, pollution sources and to mitigate their release and spread in the environment.

5.3 Outlook

While several knowledge gaps were filled based on the results of this work (Fig. 5.1), further future research needs emerged. Development of models that are capable of accurately predicting water quality deterioration also under extraordinary or extreme environmental conditions requires extensive datasets. However, recommended sampling programs cannot capture data on influential short-term events. Putting efforts in the application of automated event-triggered sampling and analysis approaches in water quality monitoring programs (Merkel et al., 2016; Zibuschka et al., 2010) to cover short-term pollution events and other extraordinary conditions could contribute to the databases needed for the development of reliable early warning systems. It remains to be tested WWTP discharge data as key explanatory variables can further enhance model performance at the studied sites. Extraordinary conditions will occur more frequently due to climate change and these conditions were shown to impact model establishment and performance. Comparable models in use probably need to be continuously checked and adapted for the future.

The selection of suitable indicators capable of adequately predicting pathogen exposure risks during water uses remains a challenge in water quality assessment, especially with regard to emerging pathogens of non-fecal origin such as *V. vulnificus* and ARG/ARB. FIB-independent water quality monitoring, such as direct multi-pathogen quantification may be a better predictor of human health risks, than solely relying on *E. coli* and intestinal enterococci (Ishii et al., 2014). Future research should focus on alternative legislative guidelines and values addressing viruses, emerging pathogens and pathogens of non-fecal origin. More

efforts should be directed into the development of tools for risk assessment and decision-making, such as the development of new models or time-effective molecular multipathogen detection assays, for example.

Most importantly, future research should focus on establishing legal critical values and monitoring guidelines for emerging risk factors like ARB in waters used for multiple purposes in order to protect human health and to find strategies to prevent the spread of antibiotic resistances in the environment. Strategies can be implemented e.g. in the field of waste water treatment technologies or optimization of using of antibiotics in human and veterinary medicine or evidence-based standards for antibiotic residues in effluents.

To support the results of this study and to advance the prediction of ARB abundance in rivers, the explanatory power of *bla*_{CTX-M} genes on actual ARB has to be investigated in greater depth to explore the reliability of *bla*_{CTX-M} genes as proxy of actual ARB in aquatic environments. Given that MLR modeling approaches based on environmental variables proved to be useful for the prediction of FIO, it should be evaluated if ARG and ARB could be integrated in similar approaches as well. However, to enable the use of such approaches in water management, further studies are needed to relate monitoring results of ARG concentrations in rivers to a risk of infection. Moreover, microbial source tracking could reliably identify the sources of ARG in lower Lahn River and measuring antibiotic residues in the water could reveal potential selection pressures. To further strengthen the results, a greater variety of selected ARG and ARG in bacterial isolates should be examined.

In general, microbial water quality has received much less attention in the framework of EU water-related legislation compared to other constituents such as nutrients or chemical substances that diminish water quality. The concept used to derive microbiological water quality standards in the EU should be reconsidered in view of the various aspects discussed above. Water bodies like rivers are often used for drinking water abstraction, irrigation and recreation. In addition, water reuse is becoming more important, due to increasing water scarcity caused by climate change (Bixio et al., 2006). One of the main barriers

identified with regard to microbial water quality and public health protection is the lack of harmonization in the regulatory framework at EU level to manage health risk related to rivers serving multiple purposes. Results of this research illustrate that a holistic view with less compartmentalization is needed to ensure a safe surface water quality with regard to the previously discussed multiple uses of flowing surface waters, amongst others the recreational use of rivers not officially designated as bathing waters. Against the background of emerging risks such as the spread of antibiotic resistances and a gradually changing climate, a stronger linkage between the BWD and the WFD is required to extend the focus of microbial water quality monitoring beyond bathing uses.

Appendix

Supplementary material to Chapter 4

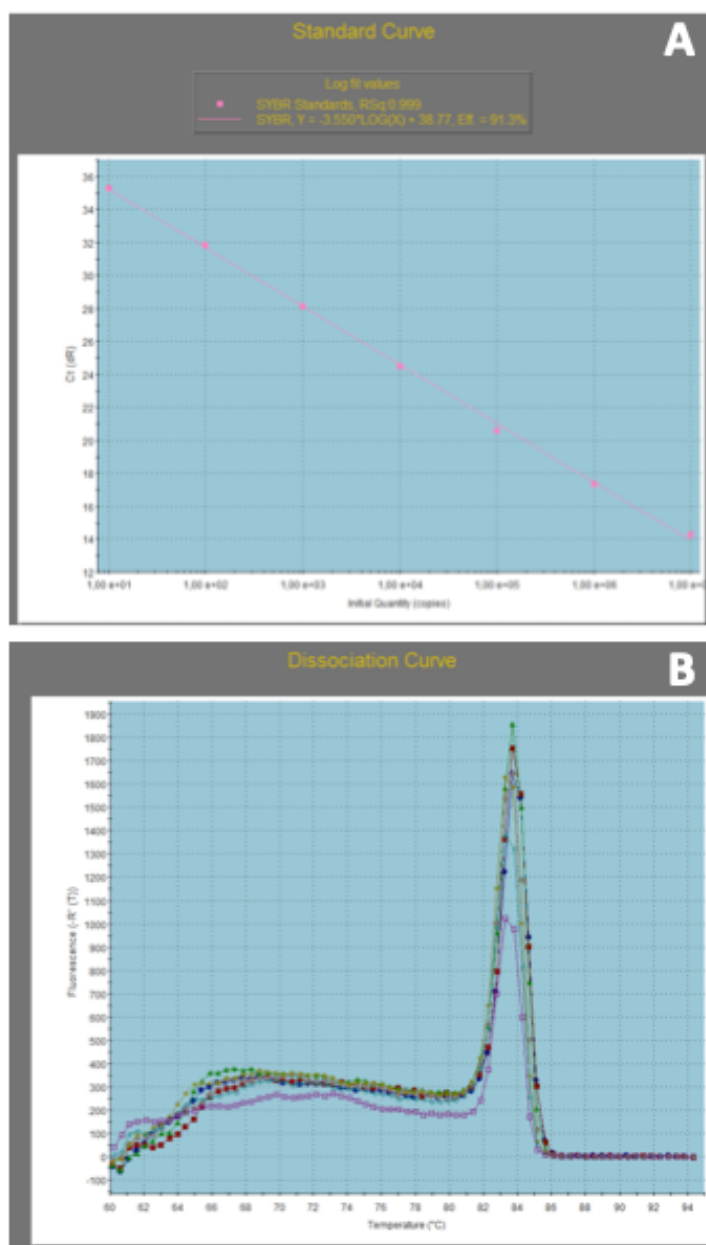


Figure A.1: Representative standard curve (A) and dissociation curves (B) of SYBR Green qPCR experiments for quantification of *bla*_{CTX-M} genes constructed of averages of triplicates by MxPro™ QPCR Software (Agilent Technologies).

Appendix

Table A.1: Numbers of total *E. coli*, antibiotic-resistant *E. coli*, ESBL-producing *E. coli* and diarrheagenic ESBL-producing *E. coli* theoretically ingested during recreational activities.

Activity	Volume of water ingested [mL/h]	Duration session [h]	Range	prevalence							ingested per hour							ingested per session						
				(1) total <i>E. coli</i> [MPN/100 mL]	(2) resistant <i>E. coli</i> [MPN/100 mL]	(3) resistant <i>E. coli</i> [MPN/100 mL]	(4) ESBL <i>E. coli</i> [MPN/100 mL]	(5) ESBL <i>E. coli</i> [MPN/100 mL]	(6) diarrheagenic ESBL <i>E. coli</i> [MPN/100 mL]	(7) diarrheagenic ESBL <i>E. coli</i> [MPN/100 mL]	(1) total <i>E. coli</i> [MPN/h]	(2) resistant <i>E. coli</i> [MPN/h]	(3) resistant <i>E. coli</i> [MPN/h]	(4) ESBL <i>E. coli</i> [MPN/h]	(5) ESBL <i>E. coli</i> [MPN/h]	(6) diarrheagenic ESBL <i>E. coli</i> [MPN/h]	(7) diarrheagenic ESBL <i>E. coli</i> [MPN/h]	(1) total <i>E. coli</i> [MPN/session]	(2) resistant <i>E. coli</i> [MPN/session]	(3) resistant <i>E. coli</i> [MPN/session]	(4) ESBL <i>E. coli</i> [MPN/session]	(5) ESBL <i>E. coli</i> [MPN/session]	(6) diarrheagenic ESBL <i>E. coli</i> [MPN/session]	(7) diarrheagenic ESBL <i>E. coli</i> [MPN/session]
Boating	3.7 ^a	4 ^b	min	15	5	7	0	0	0	0	1	0	0	0	0	0	0	2	1	1	0	0	0	0
			avg	3158	1011	1516	2	54	0	5	117	37	56	0	2	0	0	467	150	224	0	8	0	1
			max	27730	8874	13310	14	471	1	40	1026	328	492	1	17	0	1	4104	1313	1970	2	70	0	6
Canoeing	3.9 ^a	2.6 ^b	min	15	5	7	0	0	0	0	1	0	0	0	0	0	0	2	0	1	0	0	0	0
			avg	3158	1011	1516	2	54	0	5	123	39	59	0	2	0	0	320	102	154	0	5	0	0
			max	27730	8874	13310	14	471	1	40	1081	346	519	1	18	0	2	2812	900	1350	1	48	0	4
Fishing	3.6 ^a	4 ^b	min	15	5	7	0	0	0	0	1	0	0	0	0	0	0	2	1	1	0	0	0	0
			avg	3158	1011	1516	2	54	0	5	114	36	55	0	2	0	0	455	146	218	0	8	0	1
			max	27730	8874	13310	14	471	1	40	998	319	479	0	17	0	1	3993	1278	1917	2	68	0	6
Kayaking	3.8 ^a	-	min	15	5	7	0	0	0	0	1	0	0	0	0	0	0	-	-	-	-	-	-	-
			avg	3158	1011	1516	2	54	0	5	120	38	58	0	2	0	0	-	-	-	-	-	-	-
			max	27730	8874	13310	14	471	1	40	1054	337	506	1	18	0	2	-	-	-	-	-	-	-
Rowing	3.5 ^a	-	min	15	5	7	0	0	0	0	1	0	0	0	0	0	0	-	-	-	-	-	-	-
			avg	3158	1011	1516	2	54	0	5	111	35	53	0	2	0	0	-	-	-	-	-	-	-
			max	27730	8874	13310	14	471	1	40	971	311	466	0	16	0	1	-	-	-	-	-	-	-
Boating	1.9 ^b	4 ^b	min	15	5	7	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0
			avg	3158	1011	1516	2	54	0	5	60	19	29	0	1	0	0	240	77	115	0	4	0	0
			max	27730	8874	13310	14	471	1	40	527	169	253	0	9	0	1	2107	674	1012	1	36	0	3
Fishing	3.79 ^b	4 ^b	min	15	5	7	0	0	0	0	1	0	0	0	0	0	0	2	1	1	0	0	0	0
			avg	3158	1011	1516	2	54	0	5	120	38	57	0	2	0	0	479	153	230	0	8	0	1
			max	27730	8874	13310	14	471	1	40	1051	336	504	1	18	0	2	4204	1345	2018	2	71	0	6
Canoeing	7.52 ^b	2.6 ^b	min	15	5	7	0	0	0	0	1	0	1	0	0	0	0	3	1	1	0	0	0	0
			avg	3158	1011	1516	2	54	0	5	237	76	114	0	4	0	0	617	198	296	0	10	0	1
			max	27730	8874	13310	14	471	1	40	2085	667	1001	1	35	0	3	5422	1735	2602	3	92	0	8
Swimming (children)	49.33 ^c	1.32 ^d	min	15	5	7	0	0	0	0	7	2	4	0	0	0	0	10	3	5	0	0	0	0
			avg	3158	1011	1516	2	54	0	5	1558	499	748	1	26	0	2	2057	658	987	1	35	0	3
			max	27730	8874	13310	14	471	1	40	13679	4377	6566	7	233	1	20	18057	5778	8667	9	307	1	26
Swimming (adults)	21.33 ^c	1.32 ^d	min	15	5	7	0	0	0	0	3	1	2	0	0	0	0	4	1	2	0	0	0	0
			avg	3158	1011	1516	2	54	0	5	674	216	323	0	11	0	1	889	285	427	0	15	0	1
			max	27730	8874	13310	14	471	1	40	5915	1893	2839	3	101	0	9	7808	2498	3748	4	133	0	11

(1) Herrig et al. 2015
(2) calculated based on (1) and Blaak et al. 2011 (32 % of *E. coli* AB-resistant *E. coli*)
(3) calculated based on (1) and Blaak et al. 2011 (48 % of *E. coli* AB-resistant)
(4) calculated based on (1) and Blaak et al. 2014 (0.05 % of *E. coli* producing ESBL)
(5) calculated based on (1) and Haberecht et al. 2019 (1.7 % of *E. coli* producing ESBL)
(6) calculated based on (4) and Franz et al. 2015 (8.5 % of ESBL producing *E. coli* diarrheagenic)
(7) calculated based on (5) and Franz et al. 2015 (8.5 % of ESBL producing *E. coli* diarrheagenic)

a) Dorevitch et al. 2011
b) Rijal et al. 2011
c) Dufour et al. 2006
d) Schets et al. 2011

Table A.2: Numbers of *bla*_{CTX-M} genes theoretically ingested during recreational activities.

				prevalence	ingested per hour	ingested per session
Activity	Volume of water ingested [mL/h]	Duration session [h]	Range	<i>bla</i> _{CTX-M} [copies/100 mL]	<i>bla</i> _{CTX-M} [copies/h]	<i>bla</i> _{CTX-M} [copies/session]
Boating	3.7 ^a	4 ^b	min	349	13	52
			avg	3179	118	470
			max	20083	743	2972
Canoeing	3.9 ^a	2.6 ^b	min	349	14	35
			avg	3179	124	335
			max	20083	783	2115
Fishing	3.6 ^a	4 ^b	min	349	13	50
			avg	3179	114	458
			max	20083	723	2892
Kayaking	3.8 ^a	-	min	349	13	-
			avg	3179	121	-
			max	20083	763	-
Rowing	3.5 ^a	-	min	349	12	-
			avg	3179	111	-
			max	20083	703	-
Boating	1.9 ^b	4 ^b	min	349	7	27
			avg	3179	60	242
			max	20083	382	1526
Fishing	3.79 ^b	4 ^b	min	349	13	53
			avg	3179	120	482
			max	20083	761	3045
Canoeing	7.52 ^b	2.6 ^b	min	349	26	68
			avg	3179	239	622
			max	20083	1510	3927
Swimming (children)	49.33 ^c	1.32 ^d	min	349	172	227
			avg	3179	1568	2070
			max	20083	9907	13077
Swimming (adults)	21.33 ^c	1.32 ^d	min	349	74	98
			avg	3179	678	895
			max	20083	4284	5654

a) Dorevitch et al. 2011
b) Rijal et al. 2011
c) Dufour et al. 2006
d) Schets et al. 2011

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