National Institute for Public Health
and the Environment
Ministry of Health, Welfare and Sport

Prevalence of antibiotic resistant bacteria in the rivers Meuse, Rhine, and New Meuse

RIVM report 703719071/2011

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Colophon

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Abstract

Prevalence of antibiotic resistant bacteria in the rivers Meuse, Rhine and New Meuse

A high percentage of bacteria in large Dutch rivers is resistant to one or more antibiotics. This was demonstrated in an exploratory study performed at the RIVM.

Exposure through surface water
If people come into contact with contaminated surface water, they risk exposure to bacteria that are resistant to one or more antibiotics. This may occur for instance during recreation in surface water or through water used for irrigation of crops. Because antibiotics are needed for treatment of infections, such contact may involve public health risks. Antibiotic resistant bacteria can end up in surface water by different routes, for example through run off of manure from animals treated with antibiotics to surface water. Another source may be the discharge of partially treated or untreated waste water, for instance derived from hospitals where people are treated with antibiotics.

Overall, one third to half of all *Escherichia coli* and intestinal enterococci were resistant to one or more antibiotics. In some of the samples antibiotic resistant strains of *Staphylococcus aureus*, *Campylobacter* and *Salmonella* were detected. Most of these bacteria are gut bacteria; *Staphylococcus aureus* is predominantly present on people's skin and in their noses and throats.

Diverse risks
The risks can manifest themselves in different ways. Firstly, after exposure to antibiotic resistant bacteria people may develop disease caused by these bacteria, which may therefore be hard to treat. Additionally, people may not become ill themselves, but transfer the resistant bacteria to people who are more vulnerable, such as hospital patients and the elderly. Subsequently, this category of people can develop disease. Finally, there is a risk that the antibiotic resistant bacteria establish themselves in the gut and transfer resistance genes to disease-causing bacteria if these are subsequently ingested.

Further research on public health risks
Data on the prevalence of antibiotic resistant bacteria in surface water are necessary to estimate the contribution of the environment to human exposure to these bacteria. Further RIVM research is focused on this type of exposure and the associated possible public health risks.

Keywords:
antibiotic resistance, surface water, rivers, commensal bacteria, pathogenic bacteria

Rapport in het kort

Prevalentie van antibioticaresistente bacteriën in Maas, Rijn en Nieuwe Maas

In de grote Nederlandse rivieren de Maas, de Rijn en de Nieuwe Maas komen bacteriën voor waarvan hoge percentages resistent zijn tegen een of meer soorten antibiotica. Dit blijkt uit verkennend onderzoek van het RIVM.

Blootstelling via oppervlaktewater
Als mensen aan verontreinigd oppervlaktewater blootgesteld worden, kunnen zij antibioticaresistente bacteriën binnenkrijgen. Dit kan bijvoorbeeld via recreatiewater of via water dat gebruikt worden om gewassen te besproeien. Dergelijk contact kan risico's voor de volksgezondheid met zich meebrengen, omdat deze antibiotica nodig zijn om infecties te behandelen.
Antibioticaresistente bacteriën kunnen op meerdere manieren in oppervlaktewater terechtkomen, bijvoorbeeld doordat mest van dieren die met antibiotica zijn behandeld, afspoelt naar het oppervlaktewater. Een andere oorzaak kan zijn dat gedeeltelijk gezuiverd of ongezuiverd afvalwater in oppervlaktewater wordt geloosd, bijvoorbeeld afkomstig van ziekenhuizen waar mensen zijn behandeld met antibiotica.
In totaal waren gemiddeld eenderde tot de helft van alle *Escherichia coli* en van de intesintale enterococcen resistent tegen een of meer soorten antibiotica. In sommige van de monsters werden antibioticaresistente stammen van *Staphylococcus aureus*, *Campylobacter* en *Salmonella* aangetoond. De meeste van deze bacteriën zijn darmbacteriën; *Staphylococcus aureus* komt vooral voor op de huid, en in de neus en keel van mensen.

Diverse risico's
De risico's kunnen zich op verschillende manieren manifesteren. Op de eerste plaats kunnen mensen die aan antibioticaresistente bacteriën worden blootgesteld, daarvan ziek worden en vervolgens problemen krijgen bij de behandeling ervan. Daarnaast is het mogelijk dat mensen die worden blootgesteld aan de resistente bacteriën zelf niet ziek worden, maar deze overdragen aan mensen met verminderde weerstand, zoals ziekenhuispatiënten en ouderen. Deze groep mensen kan vervolgens wel ziek worden door deze bacteriën. Ten slotte is er het risico dat onschadelijke antibioticaresistente bacteriën zich in de darmen nestelen en daar genen die resistentie veroorzaken doorgeven aan andere, ziekteverwekkende bacteriën.

Nader onderzoek naar volksgezondheidsrisico's
Onderzoek naar de mate waarin antibioticaresistente bacteriën in oppervlaktwater voorkomen is van belang om te kunnen inschatten in hoeverre mensen via het milieu worden blootgesteld aan deze bacteriën. Nader RIVM-onderzoek zal hierop gericht zijn, en wat dit betekent voor de volksgezondheid.

Trefwoorden:
antibioticaresistentie, oppervlaktewater, rivieren, commensale bacteriën, pathogenen bacterien

Contents

Summary

Due to the use of antibiotics in public and animal health, the prevalence of antibiotic resistant bacteria is increasing, not only in humans and animals, but also in the environment. As a consequence, the risk of human exposure to antibiotic resistant bacteria outside a clinical setting is increasing. Antibiotic resistant bacteria excreted by humans and animals treated with antibiotics end up in the environment, for instance with the discharge of untreated or partially treated sewage or runoff of manure. Thus, the environment can be considered a collecting vessel of antibiotic resistant bacteria and resistance genes from human and animal origin. Moreover, water and soil may represent environments where resistance genes can be transferred among bacteria from different origins, among which also environmental bacterial species. This may result in the creation of novel combinations of bacteria species and specific resistance genes. People may become exposed to bacteria in surface water and soil during recreation in faecally contaminated water, when drinking inadequately treated drinking water or water from unprotected sources or when consuming fresh produce that has been irrigated with contaminated surface water or grown on contaminated soil.

In the current study, the prevalence of antibiotic resistant bacteria was studied in three large rivers: the Meuse (at Eijsden), the Rhine (at Lobith), and the New Meuse (at Brienenoord). Samples were taken every four weeks from May 2008 up to and including May 2009, and analysed for the presence of *Escherichia coli*, intestinal enterococci and *Staphylococcus aureus*. At four of the time-points samples were additionally screened for the presence of *Campylobacter* and *Salmonella.* Obtained isolates were studied with respect to their sensitivity for a panel of antibiotics that are (or have been) used in human and/or animal health care.

The prevalence of antibiotic resistance was high among *E. coli* and enterococci isolated from all three rivers. In the Meuse, 48% of *E. coli*, 54% of *E. faecium* and 56% of *E. faecalis* were resistant to at least one antibiotic*,* compared to 32% of *E. coli*, 59% of *E. faecium* and 50% of *E. faecalis* in the Rhine and 34% of *E. coli*, 58% of *E. faecium* and 18% of *E. faecalis,* in the New Meuse. Of these bacteria, a large proportion was resistant to two or more antibiotics (65%, 60% and 43% of resistant *E. coli*, *E. faecium* and *E. faecalis*, respectively). Out of eight antibiotics tested, the maximum number of resistances observed per strain was seven for *E. coli*, six for *E. faecium* and four for *E. faecalis*. On average, 10% of all isolated *E. coli* and 8% of all *E. faecium* isolated from rivers were resistant to five or more antibiotics. In some of the rivers and some of the time-points antibiotic resistant strains of *S. aureus*, *Campylobacter coli*, *Salmonella enterica* (subtypes Panama and 4,5,12:i:-) were also detected. Among the isolated bacteria were strains with clinically relevant resistances, such as ESBL-producing *E. coli*, high-level ampicillin-resistant or high level aminoglycoside-resistant enterococci, methicillin-resistant *S. aureus* (MRSA) and quinolone-resistant *Campylobacter spp*.

To illustrate the possible implications of the observed prevalence data in terms of exposure during recreation in contaminated water, we determined point estimates describing the amounts of resistant commensal bacteria ingested when accidentally swallowing river water at the study locations. Estimates were based on the average concentrations of *E. coli*, *E. faecium* and *E. faecalis* observed during the summer season, the overall prevalence of antibiotic resistance for these species, and average amounts of water that are accidentally swallowed by adult and non-adult swimmers. In the river with the highest level

of contamination with antibiotic resistant bacteria, the Meuse, an average amount of ingested water contained on average 167 resistant *E. coli* (range 68 to 281) for adults, and on average 386 (range 157-650) for children. The same 'non-adult' amount of ingested water contained on average 155 (range 63 to 260) *E. coli* resistant to five or more antibiotics. For *E. faecium* and *E. faecalis* these amounts were substantially lower: on average, one 'adult' ingestion of river water contained two resistant *E. faecium* (range 0-13) and three resistant *E. faecalis* (range 0-35), and one non-adult ingestion contained 11 resistant *E. faecium* (range 0-58) and 15 *E. faecalis* (range 0-81).

To limit the spread of antibiotic resistant bacteria and resistance genes, it is important to know the relative contribution, in terms of quantity as well as quality, of different potential transmission routes. Future research at the RIVM is aimed at determining the prevalence of antibiotic resistance in surface waters used for purposes that may lead to exposure of humans, such as recreation and irrigation. These data will then be used to more accurately determine the risks of exposure to antibiotic resistant bacteria and possible public health impacts associated with contact with contaminated surface water using Antibiotic Resistance Risk Assessments (ARRA).

1 Introduction

The use of antibiotic drugs in human and animal healthcare has resulted in the widespread development of resistance not only in humans and animals (EARSS 2008; MARAN 2009; Nethmap 2010), but also in the environmental reservoir. As a consequence, the probability of becoming exposed to antibiotic resistant bacteria outside a clinical setting increases. For instance, people may become exposed through the preparation and consumption of contaminated food, ingestion of or recreation in contaminated water or contact with healthy people in the community. Exposure to antibiotic resistant bacteria may result in infections that are difficult to treat.

1.1 Antibiotic use in human and animal health care

In the Netherlands, restricted policy with respect to the use of antibiotics in human health care has led to a relatively low prevalence of antibiotic resistance in clinical bacterial isolates in comparison with other European countries (EARSS 2008; Coenen et al. 2009). By contrast, the amounts of antibiotics used in Dutch veterinary health care are extremely high, both compared with that in other European countries (Grave et al. 2010) as well as with Dutch human health care (Geijlswijk et al. 2009). In particular, pigs, veal calves and broiler chickens receive high amounts of antibiotics, not only for the treatment of infections, but also to prevent the spread of infections among healthy animals. As a consequence, very high percentages of bacteria carried by these animals are resistant, often to multiple antibiotics (MARAN 2009). For instance, in 2009 88% of *Escherichia coli*-strains isolated from faeces from broiler chickens were resistant to at least one antibiotic. Many of the resistant bacteria isolated from food production animals are multiresistant: *E. coli*-strains from faeces from pigs, poultry and veal calves with six, seven, or eight resistances are not uncommon (MARAN 2009).

Table 1 Classes of antibiotics

Class	Examples of class members
Fluoroquinolones and Quinolones	Ciprofloxacin, Naladixic acid
Aminoglycosides	Streptomycin, Neomycin, Gentamicin
Macrolides	Erythromycin, Clarithromycin
Tetracyclines	Tetracycline
Sulfonamides	Sulfamethoxazole, Trimethoprim
Beta-lactams: Penicillins	Penicillin, Ampicillin, Oxacillin[1], Methicillin[1]
Beta-lactams: 3rd generation Cephalosporins	Cefotaxime, Ceftazidime
Beta-lactams: Carbapenems	Imipenem, Meropenem
Streptogramins	Quinupristin, Dalfopristin
Lincosamides	Clindamycin
Glycopeptides	Vancomycin

[1]Penicillase-resistant penicillins.

Antibiotics that are used in animal health care are the same as, or closely related to, those used to treat human infections. The vast majority of antibiotics used in humans and animals belongs to one of the following six classes: (fluoro)quinolones, aminoglycosides, macrolides, tetracyclines, sulfonamides/trimethoprim, and penicillins/cephalosporins (Table 1). Resistance to an antibiotic often confers resistance to other antibiotics from the same class (i.e., cross-resistance). Consequently, the development of resistance to antibiotics not used in humans by animal bacteria can have large consequences for human health care. Well-known examples are the development of vancomycin-resistant enterococci in the nineties as a result of the use of avoparcin in livestock (Van den Bogaard et al. 1997; Van den Bogaard et al. 2000), and more recently, the development of 3rd generation cephalosporin-resistant *E. coli* and *Salmonella* in poultry, presumably as a result of the (off-label) use of ceftiofur (Dutil et al. 2010).

1.2 Antibiotic resistance and public health risks

Bacteria that have acquired resistance to one or more antibiotics may present direct and indirect risks to human health. Direct risks are associated with exposure to harmful bacteria (pathogens) that are resistant to antibiotics relevant for treatment of infection caused by this pathogen, as this may directly result in disease that is difficult to treat. Indirect risks are associated with exposure to relatively harmless bacteria, such as commensals, that carry antibiotic resistance. These bacteria can colonise intestines or skin (without causing disease), and pass their resistance genes on to other bacteria that inhabit these tissues. Transfer of resistance genes occurs through a process called 'horizontal gene transfer', and can take place between bacteria of the same species as well as different species (Salyers et al. 2004; Kelly et al. 2009; Trobos et al. 2009). This way, people may become unnoticed carriers of antibiotic resistant bacteria. Upon subsequent exposure to (non-resistant) pathogens, resistance genes may be transferred to these pathogens, with difficult-to-treat infection as a consequence. Moreover, most commensal bacteria are 'opportunistic bacteria', meaning that they can cause disease in people who are more vulnerable to infection, such as hospital patients or elderly people. Carriers of antibiotic resistant bacteria may spread these bacteria to people with increased vulnerability and cause disease in this group of people. The carriers themselves are also at risk of infection if they subsequently experience a period of increased vulnerability, for instance hospitalisation. Well-known examples of antibiotic resistant commensal bacteria that can be harmful to man are those causing so-called 'hospital-associated' infections, such as methicilline-resistant *Staphylococcus aureus* (MRSA) (Falcone et al. 2009) and 'extended-spectrum beta-lactamase' (ESBL)-producing *Escherichia coli* and *Klebsiella* (Cantón et al. 2008; Coque et al. 2008). Although these bacteria cause problems in hospital patients and institutionalised people, they are also prevalent in healthy people, animals and the environment.

1.3 Antibiotic resistance in the environment

Antibiotic resistant bacteria and antibiotic resistance (AMR) genes are excreted into the environment mainly with faeces of humans and animals that are treated with antibiotics. These bacteria end up in soil and surface water through the discharge of untreated or partially treated sewage (amongst others derived from potential 'hot spots' of antibiotic resistance such as health care centres, farms and slaughterhouses), application of activated sludge from wastewater

treatment plants as fertiliser of agricultural soil, and runoff of animal manure or faeces of pasture animals. People are at risk of being exposed to these bacteria when they come into contact with contaminated environmental compartments, for instance during recreation in contaminated surface water, when consuming water produced from unprotected drinking water sources or when eating food that has been irrigated with faecally contaminated surface- or groundwater, fertilised using contaminated manure or grown in contaminated soil.

Importantly, once in the environment, bacteria of different origin come into physical contact and may exchange resistance genes with the endogenous bacterial population (Genthner et al. 1988; Coughter and Stewart 1989; Xu et al. 2007; Cattoir et al. 2008). Even though the resistant bacteria of human and animal origin may die off in the environment, the endogenous environmental bacteria may pass the acquired resistance genes on to their progeny. Despite the generally believed negative impact of acquired antibiotic resistance on fitness of the bacteria (Andersson and Levin 1999; Andersson and Hughes 2010), these genes may remain present in the environment for a long time (Andersson 2003). Besides a collecting and mixing vessel of resistance genes of anthropogenic origin, the environment is also considered to be a natural reservoir of resistance genes. Soil bacteria, especially bacteria belonging to the family of *Actinomycetes*, are known to produce antibiotic resistance proteins to protect themselves from antibiotics they produce themselves (Benveniste and Davies 1973; Cundliffe 1989; D'Costa et al. 2006; Hopwood 2007; Tahlan et al. 2007). The existence of these resistance genes, as well as genes encoding proteins that originally have alternative biochemical functions but can easily change into resistance proteins in the case of selective pressure, are considered the source of resistance in human and animal bacterial populations (D'Costa et al. 2006; D'Costa et al. 2007; Wright 2007; Chee-Sanford et al. 2009; Allen et al. 2010). The underlying idea is that bacteria excreted by humans and animals acquire these resistance genes through horizontal gene transfer, resulting in bacteria resistant to antibiotics not (yet) seen previously in human or animal bacterial isolates. When these bacteria are subsequently re-introduced in humans or animals that are treated with antibiotics, this may contribute to the rapid development of resistance.

In conclusion, in the environment bacteria excreted by man and animals may acquire resistance genes of anthropogenic as well as natural origin, facilitating the creation of novel bacteria/resistance gene combinations not seen previously in animal or man.

1.4 Aim of the study

Dutch rivers were studied for the presence of antibiotic resistant bacteria following on a previous study conducted at the RIVM in 2006 that demonstrated, for the first time, high percentages of antibiotic resistant bacteria in Dutch surface waters (Blaak et al. 2010). The previous study focused on small rivers in an area with a high density of animal farms and samples were taken from March to early May, i.e., shortly after fertilisation of the land. Because of the timing of sampling and sample locations, runoff of manure was likely to be a main source of contamination (although this was not actually determined). The present study was set up to further inventory the prevalence of antibiotic resistant bacteria in Dutch surface waters. This time, to obtain a more generalised picture of the magnitude of the prevalence of antibiotic resistant bacteria, locations were selected that are assumed to be influenced by multiple different contamination sources. Moreover, these sites were sampled at multiple time-points during a

whole year. Selected were three large rivers that flow through urban as well as rural areas, the Meuse (at Eijsden) and the Rhine (at Lobith), and the New Meuse (at Brienenoord). Rivers were sampled every four weeks from May 2008 up to and including May 2009 and analysed for the presence of the commensal bacteria *Escherichia coli*, intestinal enterococci, and *Staphylococcus aureus*. At four of the time-points, samples were additionally screened for the presence of the pathogenic bacteria *Campylobacter* and *Salmonella.* Isolated bacteria were studied with respect to their sensitivity for antibiotics that are (or have been) used in human and/or animal health care.

To illustrate the possible implications of the observed antibiotic resistance prevalence rates in terms of exposure during recreation, we determined point estimates describing the amounts of resistant *E. coli*, *E. faecium* and *E. faecalis* that could have been ingested by swimmers accidentally swallowing river water at the studied sites during the bathing season of 2008.

2 Materials and methods

2.1 Sampling and sampling locations

Samples of 20 litres were taken from the Meuse at Eijsden, the Rhine at Lobith and the New Meuse at Brienenoord, every 4 weeks from May 2008 up to and including May 2009, by RWS Waterdienst according to NEN-EN-ISO 19458 (Anonymous 2007) (see Figure 1). The New Meuse was not sampled in December 2008; Overall, thirteen samples were obtained from the New Meuse and fourteen each from the Meuse and the Rhine. Immediately after sampling, the samples were stored at 5 ± 3 °C, or if this was not feasible, stored at room temperature or in the shade for maximally three hours until being transported refrigerated to the RIVM. Upon arrival at the RIVM, samples were stored at 5 ± 3 °C and processed within 24 hours after sampling. In May 2008 at all locations and in June 2008 at the New Meuse, 'non-cooled' storage time exceeded three hours, and samples were declared unfit for quantification purposes. Nevertheless, from these samples bacteria were isolated for antibiotic analysis.

All samples were examined for the presence of *E. coli*, intestinal enterococci and *S. aureus*. At four of the sampling dates (8 Jul 2008, 30 Sep 2008, 20 Jan 2009 and 17 Mar 2009), the samples were additionally examined for the presence of *Salmonella* and *Campylobacter*.

2.2 Isolation of bacteria

With the exception of *S. aureus*, the different species/genera were isolated according to (inter)national standards (Appendix 1a). The procedure for isolation of *S. aureus* is described in paragraph 2.2.1. For the isolation of *Campylobacter* and *Salmonella* hollow fibre ultrafiltration (Kuhn and Oshima 2002) was used in parallel to membrane filtration. This technique is described in paragraph 2.2.2. Following standard procedures, *E. coli* and enteroccoci were isolated using membrane filtration and colony-count technique, while the other bacterial species were isolated according to the 'most probable number' method (MPN), and samples (either or not concentrated by membrane filtration) were pre-enriched in liquid media prior to plating (Anonymous 2005). Bacterial concentrations were determined using Mathematica software (WolframResearch, Champaign, IL, USA).

From positive samples maximally ten characteristic colonies were selected for each species or genus and streaked on solid media (Appendix 1b). After overnight incubation, isolated colonies were suspended in 1 ml buffered peptone water (BPW) and again incubated overnight at 36 ± 2 °C. To the resulting suspensions 150 µl glycerol was added and stocks were stored at –80 °C. After resuscitation, for *Campylobacter* only strains isolated from July and January appeared viable enough to determine antibiotic sensitivity profiles. For *Salmonella*, colonies derived from the New Meuse in January 2009 were inadvertently not stored.

2.2.1 Isolation of S. aureus

Water samples were filtrated using Tuffryn 0.45µm filters (Tuffryn HT450, Pall Corporation, East hills NY, VS). Filters were incubated at 36 ± 2 °C for

18 ± 2 hours, in 20 ml Mueller Hinton Broth (Oxoid, Hampshire, UK) supplemented with 6.5% NaCl (MH^+). After this initial pre-enrichment step, bacterial suspensions were homogenised and 1 ml was transferred to 9 ml Phenolred Mannitol Broth (PMB) supplemented with 75 µg/ml aztreonam (MP Biomedicals, Amsterdam, the Netherlands) to further enrich for *S. aureus*. Another 1 ml of the bacterial suspension was transferred to 9 ml PMB supplemented with 75 µg/ml Az and 4 µg/ml oxacillin (Sigma Aldrich, Zwijndrecht, the Netherlands) to selectively enrich for methicillin-resistant *S. aureus* (MRSA). Cultures were incubated at 36 ± 2 °C for 18 ± 2 hours. Of the cultures pre-enriched for *S. aureus*, 10 µl was streaked on Baird-Parker agar (BPa). Of the cultures pre-enriched for MRSA, 10 µl was streaked on BPa, blood agar (BA) and MRSA screening agar (MRSA brilliance, Oxoid). Cultures were incubated at 36 ± 2 °C for 24 ± 2 hours. Characteristic *S. aureus* colonies are black surrounded by a precipitation zone on BPa, cream-coloured to white and haemolytic on BA, and blue on MRSA screening agar. As positive controls 1 lenticule (36 colony forming units, or cfu) *S. aureus* NCTC 6571 (HPA, London UK) and 1 cfu of MRSA ATCC 43300 were used.

Figure 1 Sampling locations ('meetlocaties') the Meuse at Eijsden, the Rhine at Lobith and the New Meuse at Brienenoord.

2.2.2 Hollow fibre ultrafiltration

Part of the *Salmonella* and *Campylobacter* isolates were obtained by concentrating water samples using hollow fibre ultrafiltration (Kuhn and Oshima 2002) in parallel to membrane filtration. The method entailed pumping a 50-litre water sample across a cross-flow ultrafilter (Hemoflow HF80S; Fresenius Medical Care, Bad Homburg, Germany). Water forced through the filter is drained off and the sample is therewith concentrated. When samples were reduced to approximately 250 ml, sample containers were flushed with a 0.1 M glycine buffer. Of resulting eluates (450–500ml), multiple test portions were added to pre-enrichment media for isolation of bacteria (see Appendix 1). The remainder of the eluates was centrifuged for 12 minutes at 2000g, and pellets suspended in 20 ml physiological saline solution. Of these suspensions multiple test portions were also added to pre-enrichment media for isolation. The pre-enriched cultures were further processed according to standard procedures described in Appendix 1a.

2.3 Typing of bacterial isolates

The identity of suspected *E. coli*-isolates was confirmed using API20E strips (Biomerieux), according to the instructions of the manufacturer. Only isolates with an *E. coli* identity match of ≥ 93% (that is, with the outcome: 'good', 'very good' and 'excellent') were included for antibiotic susceptibility testing. *Campylobacter* strains were identified to species level based on catalase reaction, hippurate hydrolysis (discs from Oxoid, R21085), and indoxylacetate hydrolysis (discs from Oxoid, R21087), as described in NEN-EN-ISO 10272-1 (Anonymous 2004). Results were confirmed using 16S rDNA sequence analysis (see paragraph 2.3.1). Enterococci were also identified to species level using 16S rDNA sequence analysis. Identities of suspected *Salmonella* colonies were confirmed to genus level based on their characteristics on TSI agar, Urea agar and L-lysine Decarboxylatiemedium (LDC), in accordance with NEN-EN-ISO 1925 (Anonymous 2003). Confirmed *Salmonella* strains were identified to species level using serotyping, at the RIVM department LIS. Suspected *S. aureus* colonies were confirmed using PCR (see paragraph 2.3.2).

2.3.1 16S rDNA sequence analysis

To identify *enterococcus* and *campylobacter* strains to species level, 16S rDNA was partially sequenced and compared to reference sequences available in an international nucleotide data base (see Appendix 2). For this purpose, a 589 bp fragment of 16S rDNA was amplified by polymerase chain reaction (PCR), using PuReTaq Ready-To-Go PCR beads (GE Healthcare, Diegem, Belgium). PCRs were performed on bacterial lysates: single colonies of *Enterococcus* and *Campylobacter* cultures suspended in 100 µl demiwater and lysed by 30 minutes incubation at 100 °C. These lysates were stored at −70 °C until use. In each PCR reaction, 3 µl lysate was mixed with 18 µl primer mix (10 pmol per primer) and 22 µl beads. The following primers were used: forward primer 16S8F (5' AGA GTT TGA TCM TGG YTC AG -3') and reverse primer 16S575R (5' CTT TAC GCC CAR TRA WTC CG 3'). These primers anneal at positions 8 and 575 relative to *E. coli* 16S of the strain with accession number E05133. The resulting PCR products were analysed on agarose gel and PCR-products of the expected size were purified using QIAquick PCR purification kits (Quiagen, Venlo, the Netherlands) and stored at −20 °C. Sequence analysis of the PCR products was done using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Bleiswijk, the Netherlands) using the same primers. Per reaction, 2 µl purified PCR product was mixed with 1 µl BigDye Terminator, 7 µl sequence

buffer and 10 pmol of the PCR primers. Obtained sequences were analysed using Bionumerics 6.0 software (Applied Maths, Sint-Martens-Latem, Belgium). Phylogenetic trees were constructed from isolate- and reference sequences using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA). Because of the high homology in 16s rDNA sequences between most *Enterococcus* species, species identity was verified by comparing sequences at species-distinguishing positions described by Patel et al (Patel et al. 1998).

2.3.2 Confirmation of S. aureus and methicillin-resistance by PCR

The identity of suspected colonies was confirmed by amplification of the *S. aureus*-specific Martineau-sequence (Martineau et al. 1998). Simultaneously, the absence or presence of the methicillin resistance gene *MecA* and the virulence gene Panton Valentine Leukocidin toxin (PVL) was investigated making use of a multiplex PCR. Single colony material was suspended in 50 µl lysis buffer (100 µg lysostaphine in 1 ml 1x TE-buffer), and lysed by 35 minutes incubation at 37 °C followed by 10 minutes at 95 °C. Lysates were diluted in 200 µl 1xTE-buffer and stored at −20 °C. Of each lysate, 2 µl was analysed using (ul) PuReTaq Ready-To-Go PCR beads (GE Healthcare) and (ul) the following primers:

1) *S. aureus* confirmation (Martineau et al. 1998): Sa442-1 (5’ AAT CTT TGT CGG TAC ACG ATA TTC ACG 3’) and Sa442-2 (5’ CGT AAT GAG ATT TCA GTA GAT AAT ACA ACA 3’);
2) detection of the MecA gene: MecA-1 (5’GTT GTA GTT GTC GGG TTT GG 3’) and MecA-Cr (5’ CTT CCA CAT ACC ATC TTC TTT AAC 3’);
3) detection of the PLV-gene: SaPLV-1 (5’ ATC ATT AGG TAA AAT GTC TGG ACA TGA TCC A 3’) and SaPvL-2 (5’ GCA TCA A(GC)T GTA TTG ATA GCA AAA GC 3’).

PCR products were analysed using agarose gel electrophoresis.

2.4 Determination of antibiotic susceptibility

Antibiotic susceptibility was determined using the broth microdilution technique and Etest® method (Biomerieux, Boxtel). For micro broth dilution, the Sensititre SensiTouch® system (TREK, MCS Diagnostics, Swalmen) was used, using custom-made sensititre plates containing serial dilutions of six different antibiotics (see Appendix 3). Both methods were performed according to the manufacturers’ instructions (outlined in paragraphs 2.4.2 and 2.4.3). An overview of the antibiotics tested is shown in Table 2.

2.4.1 Minimal Inhibitory Concentration

For each antibiotic and bacterial isolate combination, the Minimal Inhibitory Concentration (MIC) was determined, defined as the lowest concentration where growth is no longer visible. In the broth microdilution method, two-fold dilutions of antibiotics are tested, while on Etest® strips in-between concentrations are included. To enable comparison between both methods, MIC values obtained using Etest® were rounded up to the nearest two-fold concentration. For *E. coli* and enterococci isolates (that were obtained by colony count method), the distributions of MIC values were determined to describe antibiotic sensitivity at the population level. For *S. aureus*, *Campylobacter* and *Salmonella* isolates (which were obtained using pre-enrichment), this type of analysis was not performed because the population of isolates obtained after pre-enrichment is not a direct reflection of the sample population (i.e., all isolates may actually descend from one or a few parental cell(s) initially present in the sample).

Table 2 Antibiotics tested per bacterial species or genus

	E. coli	***Salmo-nella***	***Campy-lobacter***	***Entero-coccus***	***S. aureus***
Sulfanomides/trimethoprim					
Sulfamethoxazole	Etest	Etest	Broth		
Trimethoprim	Broth	Broth			
Sulfa/Trim*				Broth	Broth
Penicillins					
Ampicillin			Broth	Etest	
Oxacillin					Etest
Cephalosporins					
Cefotaxime	Broth	Broth			
Aminoglycosides					
Streptomycin	Broth	Broth	Broth		
Gentamicin			Broth	Broth	
Neomycin			Broth		
Tetracyclins					
Tetracyclin	Broth	Broth	Broth	Broth	Broth
Macrolides					
Erythromycin			Broth	Broth	Broth
Clarithromycin			Broth		
Tulathromycin			Broth		
Streptogramins/ lincosamides					
Synercid*				Broth	Broth
Clindamycine					Etest
Quinolones					
Ciprofloxacin	Broth	Broth	Broth	Etest	
Naldixic Acid		Etest	Etest		
Glycopeptides					
Vancomycin				Broth	Broth
Amphenicols					
Chloramphenicol	Etest	Etest	Broth		
Ionophores					
Salinomycin				Broth	Broth

*Antibiotics tested for the different species/genera are indicated in grey. Susceptibility was tested using broth microdilution ('Broth') or Etest. * Sulfa/Trim = sulfamethoxazole and trimethoprim, Synercid = quinupristin and dalfopristin.*

2.4.2 Broth microdilution

Isolates were streaked onto culture media (Appendix 1b) and incubated at 36 ± °C for 18 to 24 hours. From fresh cultures, single colonies were picked and suspended in 5 ml sterile physiological saline solution to a density corresponding to 0.5 MacFarland. From this suspension 10 µl was transferred to 11 ml Cation-Adjusted Mueller-Hinton Broth (CAMHB; MCS Diagnostics), resulting in an inoculum of approximately 1×10^5 cfu/ml. This inoculum was added to the sensititre plates, 50 µl per well. Plates were sealed and incubated at 36 ±2 °C for 18 to 24 hours (see Figure 2). Results were interpreted using the SensiTouch® system. For all antibiotics 100% inhibition was used as endpoint, with the exception of sulfamethoxazole and trimethoprim for which an endpoint

of 80% inhibition was used (CLSI 2009). Test results were invalid if no substantial growth was observed in control wells without antibiotics.

2.4.3 *Etest®*

Isolates were streaked onto culture media (Appendix 1b) and incubated at 36 ±2 °C for 18 to 24 hours. From fresh cultures, single colonies were picked and suspended in 3 ml sterile physiological saline solution to a density corresponding to 0.5 MacFarland (or 1 MacFarland for *S. aureus*). These suspensions were evenly streaked onto Mueller-Hinton (MH) agar (Oxoid, Badhoevedorp) using cotton swabs. After 15 to 30 minutes Etest® strips (see Appendix 4) were applied using a sterile pair of tweezers. Tests were interpreted after 16 to 20 hours of incubation at 36 ±2 °C (see Figure 2). For testing of oxacillin susceptibility of *S. aureus* strains, MH agar was supplemented with 2% NaCl, and plates were incubated for 24 hours. According to the instructions of the manufacturer, the endpoints used were 100% inhibition for biocidals and 80% inhibition for biostatics.

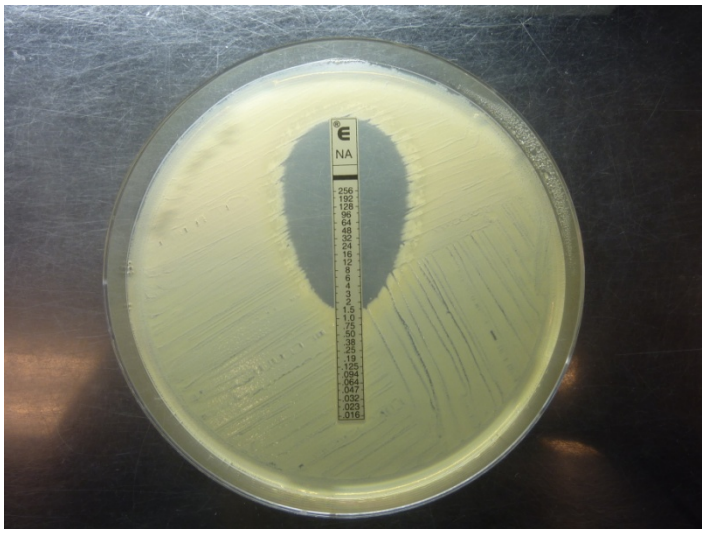

Figure 2 Etest® for antibiotic susceptibility determination. Shown is an arbitrarily selected E. coli strain tested for a biocidal antibiotic (nalidixic acid). The MIC-value in this example is 2 mg/l.

2.4.4 *Calculation of percentages of resistant strains*

To determine whether isolates are resistant to antibiotics, their MIC values are compared to so-called MIC breakpoints. Isolates growing at higher concentrations than the breakpoint concentration are considered resistant; isolates with a MIC value below the breakpoint are considered sensitive. For this purpose either epidemiological cut-off values or clinical breakpoints can be used (Dalhoff et al. 2009; EUCAST 2010). A MIC value above the epidemiological cut-off value indicates that the strain in question is less sensitive than wild-type strains of the same species. This means that the strain has developed mutations or acquired resistance genes that confer (relative) resistance to an antibiotic. By contrast, a MIC value above the clinical breakpoint indicates slim chances of successful treatment in case of infection. Clinical breakpoints are not solely based on the relative susceptibility of a bacterial strain to a specific antibiotic, but also on pharmacokinetics of the antibiotic and experience regarding treatment results. Bacterial strains resistant as compared to wild-type strains

are not necessarily resistant from a clinical perspective. For the present study the epidemiological cut-off values as determined by the European Committee on Antibiotic Susceptibility Testing (EUCAST 2010) were used.

2.4.5 Confirmation of ESBL production by E. coli strains

Cefotaxime-resistant *E. coli* strains were tested for ESBL production by means of the double disk confirmation method, according to CLSI directions (CLSI 2010). The method entails comparison of growth in the presence of the cephalosporin antibiotics cefotaxime and ceftazidime, both in the absence and presence of clavulanic acid, a compound that inhibits ESBL activity. For this purpose Sensi Disc™ susceptibility test discs (BD, Breda) were used containing 30µg/ml cefotaxim +/ - 10µg/ml clavulanic acid and discs containing 30µg/ml ceftazidime +/ - 10µg/ml clavulanic acid (see Figure 3).

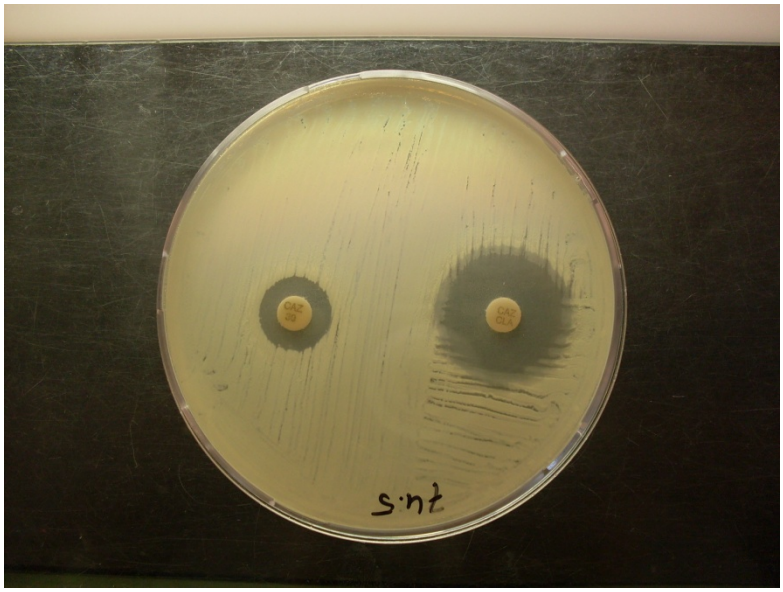

Figure 3 ESBL confirmation test using Sensi-Disc™. Shown is a cefotaxime-resistant E. coli strain tested for ESBL-production using discs containing ceftazidime (left) and ceftazidime + clavulanic acid (right). For ESBL-producing strains the diameter of the inhibition zone surrounding the disc with clavulanic acid is at least 5 mm larger than the diameter surrounding the disc without clavulanic acid (CLSI 2010). In the example the diameters are 13 and 21 mm respectively, indicating ESBL production.

2.5 Estimation of concentrations of antibiotic resistant bacteria

To estimate the average concentrations of antibiotic resistant *E. coli, E. faecium* and *E. faecalis* at the sampled locations during the bathing season, the following parameters were calculated:

A. Average *E. coli* or *Enterococcus* spp. concentrations (cfu/l): average of concentrations measured at six sampling time-points: May, June, July, August, September (2x).

B. Average proportions of *E. faecium* and *E. faecalis* among all enterococcus isolates isolated during these months (i.e., from all strains that were successfully identified to species level).

C. Average proportions of resistant *E. coli, E. faecium and E. faecalis* strains observed (i.e., based on all strains obtained during the bathing season).

Average concentrations of resistant *E. coli* were calculated for each river using the following formulas:

$$R_{E.coli\ Meuse} = \frac{A_{E.coli\ Meuse} \times C_{E.coli\ Meuse}}{100} \qquad R_{E.coli\ Rhine} = \frac{A_{E.coli\ Rhine} \times C_{E.coli\ Rhine}}{100}$$

$$R_{E.coli\ NewMeuse} = \frac{A_{E.coli\ NewMeuse} \times C_{E.coli\ NewMeuse}}{100}$$

Average concentrations of resistant *E. faecium* and *E. faecalis* were calculated for the three rivers combined using the following formulas:

$$R_{E.faecium\ all} = \frac{A_{Ent.spp.all} \times B_{E.faecium\ all} \times C_{E.faecium\ all}}{10000}$$

$$R_{E.faecalis\ all} = \frac{A_{Ent.spp.all} \times B_{E.faecalis\ all} \times C_{E.faecalis\ all}}{10000}$$

Minimum and maximum concentrations of resistant *E. coli*, *E. faecium* and *E. faecalis* were estimated by substituting the average concentration (A) in the formulas by the season's highest and lowest observed concentrations (see Appendices 9 and 10).

3 Results

3.1 Isolation of bacteria from rivers Meuse, Rhine and New Meuse

The rivers Meuse, Rhine and New Meuse were analysed for the presence of *E. coli*, enterococci and *S. aureus* every four weeks from May 2008 until May 2009. *E. coli* and enterococci were detected in nearly all samples. Exceptions were May 2008 and May 2009 samples from the Rhine and the New Meuse, which were negative for enterococci and the December 2008 sample from the Meuse in which neither bacterial species was detected.[1] *S. aureus* was detected only in the Rhine and the Meuse and only from January 2009 up to and including March (Rhine) or April (Meuse).

At four time-points the presence of *Salmonella* and *Campylobacter* was additionally investigated (July 2008, September 2008, January 2009, March 2009). *Salmonella* was detected in all three rivers in March 2009 and in the New Meuse in January 2009 as well. *Campylobacter* was detected in all three rivers in September 2008 and January 2009 and in the Meuse and the Rhine additionally in July 2008 and March 2009. In positive samples, concentrations of *S. aureus*, *Campylobacter* and *Salmonella* were on average 2- to 4-log lower than concentrations of *E. coli* and enterococci (Table 3).

Table 3 Average concentrations of Enterococcus spp., E. coli, S. aureus, Campylobacter and Salmonella in the Meuse, Rhine and New Meuse

	Average concentration (cfu/l) (range) ***number of positive samples (n)***				
	Enterococcus	*E. coli*	*S. aureus*	*Campylo-bacter*	*Salmonella*
Meuse	13,649 (1364 – 33,462) n=13	60,343 (10,773 – 130,500) n=13	132 (24 – ≥240[1]) n=4	24 (12 – 46) n=4	2.1 n=1
Rhine	6141 (4.5 – 27,500) n=13	22,488 (356 – 98,500) n=13	168 (24 – ≥240[1]) n=3	47 (11 – 134) n=4	0.5 n=1
New Meuse	741 (27 – 3818) n=10	5110 (338 – 29,500) n=11	N.A.	13 (3 – 24) n=2	0.3 (0.1 – 0.4) n=2

Indicated are average concentrations in the samples that were positive for the indicated microorganisms, with lowest and highest concentrations between brackets. Shown in grey are the numbers of positive samples. Not included in this analysis were positive samples from May 2008 and from July 2008 from the New Meuse because the samples were disallowed for quantification. N.A. = not applicable (no positive samples). [1]Value represents the lower limit since all test volumes were positive; this value was used for calculation of the average value.

[1] Colonies were obtained and stored but none appeared to be *E. coli* or enterococci in identification tests.

Concentrations of *E. coli*, enterococci and *Salmonella* were on average highest in the Meuse, while concentrations *Campylobacter* and *S. aureus* were on average highest in the Rhine.

An overview of isolated species/genera per time-point and location is given in Appendix 5. Overall, 286 *E.coli*, 318 *Enterococus*, 48 *S. aureus*, 39 *Campylobacter* and 26 *Salmonella* isolates were confirmed and identified to species level (Table 4).

Table 4 Numbers of bacterial isolates per species/genus obtained from the Meuse, Rhine and New Meuse

Bacterial Species / genus	**Number of confirmed isolates (from number of samples)**			
	Meuse	Rhine	New Meuse	Total
E. coli	92 (13)	94 (13)	100 (13)	286 (39)
Enterococcus	116 (13)	100 (13)	102 (11)	318 (37)
*S. aureus**	32 (4)	26 (3)	0 (0)	48 (7)
*Salmonella**	11 (1)	10 (1)	5 (1)	26 (3)
*Campylobacter**	20 (2)	10 (1)	9 (1)	39 (4)

** Obtained using pre-enrichment.*

3.2 Antibiotic resistant *Escherichia coli*

Overall, complete antibiotic sensitivity profiles were obtained for 277 *E. coli* river isolates. Antibiotics included were ampicillin, trimethoprim, cefotaxime, streptomycin, tetracycline, ciprofloxacin, chloramphenicol and sulfamethoxazole. For each river, the Meuse (n=90), Rhine (n=92) and New Meuse (n=95), MIC-distributions and percentages of resistant strains were determined (Table 5, Figure 4).

In each river, the most frequently detected resistances among *E. coli* were resistance to ampicillin, streptomycin, tetracyclin, trimethoprim or sulfamethoxazole, while resistance to chloramphenicol, ciprofloxacin and cefotaxime was less common. Cefotaxime-resistant *E. coli* – and therewith suspected extended spectrum beta-lactamase (ESBL)-producing strains – were only detected in the Meuse. Five out of six of these cefotaxime-resistant strains, one with a MIC of 4 mg/l and four with MICs of >8 mg/l, were indeed confirmed to produce ESBLs. *E. coli* with high MIC-values (>1 mg/l) for ciprofloxacin were more often detected in the New Meuse (4.4%) compared to the Rhine (2.2%) and the Meuse (1.1%). All ciprofloxacin-resistant *E. coli* strains were subsequently tested for sensitivity to another fluoroquinolone, nalidixic acid. All strains with MIC values of ≥ 0.25 for ciprofloxacin proved to be resistant to nalidixic acid as well, with MICs of >256 mg/l. By contrast, ciprofloxacin-resistant strains with low MICs (< 0.25 mg/l) were nalidixic acid-sensitive (data not shown).

Table 5 MIC-distributions (%) for E. coli isolates from the Meuse, the Rhine and the New Meuse May 2008 - May 2009, as well as percentages resistant isolates (%R)

	MIC distributions mg/l (%)																	
Meuse (n=90)	**0.03**	**0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**	**512**	**1024**	**2048**	%R
ampicillin						3,3	32,2	31,1	2,2	0,0	0,0	1,1	30,0					31,1
cefotaxim		86,7	5,6	1,1	1,1	0,0	0,0	1,1	0,0	4,4								6,7
tetracyclin						17,8	47,8	7,8	0,0	0,0	0,0	6,7	12,2	7,8				26,7
streptomycin							0	5,6	56,7	15,6	2,2	3,3	1,1	7,8	7,8			22,2
trimethoprim					77,8	1,1	1,1	1,1	0,0	0,0	0,0	0,0	18,9					20
ciprofloxacin	92,2	2,2	1,1	2,2	1,1	0,0	0,0	0,0	1,1									7,8
chloramphenicol	0	0	0	0	0	1,1	6,7	56,7	28,9	1,1	2,2	0	0	0	3,3			5,6
sulfamethoxazole		0	0	0	0	0	0	0	10	20	32,2	11,1	2,2	0,0	0,0	2,22	22,2	24,4

Rhine (n=92)	**0.03**	**0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**	**512**	**1024**	**2048**	%R
ampicillin						4,3	31,5	44,6	2,2	0,0	0,0	1,1	16,3					17,4
cefotaxim		92,4	6,5	1,1	0,0	0,0	0,0	0,0	0,0	0,0								0,0
tetracyclin						12,0	55,4	12,0	1,1	0,0	0,0	3,3	10,9	5,4				19,6
streptomycin							0	2,2	72,8	12,0	0,0	2,2	3,3	3,3	4,3			13,0
trimethoprim					85,9	3,3	0,0	0,0	0,0	0,0	0,0	0,0	10,9					10,9
ciprofloxacin	92,4	2,2	0,0	1,1	1,1	1,1	0,0	1,1	1,1									7,6
chloramphenicol	0	0	0	0	0	1,1	5,4	60,9	27,2	2,2	1,1	0	0	0	2,2			3,3
sulfamethoxazole		0	0	0	0	0	0	0	9,8	25,0	22,8	22,8	2,2	0,0	0,0	3,3	14,1	17,4

New Meuse (n=95)	**0.03**	**0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**	**512**	**1024**	**2048**	%R
ampicillin						3,2	35,8	38,9	3,2	0,0	1,1	0,0	17,9					18,9
cefotaxim		89,5	6,3	4,2	0,0	0,0	0,0	0,0	0,0	0,0								0,0
tetracyclin						9,5	61,1	11,6	0,0	0,0	0,0	6,3	7,4	4,2				17,9
streptomycin							0	2,1	64,2	12,6	2,1	2,1	2,1	5,3	9,5			21,1
trimethoprim					83,2	2,1	0,0	0,0	0,0	0,0	0,0	0,0	14,7					14,7
ciprofloxacin	90,5	2,1	1,1	2,1	0,0	0,0	0,0	0,0	4,2									9,5
chloramphenicol	0	0	0	0	0	0,0	10,5	61,1	23,2	0,0	0,0	0	0	0	5,3			5,3
sulfamethoxazole		0	0	0	0	0	1,053	0	5,3	22,1	27,4	15,8	6,3	0,0	0,0	4,2	17,9	22,1

White areas represent the concentrations tested for the indicated antibiotics. Results filled in at concentrations above this range indicate isolates having a MIC value above the highest concentration tested. Results filled in at the lowest test concentration indicate isolates with a MIC-value smaller than or equal to the indicated concentration. Vertical lines demonstrate the epidemiological cut-off values. Double vertical lines for ciprofloxacin and sulfamethoxazole demonstrate breakpoints used in the Maran report (2007).

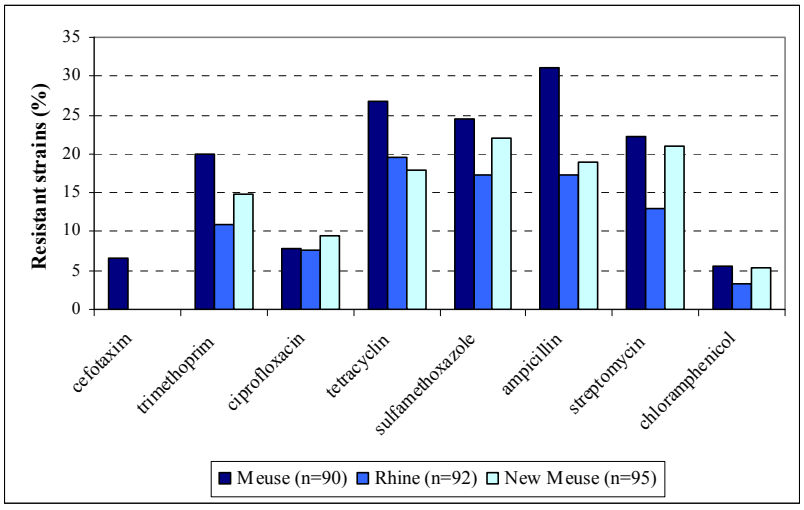

Figure 4 Percentages of antibiotic resistant E. coli strains from the Meuse, the Rhine and the New Meuse. Shown are results for isolates from all sampling time-points combined.

Almost half (48%) of all *E. coli* strains isolated from the Meuse were resistant to at least one antibiotic and of these the majority (77%) were resistant to two antibiotics or more (Figure 5). In the Rhine and the New Meuse approximately one third of all *E. coli* were resistant to at least one antibiotic (32% and 34%),

and of these a small majority (55% and 59% respectively) were resistant to two antibiotics or more. Overall, 10% of all *E. coli* isolated from the three rivers were resistant to five or more antibiotics. The maximum number of resistances per individual strain was seven, which was observed for one strain from the Meuse as well as for one strain from the New Meuse. All detected resistance phenotypes are listed in Appendix 6.

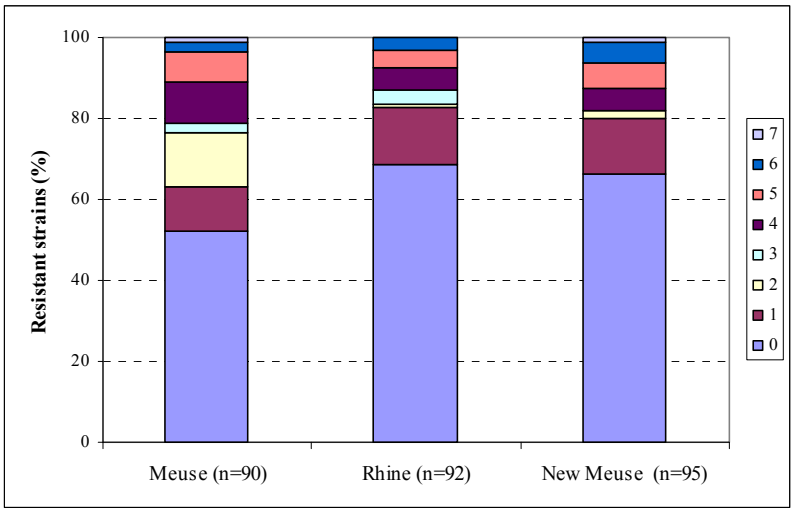

Figure 5 Percentages of sensitive, monoresistant and multiresistant E. coli strains observed in Dutch rivers

3.3 Antibiotic resistant intestinal enterococci

Complete antibiotic sensitivity profiles were obtained for 304 *Enterococcus*-isolates, for which the species distribution is shown in Table 6. The two enterococcus species that account for over 90% of all human infections, *E. faecalis* and *E. faecium,* made up 59, 49 and 41% of all isolates recovered from the Meuse, Rhine and New Meuse respectively. Of these *E. faecalis* was the most abundant species in the Meuse, while *E. faecium* was the most abundant species in the Rhine and the New Meuse. The vast majority of the remainder of recovered isolates were *E. durans* and *E. hirae* (overall 44%). Minor species were *E. mundtii* and *E. casseliflavus* (also referred to as *E. flavescens,* (Descheemaeker et al. 1997)).

Antibiotics included in the sensitivity profiles were synercid (i.e., combination of quinupristin and dalfopristin), tetracycline, salinomycin, erythromycin, vancomycin, sulfamethoxazole/trimethoprim, ampicillin and gentamicin. The antibiotic sensitivity of *E. faecium* (§3.3.1), *E. faecalis* (§3.3.2) and the other *Enterococcus* species (§3.3.3) are discussed separately in the next sections.

Table 6 Enterococcus spp. isolates with complete antibiotic susceptibility profile

Species	Meuse (%)	Rhine (%)	New Meuse (%)	Total (%)
E. faecium	28 (25)	37 (37)	26 (29)	91 (30)
E. faecalis	39 (34)	12 (12)	11 (12)	62 (20)
E. durans	25 (22)	22 (22)	21 (23)	68 (22)
E. hirae	17 (15)	17 (17)	15 (17)	49 (16)
E. mundtii	0 (0)	8 (8)	8 (9)	16 (5)
E. casseliflavus	5 (4)	2 (2)	8 (9)	16 (5)
E. gallinarum	0 (0)	0 (0)	1 (1)	1 (0.3)
Other species[1]	0 (0)	2 (2)	0 (0)	2 (1)
Total	114	100	90	304

[1]*One isolate clustered with E. moraviensis/E. sileciacus/E. caccae reference sequences, the other with E. italicus/E. camelliae. Given the low prevalence, no attempts were made to further distinguish between these species.*

3.3.1 *E. faecium isolates*

For 28, 37 and 26 *E. faecium* isolates from Meuse, Rhine and New Meuse respectively, complete antibiotic sensitivity profiles were obtained and MIC-distributions and percentages of resistant strains were determined (Table 7, Figure 6). Overall, resistance to synercid, tetracycline, erythromycin and ampicillin were most frequently detected, although differences in prevalence were observed between rivers (Figure 7). Thus, ampicillin-resistant *E. faecium* were detected twice as often in the New Meuse (33%) compared to Rhine (16%) and Meuse (14%). Resistance to erythromycin and tetracycline less frequently observed in the Meuse (11 and 25%) compared to Rhine (27 and 51%) and New Meuse (33 and 37%). In general, *E. faecium* isolates resistant to trimethoprim/sulfamethoxazole, salinomycin or gentamicin were less frequently detected and salinomycine- and gentamicin-resistance only in two of the three rivers (salinomycine-resistance in Rhine and New Meuse, gentamicin-resistance in Meuse and New Meuse). None of the isolates were vancomycine-resistant.

One *E. faecium* isolate from the Meuse and one from the New Meuse were highly resistant to gentamicin with MICs over 256 µg/ml. Because these results were indicative for high-level aminoglycoside resistance (HLAR), these isolates were retested using high-range gentamicin Etest strips and additionally tested for streptomycine-sensitivity. Both isolates had MICs of > 1024 µg/ml for both aminoglycoside antibiotics, confirming a HLAR phenotype.

In all rivers, more than half of the *E. faecium* isolates were resistant to at least one antibiotic: 54% in the Meuse, 59% in the Rhine and 58% in the New Meuse (Figure 7). In the latter two rivers, the majority of all resistant *E. faecium* strains (68 and 67%) were resistant to two or more antibiotics. In the Meuse, 40% of resistant isolates were multiresistant. Overall, 8% of all *E. faecium* river strains were resistant to five or six antibiotics. Resistances to six different antibiotics was observed for one strain that was isolated from the Rhine. An overview of all detected resistance phenotypes is given in Appendix 7.

Table 7 MIC distributions (%) for E. faecium isolates from the Meuse, the Rhine and the New Meuse May 2008 – May 2009, as well as percentages of resistant isolates (%R)

	MIC distributions mg/l (%)																	
Meuse (n=28)	**0.03**	**0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**	**512**	**1024**	**2048**	%R
synercid					32,1	25,0	17,9	25,0	0,0	0,0	0,0	0,0	0,0					42,9
tetracyclin				14,3	46,4	14,3	0,0	0,0	0,0	0,0	0,0	25,0						25,0
salinomycin					7,1	35,7	57,1	0,0	0,0	0,0	0,0	0,0						0,0
erythromycin				7,1	10,7	3,6	57,1	10,7	0,0	0,0	0,0	10,7						10,7
vancomycin					60,7	21,4	17,9	0,0	0,0	0,0	0,0	0,0	0,0					0,0
trim/sulfa*				75,0	10,7	7,1	3,6	0,0	0,0	0,0	3,6							7,1
ampicillin	0,0	0,0	7,1	14,3	21,4	32,1	3,6	7,1	3,6	7,1	0,0	3,6	0,0	0,0	0,0			14,3
gentamicin		0,0	0,0	0,0	0,0	0,0	0,0	32,1	57,1	7,1	0,0	0,0	0,0	0,0	3,6			3,6

Rhine (n=37)	**0.03**	**0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**	**512**	**1024**	**2048**	%R
synercid					37,8	16,2	10,8	29,7	5,4	0,0	0,0	0,0	0,0					45,9
tetracyclin				2,7	40,5	5,4	0,0	0,0	2,7	0,0	0,0	48,6						51,4
salinomycin					2,7	27,0	67,6	0,0	2,7	0,0	0,0	0,0						2,7
erythromycin				2,7	0,0	10,8	45,9	13,5	0,0	0,0	0,0	27,0						27,0
vancomycin					51,4	27,0	13,5	8,1	0,0	0,0	0,0	0,0	0,0					0,0
trim/sulfa*				83,8	5,4	5,4	0,0	0,0	0,0	0,0	5,4							5,4
ampicillin	0,0	0,0	5,4	13,5	10,8	27,0	5,4	21,6	2,7	0,0	2,7	5,4	0,0	2,7	2,7			16,2
gentamicin		0,0	0,0	0,0	0,0	0,0	0,0	35,1	51,4	13,5	0,0	0,0	0,0	0,0	0,0			0,0

New Meuse (n=26)	**0.03**	**0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**	**512**	**1024**	**2048**	%R
synercid					34,6	26,9	11,5	15,4	11,5	0,0	0,0	0,0	0,0					38,5
tetracyclin				3,8	30,8	26,9	0,0	0,0	0,0	0,0	3,8	34,6						38,5
salinomycin					0,0	23,1	69,2	3,8	3,8	0,0	0,0	0,0						3,8
erythromycin				11,5	0,0	23,1	26,9	3,8	0,0	0,0	3,8	30,8						34,6
vancomycin					61,5	15,4	15,4	7,7	0,0	0,0	0,0	0,0	0,0					0,0
trim/sulfa*				65,4	19,2	7,7	0,0	0,0	0,0	0,0	7,7							7,7
ampicillin	0,0	0,0	3,8	3,8	11,5	38,5	3,8	3,8	0,0	7,7	3,8	7,7	3,8	0,0	11,5			34,6
gentamicin		0,0	0,0	0,0	0,0	0,0	0,0	23,1	65,4	7,7	0,0	0,0	0,0	0,0	3,8			3,8

*White areas represent the concentrations tested for the indicated antibiotics. Results filled in at concentrations above this range indicate isolates having a MIC value above the highest concentration tested. Results filled in at the lowest test concentration indicate isolates with a MIC value smaller than or equal to the indicated concentration. Vertical lines demonstrate the epidemiological cut-off values. *Trimethoprim/sulfamethoxazole. Indicated are trimethoprim concentrations; the ratio of sulfamethoxazole to trimethoprim was 19 to 1.*

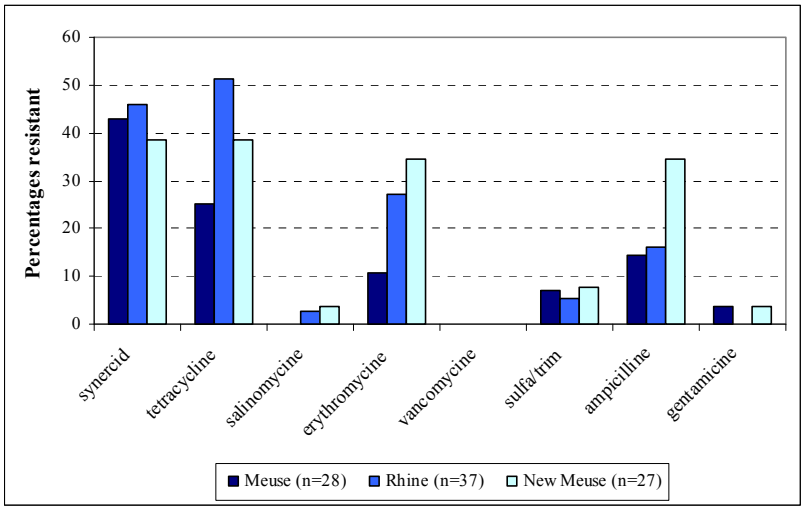

Figure 6 Percentages of antibiotic resistant E. faecium strains from the Meuse, the Rhine and the New Meuse. Shown are results for isolates from all sampling time-points combined.

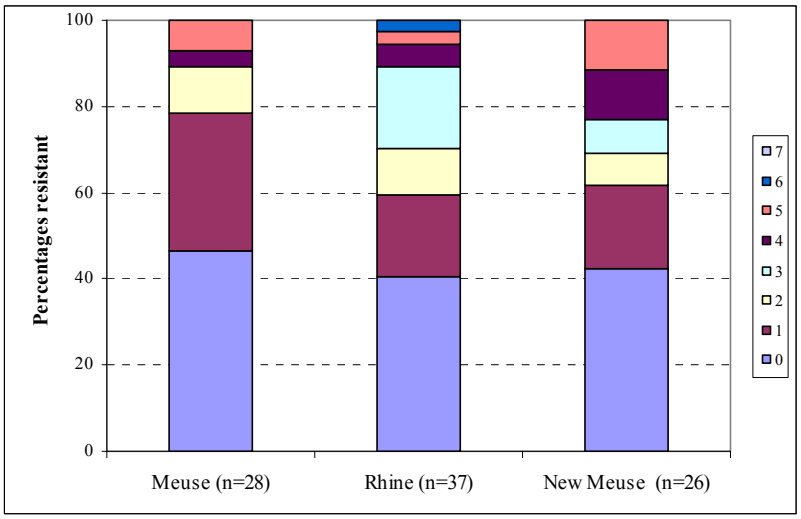

Figure 7 Percentages of sensitive, monoresistant and multiresistant E. faecium strains observed in Dutch rivers.

3.3.2 *E. faecalis isolates*

For 39, 12 and 11 *E. faecalis* isolates, from the Meuse, Rhine and New Meuse respectively, complete antibiotic sensitivity profiles were obtained and MIC distributions and percentages resistant strains determined (Table 8, Figure 8). For *E. faecalis* epidemiological cut-off values for sulfamethoxazole/trimethoprim and synercid have not been documented. For synercid, a cut-off value of 32 µg/ml was used, according to recommendations of the European Food Safety Authority (EFSA 2008). This relatively high breakpoint value is related to the intrinsic resistance of *E. faecalis* to streptogramins (Bouanchaud 1997; Williams et al. 1997; Allington and Rivey 2001; Duh et al. 2001). For trimethoprim and sulfamethoxazole the cut-off value of *E. faecium* (1/ 19 µg/ml) was used as breakpoint.

The most commonly detected resistance for *E. faecalis* was tetracycline-resistance. In the New Meuse this was the only resistance detected for this species, with a prevalence of 18%. In the Meuse and Rhine respectively 56% and 42% of *E. faecalis* were tetracycline-resistant; In these two rivers, erythromycine-resistance was also common (25–26% respectively). Small proportions of Meuse- and Rhine-isolates were resistant to trimethoprim/sulfamethoxazole (8% in both rivers) or to gentamicin (3 and 8% respectively). Resistance to salinomycin, vancomycine or ampicillin was not detected, nor was resistance to synercid.

The gentamicin-resistant *E. faecalis* strains from the Meuse and the Rhine (one from each river) were retested using high-range gentamicin Etest strips and additionally tested for streptomycine-sensitivity. Both isolates had MICs of > 1024 µg/ml for both aminoglycoside antibiotics, confirming a HLAR phenotype.

Table 8 MIC-distributions (%) for E. faecalis isolates from the Meuse, the Rhine and the New Meuse May 2008 - May 2009, as well as percentages resistant isolates (%R)

	MIC distributions mg/l (%)																	
Meuse (n=39)	**0.03**	**0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**	**512**	**1024**	**2048**	%R
synercid					2,6	0,0	15,4	12,8	30,8	33,3	5,1	0,0	0,0					0,0
tetracyclin				2,6	10,3	23,1	7,7	0,0	0,0	0,0	0,0	56,4						56,4
salinomycin					12,8	82,1	5,1	0,0	0,0	0,0	0,0	0,0						0,0
erythromycin				0,0	10,3	35,9	12,8	15,4	0,0	0,0	0,0	25,6						25,6
vancomycin					2,6	61,5	28,2	7,7	0,0	0,0	0,0	0,0	0,0					0,0
trim/sulfa*				92,3	0,0	0,0	0,0	0,0	0,0	0,0	7,7							7,7
ampicillin	0,0	0,0	0,0	0,0	25,6	66,7	7,7	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0			0,0
gentamicin		0,0	0,0	0,0	0,0	0,0	0,0	0,0	2,6	64,1	30,8	0,0	0,0	0,0	2,6			2,6

Rhine (n=12)	**0.03**	**0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**	**512**	**1024**	**2048**	%R
synercid					0,0	0,0	0,0	16,7	16,7	66,7	0,0	0,0	0,0					0,0
tetracyclin				0,0	16,7	33,3	8,3	0,0	0,0	0,0	0,0	41,7						41,7
salinomycin					0,0	100,0	0,0	0,0	0,0	0,0	0,0	0,0						0,0
erythromycin				8,3	16,7	25,0	8,3	16,7	0,0	0,0	0,0	25,0						25,0
vancomycin					0,0	58,3	33,3	8,3	0,0	0,0	0,0	0,0	0,0					0,0
trim/sulfa*				91,7	0,0	0,0	0,0	0,0	0,0	0,0	8,3							8,3
ampicillin	0,0	0,0	0,0	0,0	16,7	75,0	8,3	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0			0,0
gentamicin		0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	58,3	33,3	0,0	0,0	0,0	8,3			8,3

New Meuse (n=11)	**0.03**	**0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**	**512**	**1024**	**2048**	%R
synercid					0,0	0,0	9,1	9,1	54,5	27,3	0,0	0,0	0,0					0,0
tetracyclin				0,0	18,2	36,4	27,3	0,0	0,0	0,0	0,0	18,2						18,2
salinomycin					9,1	90,9	0,0	0,0	0,0	0,0	0,0	0,0						0,0
erythromycin				18,2	27,3	18,2	18,2	18,2	0,0	0,0	0,0	0,0						0,0
vancomycin					9,1	45,5	36,4	9,1	0,0	0,0	0,0	0,0	0,0					0,0
trim/sulfa*				100,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0							0,0
ampicillin	0,0	0,0	0,0	0,0	27,3	63,6	9,1	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0			0,0
gentamicin		0,0	0,0	0,0	0,0	0,0	0,0	0,0	18,2	27,3	54,5	0,0	0,0	0,0	0,0			0,0

White areas represent the concentrations tested for the indicated antibiotics. Results filled in at concentrations above this range indicate isolates having a MIC value above the highest concentration tested. Results filled in at the lowest test concentration indicate isolates with a MIC-value smaller than or equal to the indicated concentration. Vertical lines demonstrate the EUCAST epidemiological cut-off values and the EFSA breakpoint for synercid. For synercid, the CLSI clinical break-point is indicated as well (dashed line). For trimethoprim/ sulfamethoxazole the EUCAST epidemiological cut-off values of E. faecium is indicated (double lines).
**Trimethoprim/sulfamethoxazole. Indicated are trimethoprim concentrations; the ratio of sulfamethoxazole to trimethoprim was 19 to 1.*

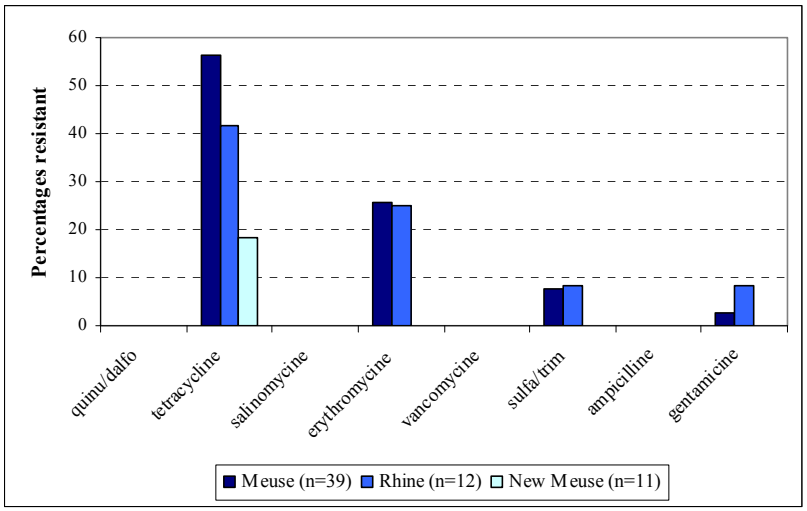

Figure 8 Percentages of antibiotic resistant E. faecalis strains from the Meuse, the Rhine and the New Meuse. Shown are results for isolates from all time-points combined.

In the Meuse and the Rhine, more than half of *E. faecalis* strains were resistant to at least one antibiotic (54 and 59%) (Figure 9). Of these resistant strains, 45% and 50% in the Meuse and Rhine respectively, were resistant to two or more antibiotics. In the New Meuse, resistance among *E. faecalis* was less common. In this river 18% of strains was resistant and all were resistant to just one of the tested antibiotics (tetracycline). The maximum number of resistances carried by an individual strain was four; this was observed for three isolates from the Meuse. An overview of all detected resistance phenotypes is given in Appendix 7.

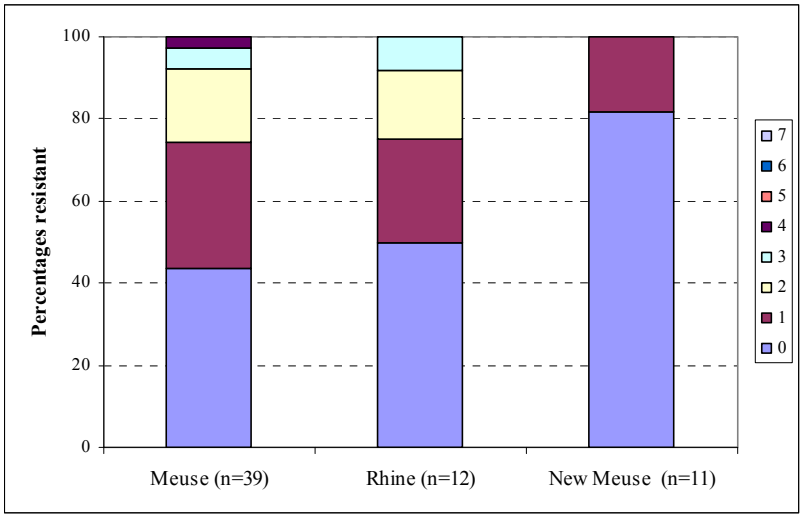

Figure 9 Percentages of sensitive, monoresistant and multiresistant E. faecalis strains observed in Dutch rivers.

3.3.3 Non-E. faecium, non-E. faecalis species

Complete antibiotic sensitivity profiles were obtained for 151 *Enterococcus* isolates of species other than *E. faecium* and *E. faecalis.* The majority of these isolates were identified as *E. durans* (n=68) and *E. hirae* (n=49) species. The remainder were mostly *E. mundtii* (n=16) and *E. casseliflavus* (n=16) strains. MIC distributions were determined for isolates from these four species, combining results from all three rivers (Table 9). However, for most species/antibiotic combinations, epidemiological cut-off values are not documented. To allow comparison of antibiotic sensitivity across all *Enterococcus* species, the epidemiological cut-off values of *E. faecium* were used to calculate percentages of resistant strains (Table 9, Figure 10). Because synercid breakpoints are highly divergent for *E. faecium* and *E. faecalis* (1 vs. 32 µg/ml), for the sake of comparison between species (Figure 10), the clinical breakpoint of *Enterococcus* spp. as recommended by CLSI was used (CLSI 2010).

Table 9 MIC-distributions (%) for non-E. faecium, non-E. faecalis isolates from the Meuse, the Rhine and the New Meuse May 2008 - May 2009, as well as percentages of resistant isolates (%R)

	MIC distributions mg/l (%)																	
E. durans (n=68)	**0.03**	**0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**	**512**	**1024**	**2048**	%R
quinu/dalfo *					14,7	7,4	10,3	67,6	0,0	0,0	0,0	0,0	0,0					67,6
tetracyclin				4,4	36,8	45,6	4,4	1,5	0,0	0,0	0,0	7,4						7,4
salinomycin					0,0	22,1	61,8	16,2	0,0	0,0	0,0	0,0						0,0
erythromycin				23,5	4,4	0,0	4,4	35,3	27,9	4,4	0,0	0,0						32,4
vancomycin					58,8	39,7	0,0	1,5	0,0	0,0	0,0	0,0	0,0					0,0
sulfa/trim *				82,4	16,2	0,0	0,0	0,0	0,0	0,0	1,5							1,5
ampicillin	0,0	0,0	4,4	2,9	19,1	33,8	30,9	8,8	0,0	0,0	0,0	0,0	0,0	0,0	0,0			0,0
gentamicin		0,0	0,0	0,0	0,0	0,0	5,9	19,1	55,9	14,7	4,4	0,0	0,0	0,0	0,0			0,0

	MIC distributions mg/l (%)																	
E. hirae (n=49)	**0.03**	**0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**	**512**	**1024**	**2048**	%R
quinu/dalfo *					6,1	14,3	22,4	55,1	2,0	0,0	0,0	0,0	0,0					57,1
tetracyclin				8,2	24,5	42,9	0,0	0,0	0,0	0,0	0,0	24,5						24,5
salinomycin					8,2	26,5	36,7	28,6	0,0	0,0	0,0	0,0						0,0
erythromycin				83,7	2,0	0,0	2,0	0,0	2,0	0,0	0,0	10,2						12,2
vancomycin					38,8	57,1	4,1	0,0	0,0	0,0	0,0	0,0	0,0					0,0
sulfa/trim *				63,3	32,7	2,0	0,0	0,0	0,0	0,0	2,0							2,0
ampicillin	0,0	0,0	4,2	12,5	35,4	31,3	10,4	6,3	0,0	0,0	0,0	0,0	0,0	0,0	0,0			0,0
gentamicin		0,0	0,0	0,0	0,0	0,0	0,0	14,3	53,1	28,6	4,1	0,0	0,0	0,0	0,0			0,0

	MIC distributions mg/l (%)																	
E. casseliflavus (n=15)	**0.03**	**0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**	**512**	**1024**	**2048**	%R
quinu/dalfo *					0,0	13,3	6,7	80,0	0,0	0,0	0,0	0,0	0,0					80,0
tetracyclin				0,0	0,0	6,7	86,7	0,0	0,0	0,0	0,0	6,7						6,7
salinomycin					6,7	6,7	80,0	6,7	0,0	0,0	0,0	0,0						0,0
erythromycin				13,3	0,0	6,7	26,7	53,3	0,0	0,0	0,0	0,0						0,0
vancomycin					0,0	0,0	0,0	13,3	80,0	6,7	0,0	0,0	0,0					86,7
sulfa/trim *				93,3	6,7	0,0	0,0	0,0	0,0	0,0	0,0							0,0
ampicillin	0,0	0,0	0,0	6,7	46,7	33,3	13,3	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0			0,0
gentamicin		0,0	0,0	0,0	0,0	0,0	0,0	66,7	20,0	13,3	0,0	0,0	0,0	0,0	0,0			0,0

	MIC distributions mg/l (%)																	
E. mundtii (n=16)	**0.03**	**0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**	**512**	**1024**	**2048**	%R
quinu/dalfo *					0,0	0,0	50,0	50,0	0,0	0,0	0,0	0,0	0,0					50,0
tetracyclin				0,0	6,3	68,8	25,0	0,0	0,0	0,0	0,0	0,0						0,0
salinomycin					6,3	12,5	81,3	0,0	0,0	0,0	0,0	0,0						0,0
erythromycin				6,3	56,3	31,3	6,3	0,0	0,0	0,0	0,0	0,0						0,0
vancomycin					43,8	56,3	0,0	0,0	0,0	0,0	0,0	0,0	0,0					0,0
sulfa/trim *				100,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0							0,0
ampicillin	0,0	6,3	12,5	18,8	43,8	18,8	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0			0,0
gentamicin		0,0	0,0	0,0	0,0	0,0	0,0	6,3	68,8	25,0	0,0	0,0	0,0	0,0	0,0			0,0

*White areas represent the concentrations tested for the indicated antibiotics. Results filled in at concentrations above this range indicate isolates having a MIC value above the highest concentration tested. Results filled in at the lowest test concentration indicate isolates with a MIC value smaller than or equal to the indicated concentration. Vertical lines indicate EUCAST epidemiological cut-off values of E. faecium and the clinical breakpoint for synercid. These values were used to calculate percentages of resistant strains. For synercid, the epidemiological cut-off of E. faecium is indicated as well (dashed line). *Trimethoprim/sulfamethoxazole. Indicated are trimethoprim concentrations; sulfamethoxazole was added in a ratio of 19 to 1.*

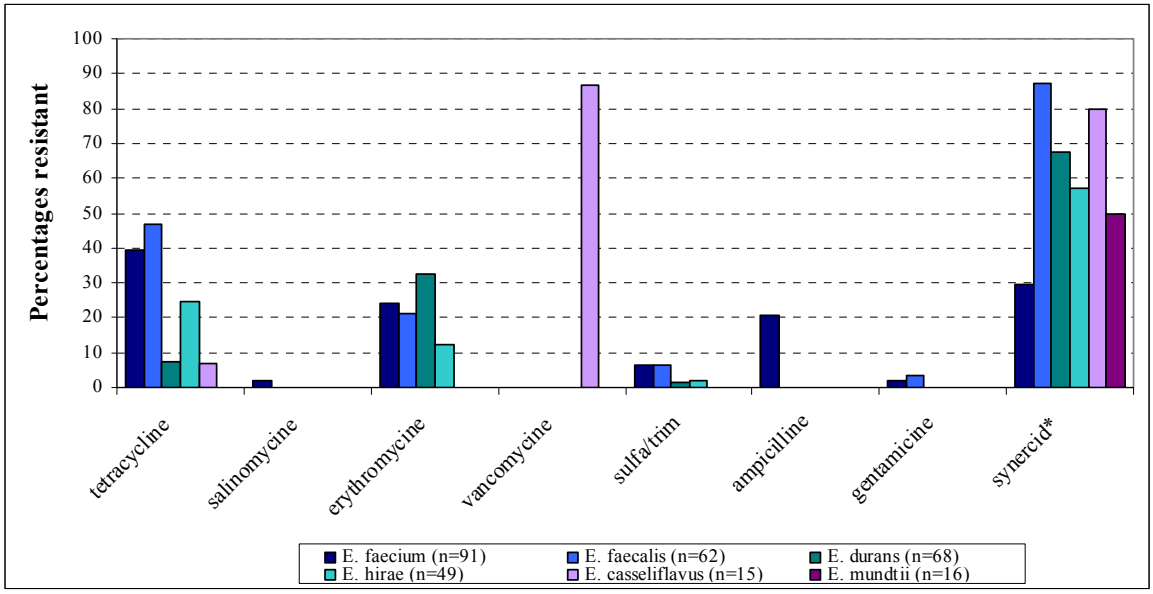

*Figure 10 Antibiotic resistance for different Enterococcus species isolated from the Meuse, Rhine and New Meuse. *For synercid, clinical resistance (MIC ≥4µg/ml) is shown.*

As observed for *E. faecium* and *E. faecalis*, the most frequently occurring resistances among the other *enterococcus* species were resistances to tetracycline and erythromycine. Tetracycline resistance was most frequently detected for *E. hirae* and to a lesser extent, also for *E. durans* and *E. casseliflavus*. Erythromycine resistance and resistance to sulfamethoxazole/trimethoprom was only observed for *E. hirae* and *E. durans*. Thirteen of the 15 *E. casseliflavus* isolates had low-level resistance to vancomycine (MICs of 8 and 16 µg/ml), in agreement with the described intrinsic low sensitivity to vancomycine of this species due to the presence of the *vanC* gene (Vincent et al. 1991; Clark et al. 1998). None of the non-*E. faecium* non-*E. faecalis* strains were resistant to ampicillin or gentamicin. Clinical resistance to synercid was nearly as common for *E. casseliflavus* as for *E. faecalis* (81% vs. 87%), and intermediate for the other non-*E. faecium* non-*E. faecalis* strains (50-68%) relative to *E. faecalis* and *E. faecium* (30%).

3.4 Antibiotic resistant *Staphylococcus aureus*

For thirty-two confirmed *S. aureus* isolates from the Meuse (derived from four different samples) and 26 isolates from the Rhine (derived from three samples), antibiotic sensitivity profiles were determined. Antibiotics included were synercid, tetracycline, salinomycin, erythromycin, vancomycin, trimethoprim/sulfamethoxazole, ciprofloxacin, oxacillin and clindamycin. Observed resistance phenotypes are shown in Table 10.

In one of the samples from the Meuse (from 17 February 2009) at least two different strains of *S. aureus* were simultaneously present: one MRSA and one MSSA. MRSA were also recovered from one of the Rhine samples (20 January 2009), in which case it was the only phenotype observed. All MRSA isolates were confirmed to carry the *MecA* gene by PCR. MRSA from both rivers were resistant to oxacillin, ciprofloxacin and erythromycin and the strain recovered from the Meuse was additionally resistant to clindamycine. All other *S. aureus* isolates that were recovered had wild-type sensitivity to all antibiotics tested.

Table 10 Observed resistance phenotypes of S. aureus isolates from the Meuse and the Rhine

	Meuse					*Rhine*		
	20-Jan-09 (n=5)	17-Feb-09[1] (n=8)	17-Feb-09[1] (n=2)	7-Mar-09 (n=7)	14-Apr-09 (n=10)	20-Jan-09 (n=6)	17-Feb-09 (n=10)	07-Mar-09 (n=10)
Syn	≤0.05	≤0.05	1	≤0.05	≤0.05/1	≤0.05/1	≤0.05	≤0.05
Tet	0.5	0.5/1	1	≤0.25/0.5	0.5/1	0.5/1	0.5	0.5
Sal	1	1	1/2	1	1	2	1	1/2
Ery	0.5	0.5	>32	0.5	0.5	>32	0.5	≤0.25/0.5
Van	1	1/2	1	1	1	1	1	1
Sxt	≤0.25	≤0.25/0.5	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25/0.5
Cip	0.5/1	0.5/1	>32	0.25-1	0.25-1	>32	0.5/1	0.25/0.5
Oxa	0.25	0.5/1	>256	0.5/1	0.25	>256	1/2	0.25
Cli	0.125	0.125	>256	0.125	0.125	0.125	0.125	0.125/0.25

Each column represents one of the phenotypes observed in a sample, with numbers between brackets representing the number of colonies with the indicated phenotype. Values are the observed MIC values (µg/ml) per phenotype. Identical phenotypes were defined as phenotypes with the same MICs or MICs maximally 2 dilution steps apart for each tested antibiotic; Syn = synercid, Tet = tetracycline, Sal = salinomycin, Ery = erythromycin, Van = vancomycin; Sxt = trimethoprim/sulfamethoxazole, Cip = ciprofloxacin, Oxa = oxacillin, Cli = clindamycin; Resistance is indicated in orange, break-points used were epidemiological cut-off values from EUCAST (2010), see Appendix 8.[1] From this sample, two different resistance phenotypes were identified.

3.5 Antibiotic resistant *Campylobacter*

Nineteen *Campylobacter* isolates from the Meuse, ten from the Rhine and nine from the New Meuse were analysed for antibiotic sensitivity. All isolates were identified as *Campylobacter coli*. Antibiotics included were: ampicillin, clarithromycin, ciprofloxacin, erythromycine, gentamicin, nalidixic acid, neomycin, streptomycin, sulfamethoxazole, tetracycline, tulathromycin, chloramphenicol. Observed resistance phenotypes are shown in Table 11.

In one of the samples from Meuse and New Meuse (both of 20 January 2009), two different resistance phenotypes were detected simultaneously: one pan-susceptible and the other with acquired resistance. The resistant strain from the Meuse was resistant to seven antibiotics, among which quinolones (ciprofloxacin and nalidixic acid) and macrolides (erythromycin, clarithromycin and tulathromycin). The resistant strain from the Rhine was resistant to one antibiotic: streptomycin. In the other samples all isolates were pan-susceptible.

In both river samples containing resistant *C. coli*, three of nine tested colonies were resistant. Assuming that during pre-enrichment no phenotype has a selective advantage over another, the prevalence of quinolone-/ macrolide-resistant *C. coli* in the Meuse and streptomycine-resistant *C. coli* in the Rhine was roughly 33% in the water sampled on 20 January 2009.

Table 11 Observed resistance phenotypes of C. coli isolates from the Meuse, Rhine and New Meuse

	Meuse			*Rhine*	*New Meuse*	
	8-Jul-08 (n=10)	20-Jan-09 (n=6)	20-Jan-09 (n=3)	20-Jan-09 (n=10)	20-Jan-09 (n=6)	20-Jan-09 (n=3)
Amp	1 / 2	1 / 2	>32	1 – 4	≤ 0.25 / 2	8
Cla	≤ 0.5	≤ 0.5 / 2	>64	≤ 0.5 / 1	≤ 0.5 / 1	≤ 0.5
Cip	≤ 0.12	≤ 0.12	16	≤ 0.12 / 0.25	≤ 0.12	≤ 0.12
Ery	≤ 0.5 / 1	≤ 0.5 / 1	>64	≤ 0.5 / 1	≤ 0.5 / 1	≤ 0.5
Gen	1	1	1	1	0.5 / 1	1
Nal	2 / 4	4	64	2 / 4	2 / 4	4 / 8
Neo	1 /2	2	1 /2	1 / 2	1 / 2	1 / 2
Str	2	2 / 4	2	2 – 8[1]	≤ 1 – 4	64
Smx	32 / 64	16 – 64	256	32 – 128	64 / 128	32
Tet	≤ 0.5	≤ 0.5	>64	≤ 0.5	≤ 0.5 / 1	≤ 0.5
Tul	≤ 0.5	≤ 0.5 / 2	64 / >64	≤ 0.5 / 1	≤ 0.5 / 1	≤ 0.5
Chl	≤ 2 / 4	≤ 2 / 4	4	≤ 2 / 4	≤ 2 / 4	4

Each column shows the phenotypes observed in a sample, with numbers between brackets representing the number of colonies with the indicated phenotype. Values are the observed MIC values (µg/ml) per phenotype. Identical phenotypes were defined as phenotypes with the same MICs or MICs maximally two dilution steps apart for each tested antibiotic;
Amp = ampicillin, Cla = clarithromycin, Cip = ciprofloxacin, Ery = erythromycin,
Gen = gentamicin, Nal = nalidixic acid, Neo = neomycin, Str = streptomycin,
Smx = sulfamethoxazole, Tet = tetracycline, Tul = tulathromycin, Chl = chloramphenicol;
Resistance is indicated in orange, break-points used were epidemiological cut-off values from EUCAST (2010) and MARAN (2007), see Appendix 7.[1] *For streptomycine, the observed range of maximally two steps apart includes a MIC just above break-point value (8 µg/ml); this value was observed once out of 10 times and may indicate the presence of two slightly deviating phenotypes rather than one.*

3.6 Antibiotic resistant *Salmonella*

Overall, 26 *Salmonella* isolates were analysed for antibiotic sensitivity. All isolates were derived from one time-point (March 2009). Among these isolates were different *Salmonella enterica* serotypes depending on river of origin. From the Meuse were recovered: *S.* Panama, *S.* Anatum, *S.* Derby, and *S.* 4, [5], 12:i:- ; from the Rhine: *S.* Typhimurium and *S.* Enteritidis (phage type 8); from the New Meuse: *S.* Enteritidis (phage type 21). Antibiotics included in the sensitivity profile were: streptomycin, tetracycline, cefotaxime, ciprofloxacin, trimethoprim, ampicillin, sulfamethoxazole, chloramphenicol and nalidixic acid. For each serovar, one resistance phenotype was observed, shown in Table 12.

Antibiotic resistant *Salmonella* were only detected in the Meuse: *S.* Panama resistant to ampicillin and *S.* 4, [5], 12:i:- resistant to ampicillin, streptomycin and sulfamethoxazole. No resistance was observed for the other recovered *Salmonella* serovars from the Meuse and the other two rivers.

Table 12 Observed resistance phenotypes of Salmonella enterica subspecies enterica serovars isolated from the Meuse, Rhine and New Meuse

	Meuse				*Rhine*		*New Meuse*
	S. Panama (n=2)	*S.* Anatum (n=1)	*S.* Derby (n=5)	*S.* 4,[5],12:i:- (n=3)	*S.* typhimurium (n=5)	*S.* enteritidis Pt. 8 (n=5)[1]	*S.* enteritidis Pt. 21 (n=5)
Amp	>64	≤1	2	>64	≤1	≤1/ 2	2
Fot	≤0.06 / 0.12	0.12	0.12	≤0.06 / 0.12	≤0.06 / 0.12	≤0.06 / 0.12	0.12
Tet	2	2	2/4	2	2 /4	2 / 4	4
Str	8/16	16	8 - 32	128 - >256	8 / 16	4	≤2 / 4
Tmp	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5
Cip	≤0.03	≤0.03	≤0.03	≤0.03	≤0.03	≤0.03	≤0.03
Chl	4	4	4 / 8	4	4	2 / 4	2 / 4
Smx	64 / 128	64	64 / 128	>1024	64 / 128	128	128
Nal	8	4	4	8	4	2 / 4	4

Each column shows the observed phenotypes for a serovar, with numbers between brackets representing the number of colonies of the indicated serovar and with indicated phenotype. Values are the observed MIC-values (µg/ml) per phenotype. Identical phenotypes were defined as phenotypes with the same MICs or MICs maximally 2 dilution steps apart for all each tested antibiotic;
Amp = ampicillin, Fot = cefotaxime; Tet = tetracycline, Str = streptomycin, Tmp = trimethoprim, Cip = ciprofloxacin; Chl = chloramphenicol, Smx = sulfamethoxazole, Nal = nalidixic acid; Resistance is indicated in orange, break-points used were epidemiological cut-off values from EUCAST (2010) and MARAN (2007), see Appendix 7.[1] For one of the colonies the phage type could not be determined but the phenotype was similar to the phage type 8 isolates.

3.7 Seasonal prevalence and exposure through swimming

To illustrate the possible implications of the observed prevalence data in terms of exposure during recreation, we determined point estimates describing the amounts of resistant commensal bacteria ingested when accidentally swallowing river water. For this purpose, we took as a starting point the observed prevalence of antibiotic resistant bacteria during the official bathing season, which in the Netherlands lasts from May to October. The current study included the entire bathing season of 2008. During this season, *E. coli* and enterococci were present at all sampled time-points in all three rivers (see Appendix 5). Of *S. aureus*, *Salmonella* and *Campylobacter*, only the latter was detected at some time-points during the bathing season. However, of these 'summer' isolates, only *Campylobacter* isolates from the Meuse (isolated in July) were vital enough to allow analyses of antibiotic sensitivity and these isolates were all pan-susceptible. For *E. coli*, *E. faecium* and *E. faecalis*, sufficient isolates were retrieved to describe prevalence during bathing and winter seasons separately and to estimate the average level of exposure to antibiotic resistant strains for swimmers at these sites during the bathing season of 2008.

3.7.1 Bathing season versus autumn and winter season

Overall, percentages of resistant *E. coli*, *E. faecium* and *E. faecalis* were lower during the bathing season compared to the winter season (Figure 11). Nevertheless, resistance was observed and generally to the same antibiotics as during the winter season. As an exception to this, salinomycin- or

sulfamethoxazole/trimethoprim-resistant *E. faecium* were not observed during the bathing season.

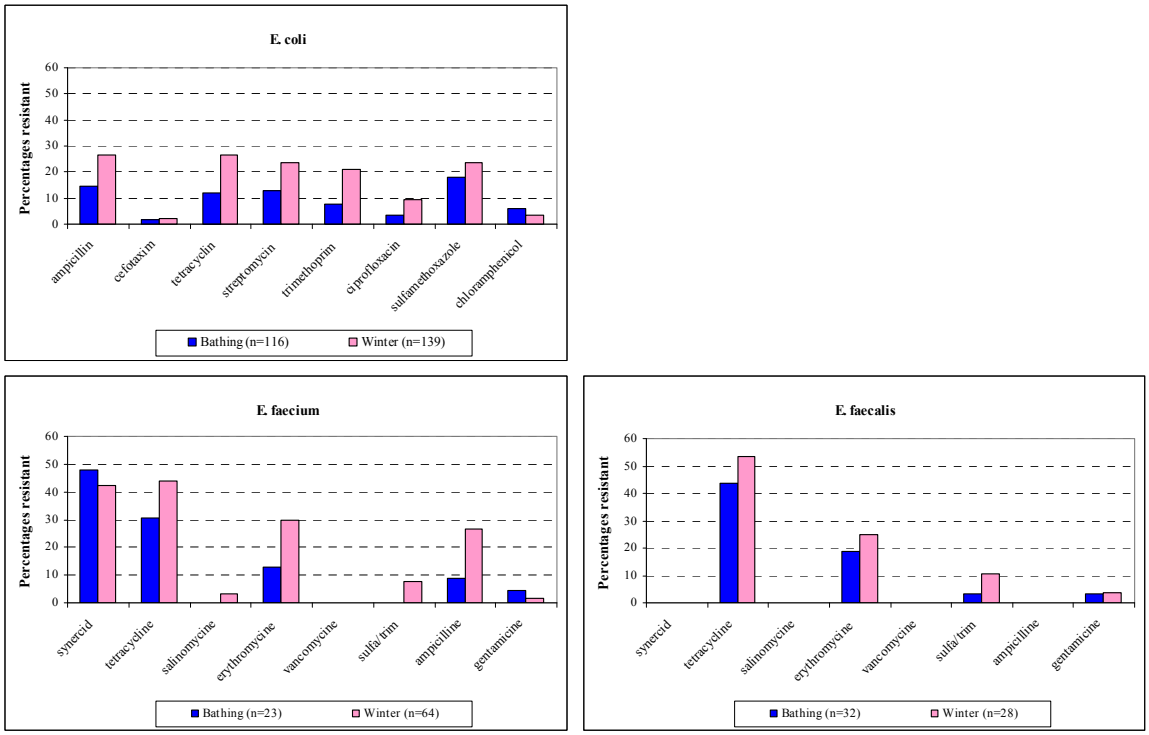

Figure 11 Percentages of antimicrobial resistant E. coli, E. faecium and E. faecalis isolates during bathing season and subsequent autumn and winter season.

Bathing season includes all strains isolated from May 2008 up to and including September 2008, winter season included strains isolated from October 2008 up and to April 2009. Strains isolated in May 2009 were not included in the analysis.

Of all *E. coli* strains isolated during the bathing season, 28% were resistant to one or more antibiotic: 39% of strains from the Meuse, 21% from the Rhine and 23% from the New Meuse (Figure 12). The majority of resistant strains isolated from the Meuse and the Rhine were multiresistant: 67% and 63% respectively. In the New Meuse, 33% of the resistant strains was multiresistant. On average, 16% of *E. coli* from the Meuse, 3% from the Rhine and 5% from the New Meuse were resistant to five or more antibiotics.

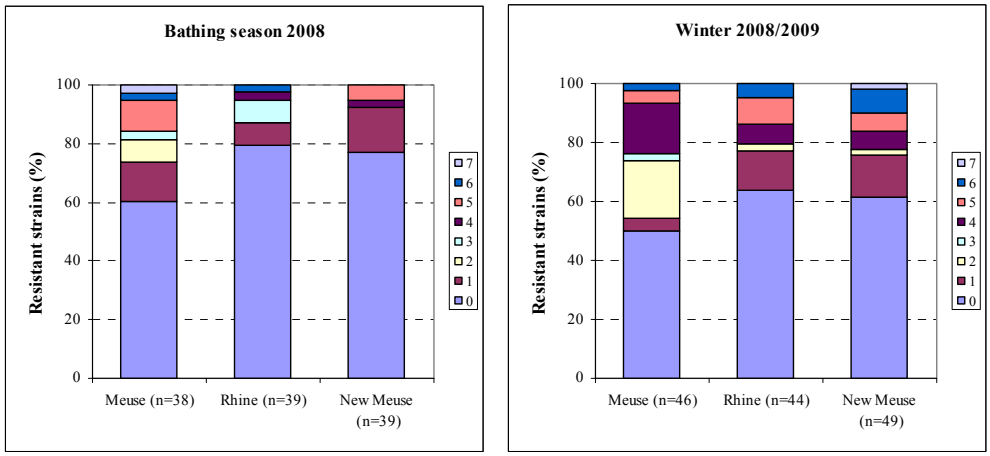

Figure 12 Percentages of sensitive, monoresistant and multiresistant E. coli strains during the bathing season of 2008 and the subsequent autumn and winter season.

During the bathing season, 52% of all *E. faecium* strains and 47% of all *E. faecalis* strains were resistant to one or more antibiotic (Figure 13). Because only a few *E. faecium* and *E. faecalis* strains were retrieved from the Rhine (six and four respectively) and the New Meuse (five and six respectively), resistance prevalence was not determined for each river individually. Instead, calculations were based on averages for all river isolates. During the bathing season, overall percentages of resistant and multiresistant *E. faecium* and *E. faecalis* strains were slightly lower than during the fall/winter bathing season (Figure 13). Of resistant *E. faecium* strains isolated during the bathing season, 50% was multiresistant, compared to 33% of resistant *E. faecalis* strains. Strains with five resistances were only observed for *E. faecium*; during the bathing season it concerned one isolate or 4% of all strains.

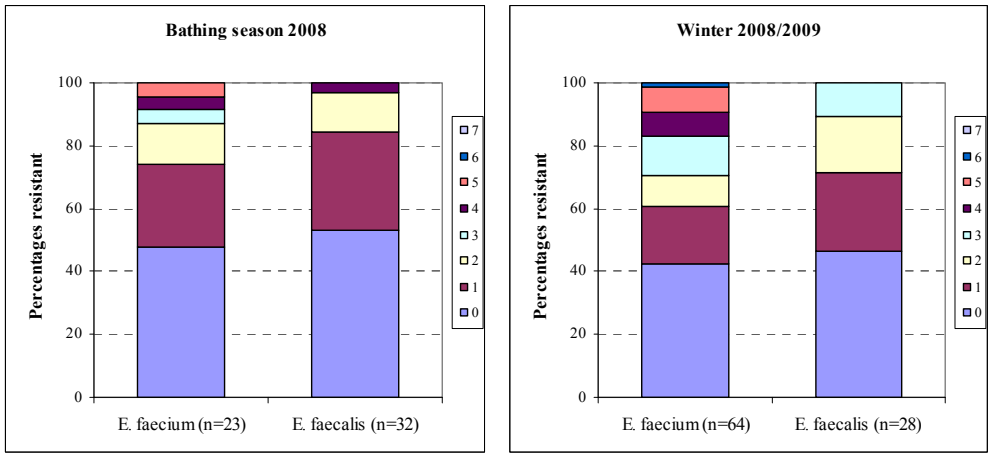

Figure 13 Percentages of sensitive, monoresistant and multiresistant E. faecium and E. faecalis strains during the bathing season of 2008 and the subsequent autumn and winter season.

3.7.2 *Potential exposure through accidental ingestion*

The amount of water that is accidentally ingested during (45 minutes of) swimming has been estimated to be on average 16 ml for adults and 37 ml for non-adult recreational swimmers (Dufour et al. 2006). These amounts were

used to estimate the amounts of antibiotic resistant *E. coli*, *E. faecium* and *E. faecalis* that might accidentally be swallowed during swimming in the rivers studied, based on average, peak and low concentrations of total *E. coli* and enterococci during the bathing season and the season's average prevalence of antibiotic resistance (see Appendices 9 and 10).

E. coli

In the Meuse, the river with the highest level of contamination of the three rivers, one 'adult' swallow of water contained on average 167 cfu antibiotic resistant *E. coli*, with a minimum of 68, assuming the lowest *E. coli* concentration of the season and a maximum of 281, assuming the highest *E. coli* concentration of the season. By contrast, in the New Meuse the same exposure contained on average 11 (range 7-22) resistant *E. coli*. One 'non-adult' ingestion of Meuse water contained on average 386 (range 157-650) antimicrobial resistant *E. coli* and 155 (range 63 to 260) *E. coli* with resistance to 5 or more antibiotics.

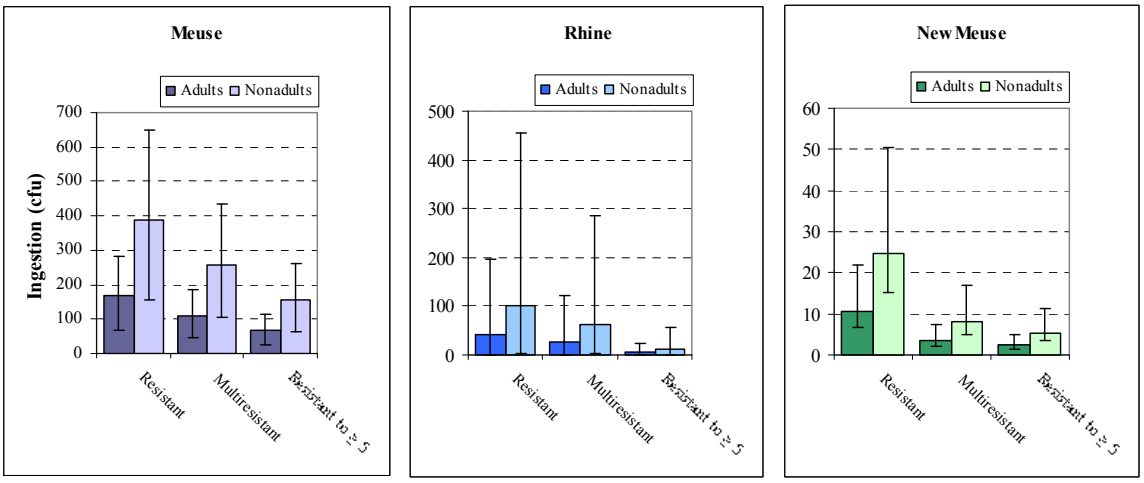

Figure 14 Amounts of resistant and multiresistant E. coli in one average accidental intake of water.

Values (colony forming units, or cfu) were derived from the average concentrations during the bathing season, the proportion of resistant and multiresistant strains and strains resistant to 5 or more antibiotics (Appendix 8) and assuming accidental ingestion of 16 ml (adults) and 37 ml (non-adults) of water. The error bars represent the number of resistant bacteria assuming the highest and lowest *E. coli* concentrations measured during this season.

E. faecium and E. faecalis

Overall 18% of *Enterococcus* isolates derived from samples taken during the bathing season 2008 were identified as *E. faecium* and 25% as *E. faecalis* (Appendix 9), corresponding to, on average, 299 cfu *E. faecium* and 416 cfu *E. faecalis* per litre of river water. On average, one 'adult' swallow of river water contained two resistant *E. faecium* (range 0-13) and three resistant *E. faecalis* (range 0-35). One non-adult swallow contained 11 resistant *E. faecium* (range 0-58) and 15 resistant *E. faecalis* (range 0-81).

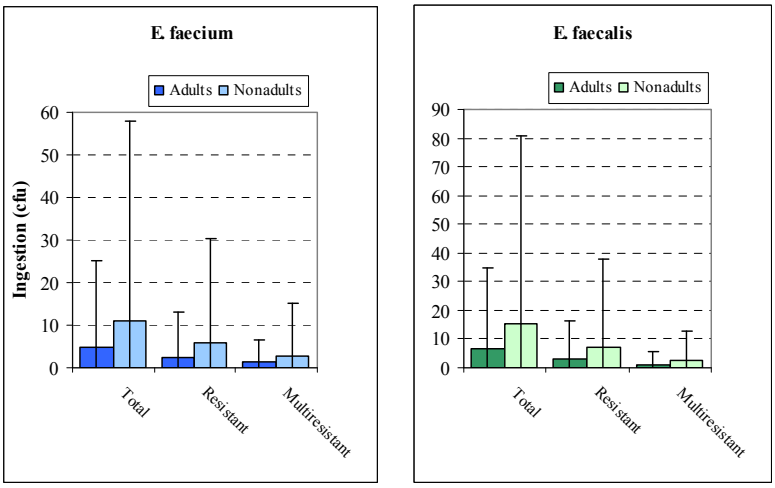

Figure 15 Amounts of resistant and multiresistant E. faecium and E. faecalis in an average accidental intake of water.

Values (colony forming units or cfu) were derived from the average concentrations of enterococci during the bathing season, the proportion of each species and the proportions of resistant and multiresistant strains (Appendix 9), and assuming accidental ingestion of 16 ml (adults) and 37ml (non-adults) of water. The error bars represent the number of resistant bacteria assuming the highest and lowest *E. faecium* and *E. faecalis* concentrations measured during this season.

4 Discussion

In the rivers Meuse, Rhine and New Meuse, high percentages of *E. coli* and *Enterococcus* spp. were resistant to one or more antibiotics. In the Meuse, of the three rivers the one with the highest level of bacterial contamination, 48% of *E. coli*, 54% of *E. faecium* and 56% of *E. faecalis* were resistant to at least one antibiotic. In the Rhine 32% of *E. coli*, 59% of *E. faecium* and 50% of *E. faecalis* were resistant and in the New Meuse 34% of *E. coli*, 58% of *E. faecium* and 18% of *E. faecalis*. Previously, we determined the prevalence of antibiotic resistant bacteria in small rivers in an area with intensive husbandry (Blaak et al. 2010). In those rivers the prevalence of ABR *E. coli*, *E. faecium* and *E. faecalis* were 44%, 80% and 47% respectively. In other words, the prevalence of ABR *E. coli* in the three rivers was similar (Meuse) or slightly lower (Rhine, New Meuse) compared to that in the small rivers in the area with intensive husbandry, while the prevalence of ABR *Enterococcus spp*. appeared to be considerably lower in the three rivers. Comparable prevalence numbers have been reported in the Seine in France between 2005 and 2007 (Servais and Passerat 2009). In the Seine, 42% of *E. coli* and 83% of intestinal enterococci were resistant to at least one antibiotic.

A large proportion of the resistant *E. coli* and enterococci from the three rivers was resistant to minimally two antibiotics: 65%, 60% and 43% of resistant *E. coli*, *E. faecium* and *E. faecalis*, respectively. The maximum number of resistances out of eight tested antibiotics observed per strain was seven for *E. coli*, six for *E. faecium* and four for *E. faecalis*. On average, 10% of all isolated *E. coli* and 8% of all *E. faecium* isolated from rivers were resistant to five or more antibiotics. For comparison, in small rivers in the area with intensive husbandry, 12% of *E. coli* and 3% of *E. faecium,* and in the Seine 2% of *E. coli* and intestinal enterococci (Servais and Passerat 2009), were resistant to five or more antibiotics. For *E. coli* strains from Dutch food-producing animals prevalence data on multiresistance are also available (MARAN 2009). In 2009, the prevalence of *E. coli* resistant to at least one antibiotic was 26% for dairy cattle, 68% for veal calves, 81% for slaughter pigs and 88% for broiler chickens. In these animals approximately 5%, 25%, 13% and 45%, respectively, were resistant to at least five different antibiotics (MARAN 2009). Thus, the level of prevalence of resistant and multiresistant *E. coli* in Dutch rivers is slightly below average compared to the situation in Dutch food-producing animals. To the author's knowledge, similar data are currently not known for *E. coli* in the human community.

Besides antibiotic resistant *E. coli* and enterococci, antibiotic resistant strains of *S. aureus*, *Campylobacter coli* and *Salmonella enterica* subtypes Panama and 4,5,12:i:- were also detected in the Meuse at some of the time-points. In the Rhine, resistant *S. aureus* was detected once and in the New Meuse *C. coli*. Among the detected antibiotic resistant bacteria were strains with clinically relevant resistances, such as ESBL-producing *E. coli* (Meuse), high-level ampicillin-resistant *E. faecium* (Rhine, New Meuse), high-level aminoglycoside-resistant *E. faecium* or *E. faecalis* (all rivers), MRSA (Meuse, Rhine) and quinolone-resistant *Campylobacter spp* (Meuse).

The origin of the resistant bacteria was not determined and is likely to be mixed; for all three rivers possible contamination sources are agriculture (e.g., run off of manure), households and health care facilities (e.g., waste water treatment plants) and shipping traffic (e.g., waste water, ballast water). The observed

prevalence of resistant *E. coli* and enterococci is comparable to that observed previously in small rivers in an area with intensive husbandry (Blaak et al. 2010). These findings indicate that antibiotic resistant bacteria are omnipresent in the aquatic environment. However, to draw firm conclusions, more waters in different geographical areas should be studied.

In the Netherlands, the most likely way for people to become exposed to contaminated surface water is presumably through recreation. To illustrate the implications of the observed prevalence of antibiotic resistance in terms of exposure, we estimated the intake of resistant *E. coli* and enterococci via accidental ingestion of water during recreational swimming. Estimates were based on the average bacterial concentrations and proportions of antibiotic resistant variants during the bathing season 2008 at the studied sites. In the Meuse, one average adult swallow of water, assumed to be 16 ml per swimming event as described by Dufour et al. in 2006 (Dufour et al. 2006), contained on average 167 resistant *E. coli* (range 68-281) and one non-adult swallow (of 37 ml) on average 386 (range 157-650). The same 'non-adult' swallow of water contains on average 155 (range 63-260) *E. coli* resistant to five or more antibiotics. In the other two rivers, the ingestion of antibiotic resistant bacteria with one swallow of water was lower, for adults on average 43 (range 42-154) in the Rhine and 11 (range 4-11) in the New Meuse and for non-adults 100 (range 97-356) and 25 (range 9-26), respectively. For *E. faecium* and *E. faecalis* the amounts of accidentally ingested resistant bacteria were calculated based on the average prevalence data of the three rivers combined. The amounts were substantially lower than for *E. coli*: on average, one 'adult' swallow of river water contained two resistant *E. faecium* (range 0-13) and three resistant *E. faecalis* (range 0-35), one non-adult swallow contained 11 resistant *E. faecium* (range 0-58) and 15 *E. faecalis* (range 0-81). The prevalence of *S. aureus* and the pathogens *Salmonella* and *Campylobacter* in the rivers was low compared to that of *E. coli* and *Enterococcus* spp. and the chance of encountering resistant strains of these (opportunistic) pathogens through contact with water is therefore lower.

The magnitude of the health risk associated with this level of exposure has not yet been established and depends on multiple parameters. Ingestion of antibiotic resistant commensal bacteria such as *E. coli* and intestinal enterococci, may result in colonisation, and therewith carriage of antibiotic resistant gut flora. These bacteria may transfer the resistance genes to pathogens encountered in the future. As shown in this and other studies, commensal bacteria such as *E. coli* and enterococci are often resistant to multiple antibiotics, which increases the chance that a pathogen will acquire resistance genes that are relevant for treatment. Most commensal bacteria are opportunistic pathogens and asymptomatic carriage may enable passage to individuals that are vulnerable to infection, or be the cause of difficult-to-treat infections at later stages in the carrier's life. The health risks associated with ingestion of antibiotic resistant commensal bacteria are therefore dependent on the efficiency of colonisation (or the duration of passage in the absence of colonisation), the efficiency of transfer of genes to other intestinal bacteria, the stability in the gut of the resistance genes in the absence of antibiotics and the relevance of the resistances for treatment of infections caused by the ingested bacteria or the pathogens that acquire the resistance(s) in the gut. Ingestion of antibiotic resistant pathogens, such *Salmonella* and *Campylobacter* may directly result in difficult-to-treat infections. The impact of exposure then depends on the level of pathogenicity of the pathogen (i.e., whether treatment is required), the minimum dose of bacteria required to establish infection (dose-response relationship) and whether the resistances the pathogen carries are relevant for treatment.

For enterococci, only the amounts of ingested resistant *E. faecium* and *E. faecalis* strains were calculated. These two species are the most important species from a human health perspective because they are responsible for nearly all human *Enterococcus* infections (Murray 1990; Ruoff et al. 1990; Moellering 1992; Stern et al. 1994; Chaudhary et al. 2007). Although a substantial proportion of the enterococci isolated from river water during the bathing season were *E. faecium* (18%) and *E. faecalis* (25%), a small majority of 56% of isolates were actually non-faecium, non-faecalis species. These species, among which *E. durans* (25%), *E. hirae* (17%), *E. mundtii* (10%) and *E. casseliflavus* (4%), also carried resistance and are known to be able to colonise and infect humans (Ruoff et al. 1990; Kaufhold and Ferrieri 1991; Stern et al. 1994; Chaudhary et al. 2007). Antibiotic strains of these non-faecium, non-faecalis species might therefore also contribute to the exposure risk associated with ingestion of river water.

The presented estimates for accidental intake are indicative of exposure risks associated with recreation in waters that are faecally contaminated in the same order of magnitude as the three rivers studied here. The water quality of official recreation waters is regulated according to the European Bathing Water Directives 76/100/EC and 2006/7/EC (Anonymous 1976, 2006). According to the latter directive, the water quality of inland waters is considered of 'sufficient' quality when the 90-percentile value of the concentrations of faecal indicators based on minimally 16 observations during four subsequent bathing seasons, does not exceed 900 colony forming units (cfu) *E. coli* and 330 cfu enterococci per 100 ml of water. For comparison, the average and 90% percentile values of faecal indicators during the bathing season of 2008 were 2645 (90% percentile 3370) *E. coli* and 354 (90% percentile 657) enterococci for the Meuse, 1314 (90% percentile 3682) *E. coli* and 122 (90% percentile 340) enterococci for the Rhine, and 287 (90% percentile 474) *E. coli* and 17 (90% percentile 30) enterococci for the New Meuse. In other words, based on the official criteria, all sampled locations were of poor quality (note however that values were based on only five observations made during one season). Nevertheless, people do not swim in allocated recreational waters only and swimming in surface waters of (unregistered) poor microbiological quality may be associated with a substantial risk of exposure to antibiotic resistant bacteria.

Besides the level of faecal contamination, another factor influencing the risk of exposure to antibiotic resistant bacteria is the proportion of resistant bacteria in the contamination source. This is likely to differ between locations. Higher proportions of antibiotic resistant bacteria may be expected for instance in areas with high densities of animal husbandry or downstream of waste water treatment plants (WWTP) that process the waste water of large hospitals or other health care facilities. Future research at the RIVM will be aimed at assessing exposure risks associated with different types of recreational water with respect to microbiological quality and their location relative to possible contamination sources to identify mitigation options.

Currently, the relative contribution of surface water as a source of human exposure to antibiotic resistant bacteria is not known. Presumably, the risk of exposure to antibiotic resistant bacteria in environmental compartments is lower compared to direct contact with food animals and the consumption of animal products. The rationale for this is amongst other things the lower numbers of viable resistant bacteria in the environment compared to (excreta of) food animals and the fact that people come into contact with potentially contaminated water less frequently than they consume food. In the past, the risk of exposure to *Campylobacter* (of unknown resistance profile) via recreational water was

estimated to be on average 33 and 56 times lower compared to consumption of food of animal origin and direct contact with animals, respectively (Evers et al. 2008). Yet, due to the necessity to make assumptions regarding some of the parameters, confidence intervals were large and value ranges largely overlapped for risks associated with recreation in water and for instance, consumption of cooked chicken or raw vegetables as well as direct contact with cows and calves.

Given the high prevalence of antibiotic resistant commensal bacteria in Dutch waters, the risk of water recreants being exposed to these bacteria appears to be credible. The magnitude of this risk likely depends on the microbiologic quality of the water as well as the source of the faecal contamination. In addition to recreation, contaminated surface water may also be a source of exposure to antibiotic resistant bacteria if it is used for irrigation of vegetables and fruits that are consumed uncooked. Even though the *E. coli* and enterococci do not directly cause disease in the majority of the exposed, the ingestion of antibiotic strains likely indirectly poses health risks. Moreover, exposure to antibiotic resistant bacteria via environmental components may pose additional risks compared to other exposure routes, considering the possibility of exposure to novel mixtures of bacteria and resistance genes. To be able to reduce the prevalence of antibiotic bacteria in humans, the relative contributions of different exposure routes should be assessed, both in terms of quantity and quality.

Conclusions

The rivers Meuse, Rhine and New Meuse contain high percentages of antibiotic resistant and multiresistant *E. coli* and *Enterococcus* spp.

Antibiotic resistant *S. aureus* strains were detected in the Meuse and the Rhine, antibiotic resistant *C. coli* in the Meuse and the New Meuse and antibiotic resistant *Salmonella enterica* subtypes Panama and 4,5,12:i:- in the Meuse.

Among the detected antibiotic resistant bacteria were strains with clinically relevant resistances, such as ESBL-producing *E. coli*, high-level ampicillin-resistant or high-level aminoglycoside-resistant enterococci, MRSA and quinolone-resistant *C. coli*.

The presence of antibiotic resistant bacteria in surface waters used for recreation may pose a health risk and needs to be more accurately assessed. Moreover, possible public health risks from this route should be compared with other possible exposure routes for these antibiotic resistant bacteria.

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Appendix 1a Isolation methods, media and analysed volumes

Micro-organism	Procedure	Media (manufacturer, nr.)	Volume (ml)
E. coli	NEN-EN-ISO 9308-1 rapid test (Anonymous 2000b)	Trypton Soya agar (Oxoid, PO5012A) Trypton Bile agar (Oxoid, PO5017A)	1; 10; 100
Entero-coccen	NEN-EN-ISO 7899-2 (Anonymous 2000a)	Slanetz and Bartley agar (Oxoid, PO5018A) Bile Aesculin Azide agar (Oxoid, PO5062A)	1; 10; 100
Salmonella	NEN-EN-ISO 19250 (Anonymous 2010)	Buffered Peptone Water (NVI, E4900F) Rappaport-Vassiliadis Soya Peptone broth (Oxoid, CM0866) Rappaport-Vassiliadis (MSRV) medium (Difco 218681) Xylose Lysine Deoxycholate agar (Oxoid, PO5057A) Brilliant Green Agar (Oxoid PO5062A)	10; 100; 500* (1, 5, 10, 100 mar) 0.1; 1; 10‡ 0.1; 1; 5§
Campylo-bacter	NEN 6269 (Anonymous 1996)	Preston Broth: Nutrient Broth no.2 (Oxoid, CM0067B) Laked Horse Blood (Oxoid, SR0048C) Campylobacter Growth Supplement (Oxoid, SR0232E) Campylobacter Selective Supplement (Oxoid, SR0117E) Karmali agar (Oxoid PO5041A)	10; 100; 500* 0.1; 1; 10‡ 0.1; 1; 5§
S. aureus	Protocol §2.2.1	Mueller-Hinton Broth (Oxoid, CM0337) Phenolred Mannitol Broth (Biomerieux, NL020) Colombian Agar with Sheep blood (PO5008A) Baird Parker agar (Oxoid, CM0961) Rabbit Plasma Fibrinogen-supplement (Oxoid, SR0122A) Brilliance MRSA Agar (Oxoid, PO5196A)	10; 100

* Water samples (membrane filtrated)
‡ Samples of eluate resulting after glass fibre ultrafiltration
§ Samples of pellet suspension derived from centrifuged eluate

Appendix 1b Solid media used for propagation of isolates

Media	**Species/genus**
Brain Heart Infusion agar	Enterococci
Colombian agar with Sheep Blood	*S. aureus*, *Campylobacter, Salmonella*
Trypton Soya Agar	*E. coli*, *Salmonella*

Appendix 2 Reference sequences used in 16S rDNA sequence analysis

Species	GenBank number
E. faecium	EU547780; AF039901; Y18294; AJ276355; AF070223; AJ301830
E. faecalis	AJ420803; AB012212; AJ301831
E. durans	AF061000; Y18359; AB018210; AJ276354
E. hirae	AF061011; AJ276356; AJ554205; Y17302
E. mundii	AF061013; Y18340; AJ301836
E. casseliflavus	Y18161; AJ301826; AJ420804
E. flavescens	Y18295; AJ420802; AJ301832
E. gallinarum	AJ301833; Y18160; AJ420805
E. moraviensis	AF286831
E. silesiacus	AM039966; AM039967
E. caccae	AY943820
E. camelliae	EF154454
E. italicus	AJ582753

Appendix 3 Composition of Sensititre MIC plates

Plates used for *E. coli* and *Salmonella*:

Plate Code: **NLV80** Date: **15-jun-09**

	1	2	3	4	5	6	7	8	9	10	11	12
A	**AMP**	**FOT**	**TET**	**STR**	**TMP**	**CIP**	**AMP**	**FOT**	**TET**	**STR**	**TMP**	**CIP**
	1	0.06	1	2	0.5	0.03	1	0.06	1	2	0.5	0.03
B	AMP	FOT	TET	STR	TMP	CIP	AMP	FOT	TET	STR	TMP	CIP
	2	0.12	2	4	1	0.06	2	0.12	2	4	1	0.06
C	AMP	FOT	TET	STR	TMP	CIP	AMP	FOT	TET	STR	TMP	CIP
	4	0.25	4	8	2	0.12	4	0.25	4	8	2	0.12
D	AMP	FOT	TET	STR	TMP	CIP	AMP	FOT	TET	STR	TMP	CIP
	8	0.5	8	16	4	0.25	8	0.5	8	16	4	0.25
E	AMP	FOT	TET	STR	TMP	CIP	AMP	FOT	TET	STR	TMP	CIP
	16	1	16	32	8	0.5	16	1	16	32	8	0.5
F	AMP	FOT	TET	STR	TMP	CIP	AMP	FOT	TET	STR	TMP	CIP
	32	2	32	64	16	1	32	2	32	64	16	1
G	AMP	FOT	TET	STR	TMP	CIP	AMP	FOT	TET	STR	TMP	CIP
	64	4	64	128	32	2	64	4	64	128	32	2
H	**POS**	FOT	TET	STR	TMP	CIP	**POS**	FOT	TET	STR	TMP	CIP
	CON	8	128	256	64	4	**CON**	8	128	256	64	4

	ANTIMICROBIALS
AMP	Ampicillin
POS	Positive Control
FOT	Cefotaxime
TET	Tetracycline
STR	Streptomycin
TMP	Trimethoprim
CIP	Ciprofloxacin

Plates used for *Enterococcus* and *S. aureus*:

Plate Code: **NLV81** Date: **15-jun-09**

	1	2	3	4	5	6	7	8	9	10	11	12
A	**SYN**	**TET**	**SAL**	**ERY**	**VAN**	**SXT**	**SYN**	**TET**	**SAL**	**ERY**	**VAN**	**SXT**
	0.5	0.25	0.5	0.25	0.5	0.25/4.75	0.5	0.25	0.5	0.25	0.5	0.25/4.75
B	SYN	TET	SAL	ERY	VAN	SXT	SYN	TET	SAL	ERY	VAN	SXT
	1	0.5	1	0.5	1	0.5/9.5	1	0.5	1	0.5	1	0.5/9.5
C	SYN	TET	SAL	ERY	VAN	SXT	SYN	TET	SAL	ERY	VAN	SXT
	2	1	2	1	2	1/19	2	1	2	1	2	1/19
D	SYN	TET	SAL	ERY	VAN	SXT	SYN	TET	SAL	ERY	VAN	SXT
	4	2	4	2	4	2/38	4	2	4	2	4	2/38
E	SYN	TET	SAL	ERY	VAN	SXT	SYN	TET	SAL	ERY	VAN	SXT
	8	4	8	4	8	4/76	8	4	8	4	8	4/76
F	SYN	TET	SAL	ERY	VAN	SXT	SYN	TET	SAL	ERY	VAN	SXT
	16	8	16	8	16	8/152	16	8	16	8	16	8/152
G	SYN	TET	SAL	ERY	VAN	SXT	SYN	TET	SAL	ERY	VAN	SXT
	32	16	32	16	32	16/304	32	16	32	16	32	16/304
H	SYN	TET	SAL	ERY	VAN	**POS**	SYN	TET	SAL	ERY	VAN	**POS**
	64	32	64	32	64	**CON**	64	32	64	32	64	**CON**

	ANTIMICROBIALS
SYN	Quinupristin / dalfopristin
TET	Tetracycline
SAL	Salinomycin
ERY	Erythromycin
VAN	Vancomycin
SXT	Trimethoprim / sulfamethoxazo
POS	Positive Control

Plates used for *Campylobacter*:

Plate Code: **NLV64** Date: **6-feb-07**

	1	2	3	4	5	6	7	8	9	10	11	12
A	**AMP**	**CLA**	**CIP**	**ERY**	**GEN**	**NAL**	**NEO**	**STR**	**SMX**	**TET**	**TUL**	**CHL**
	0.25	0.5	0.12	0.5	0.25	1	0.5	1	8	0.5	0.5	2
B	AMP	CLA	CIP	ERY	GEN	NAL	NEO	STR	SMX	TET	TUL	CHL
	0.5	1	0.25	1	0.5	2	1	2	16	1	1	4
C	AMP	CLA	CIP	ERY	GEN	NAL	NEO	STR	SMX	TET	TUL	CHL
	1	2	0.5	2	1	4	2	4	32	2	2	8
D	AMP	CLA	CIP	ERY	GEN	NAL	NEO	STR	SMX	TET	TUL	CHL
	2	4	1	4	2	8	4	8	64	4	4	16
E	AMP	CLA	CIP	ERY	GEN	NAL	NEO	STR	SMX	TET	TUL	CHL
	4	8	2	8	4	16	8	16	128	8	8	32
F	AMP	CLA	CIP	ERY	GEN	NAL	NEO	STR	SMX	TET	TUL	CHL
	8	16	4	16	8	32	16	32	256	16	16	64
G	AMP	CLA	CIP	ERY	GEN	NAL	NEO	STR	SMX	TET	TUL	CHL
	16	32	8	32	16	64	32	64	512	32	32	128
H	AMP	CLA	CIP	ERY	GEN	NAL	NEO	STR	SMX	TET	TUL	**POS**
	32	64	16	64	32	128	64	128	1024	64	64	**CON**

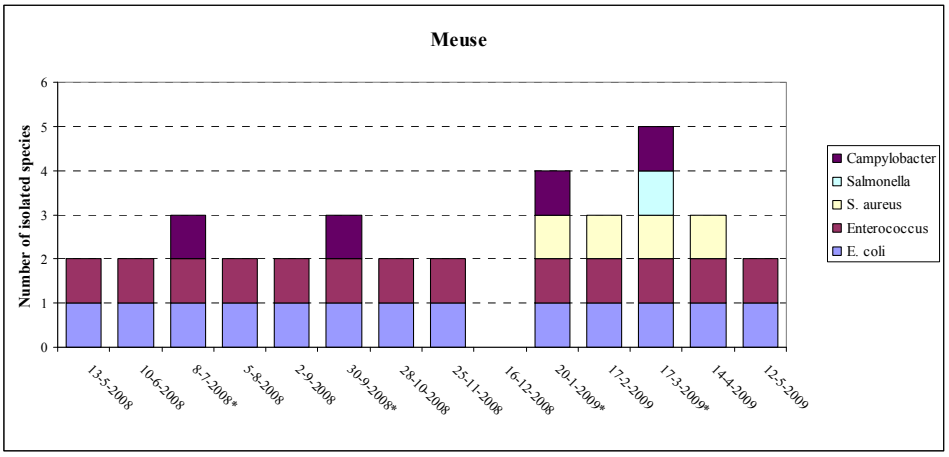
	ANTIMICROBIALS
AMP	Ampicillin
CLA	Clarithromycin
CIP	Ciprofloxacin
ERY	Erythromycin
GEN	Gentamicin
NAL	Nalidixic Acid
NEO	Neomycin
STR	Streptomycin
SMX	Sulfamethoxazole
TET	Tetracycline
TUL	Tulathromycin
CHL	Chloramphenicol
POS	Positive Control

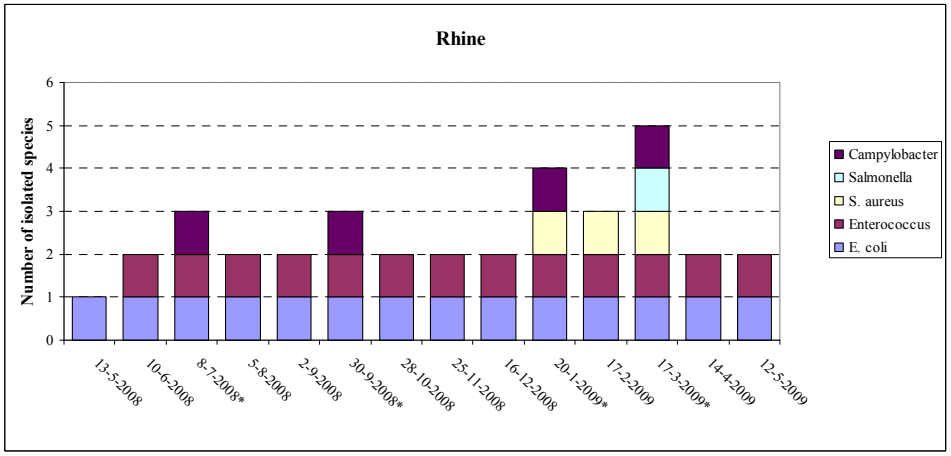
Appendix 4 Used Etest® strips and concentrations

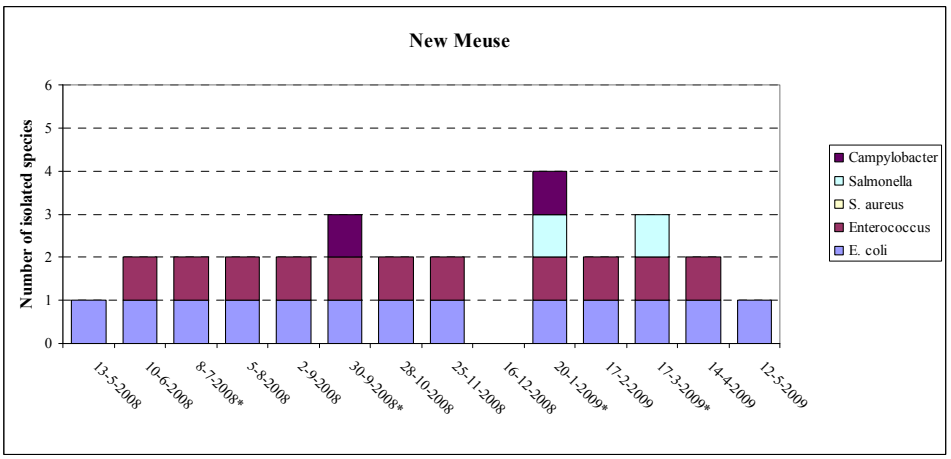
Antibiotic	Bacterial species	Tested concentrations (µg/ml)
Chloramphenicol	*E. coli / Salmonella*	0.016 – 256
Sulfamethoxazole	*E. coli / Salmonella*	0.064 – 1024
Nalidixic acid	*E. coli / Salmonella*	0.016 – 256
Ampicillin	*Enterococcus*	0.016 – 256
Gentamicin	*Enterococcus*	0.016 – 256 / 0.064 – 024
Clindamycin	*S. aureus*	0.016 – 256
Oxacillin	*S. aureus*	0.016 – 256
Ciprofloxacin	*S. aureus*	0.002 – 32

Appendix 5 Overview of bacterial species isolated per time-point and location

All samples were screened for the presence of *E.coli*, enterococcen and *S. aureus*. Asterixes indicate samples additionally screened for *Salmonella* and *Campylobacter*. In December 2008 the New Meuse was not sampled (NS).







Appendix 6 Resistance phenotypes of *E. coli* strains

AMP	TET	STR	TMP	SMX	CTX	CIP	CHL	Maas n=43	Rijn n=29	Nwe Maas n=32
								1	1	2
								4	4	1
								0	0	3
								2	0	0
								1	3	4
								1	0	0
								1	4	3
								0	1	0
								1	0	0
								2	0	0
								4	0	0
								1	0	0
								2	0	0
								1	1	0
								0	0	1
								1	0	0
								0	0	1
								0	2	0
								1	0	0
								0	1	0
								1	0	0
								1	0	1
								4	1	2
								0	0	1
								2	2	0
								1	0	0
								1	0	0
								0	1	0
								0	1	0
								0	0	1
								4	4	5
								1	0	0
								2	0	0
								0	0	1
								1	1	2
								1	2	3
								1	0	1

The table shows all phenotypes that were found in the Meuse, the Rhine and the New Meuse from May 2008 until May 2009. Each row represents a phenotype with green indicating resistance and white susceptibility; numbers behind each row indicate the number of isolates with the respective phenotypes. AMP = ampicillin; TET = tetracyclin; STR = streptomycin; TMP = trimethoprim ; SMX = sulfametoxazole; CTX = cefotaxime; CIP = ciprofloxacin; CHL = chloramphenicol.

Appendix 7 Resistance phenotypes of *E. Faecium*- and *E. faecalis*-strains

A. *E. faecium*

SYN	TET	SXT	AMP	SAL	ERY	VAN	GEN	Meuse n=15	Rhine n=22	New Meuse n=15
								6	3	2
								2	4	2
								1	0	0
								0	0	1
								2	4	1
								0	0	0
								1	0	0
								0	0	1
								0	0	1
								0	1	0
								0	5	0
								0	1	0
								0	0	1
								1	2	3
								1	1	1
								1	0	1
								0	0	1
								0	1	0

B. E. faecalis

SYN	TET	SXT	AMP	SAL	ERY	VAN	GEN	Meuse n=22	Rhine n=6	New Meuse n=2
								12	2	2
								0	1	0
								7	1	0
								0	1	0
								2	0	0
								0	1	0
								1	0	0

The table shows all phenotypes that were found in the Meuse, the Rhine and the New Meuse from May 2008 until May 2009. Each row represents a phenotype with green indicating resistance and white susceptibility; numbers behind each row indicate the number of isolates with the respective phenotypes. SYN = synercid; TET = tetracyclin; STX = sulfamethoxazole/trimethoprim; AMP = ampicillin; SAL = salinomycin; ERY = erythromycin; VAN = vancomycin; GEN = gentamicin.

Appendix 8 Cut-off values for *S. aureus*, *C. coli* and *Salmonella* spp.

S. aureus

	Epidemiological cut-off: WT ≤ x mg/L	Source
Erythromycin	1	EUCAST, 2010
Vancomycin	2	EUCAST, 2010
Trimethoprim / sulfamethoxazole	0.5/9.5	EUCAST, 2010
Tetracyclin	1	EUCAST, 2010
Oxacillin	2	EUCAST, 2010
Salinomycin[1]	-	-
Ciprofloxacin	1	EUCAST, 2010
Clindamycin	0.25	EUCAST, 2010

Resistant variants have MICs > the denoted MICs. WT = wild type.[1] Break-points have not been described.

C. coli

	Epidemiological cut-off: WT ≤ x mg/L	Source
Erythromycin	16	EUCAST, 2010
Gentamicin	2	EUCAST, 2010
Streptomycin	4	EUCAST, 2010
Neomycin	2	EUCAST, 2010
Tetracyclin	2	EUCAST, 2010
Ciprofloxacin	1	EUCAST, 2010
Nalidixic acid	32	EUCAST, 2010
Tulathromycin[1]	16	MARAN, 2007
Ampicillin	16	EUCAST, 2010
Clarithromycin[1]	32	MARAN, 2007
Sulfamethoxazole[1]	256	MARAN, 2007
Chloramphenicol[1]	16	MARAN, 2007

Resistant variants have MICs > the denoted MICs. WT = wild type.[1] No cut-off values are given by EUCAST and break-points described in the MARAN 2007 report were used.

Salmonella spp.

	Epidemiological cut-off: WT ≤ x mg/L	Source
Ampicillin	8	EUCAST, 2010
Cefotaxime	0.5	EUCAST, 2010
Tetracyclin	8	EUCAST, 2010
Streptomycine	16	EUCAST, 2010
Trimethoprim	2	EUCAST, 2010
Ciprofloxacin	0.064	EUCAST, 2010
Chloramphenicol	16	EUCAST, 2010
Sulfamethoxazole[1]	256	MARAN, 2007
Naldixic acid	16	EUCAST, 2010

Resistant variants have MICs > the denoted MICs. WT = wild type.[1] No cut-off values are given by EUCAST and break-points described in the MARAN 2007 report were used.

Appendix 9 Concentrations of *E. coli* during bathing and winter seasons and prevalence of resistant strains

A. Concentrations of total *E. coli*

	Bathing season (x10^3 cfu/l)		*Winter season (x10^3 cfu/l)*	
	Average	Range	Average	Range
Meuse	26 (n=5)	11 – 44	83 (n=7)	36 – 130
Rhine	13 (n=5)	0.4 – 60	32 (n=7)	1.1 – 99
New Meuse	2.9 (n=4)	1.8 – 5.9	7.4 (n=6)	0.5 – 29
Total	14 (n=14)	0.4 – 60	38 (n=20)	0.5 – 130

The bathing season included 6 sampling time-points: May-08, June-08, July-08, August-08, and twice in September-08. The winter season included 7 sampling points: October-08, November-08, December-08, January-09, Februari-09, March-09 and April-09. Concentrations *E. coli and Enterococus* spp. could not be determined for the May samples and June sample from the New Meuse, in December the New Meuse was not sampled; these dates are therefore not included in the average concentration.

B. Prevalence of resistant *E. coli* strains

	Bathing season (%)			*Winter season (%)*		
	Resistant	Multiresistant	≥ 5 AM*	Resistant	Multiresistant	≥ 5 AM*
Meuse	39.5	26.3	15.8	53.8	44.2	6.5
Rhine	20.5	12.8	2.6	39.6	20.8	13.6
New Meuse	23.1	7.7	5.1	41.1	28.6	12.2
Total	27.6	15.5	7.8	44.7	31.1	12.2

* With resistance to 5 or more antibiotics (AM).

Appendix 10 Concentrations of *E. faecium* and *E. faecalis* during bathing and winter seasons and prevalence of resistant strains

A. Concentrations of total *Enterococcus* spp.

	Bathing season (x10^3 cfu/l)		*Winter season (x10^3 cfu/l)*	
	Average	Range	Average	Range
Meuse	3.5 (n=5)	1.4 – 8.6	21 (n=7)	4.4 – 33
Rhine	1.2 (n=5)	0.005 – 5.3	11 (n=7)	0.04 – 27
New Meuse	0.17 (n=4)	0.03 – 0.3	1.1 (n=6)	0.04 – 3.8
Total	1.6 (n=14)	0.005 – 8.6	11 (n=20)	0.04 – 33

The bathing season included 6 sampling time-points: May-08, June-08, July-08, August-08, and twice in September-08. The winter season included 7 sampling points: October-08, November-08, December-08, January-09, Februari-09, March-09 and April-09. Concentrations of *E. coli and Enterococus* spp. could not be determined for the May samples and June sample from the New Meuse, in December the New Meuse was not sampled; these dates are therefore not included in the average concentration.

B. Prevalence of *E. faecium* and *E. faecalis*

	Bathing season (%)	*Winter season (%)*
E. faecium	18.3	39.3
E. faecalis	25.4	17.2
Non-faecium, non-faecalis	56.3	43.6

Isolates obtained from sampling time-points indicated in table A; isolates from May 2009 are not included.
Percentages are calculated from all strains that could be identified to species level.

C. Prevalence of resistant *E. faecium* and *E. faecalis*

	Bathing season (%)			*Winter season (%)*		
	Resistant	Multiresistant	≥ 5 AM*	Resistant	Multiresistant	≥ 5 AM*
E. faecium	52.2	26.1	0	59.4	39.1	10.9
E. faecalis	46.9	15.6	0	53.6	28.6	0

Isolates obtained from sampling time-points indicated in table A; isolates from May 2009 are not included.

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