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Fate of Extended-Spectrum β-Lactamase-Producing *Escherichia coli* from Faecal Sources in Surface Water and Probability of Human Exposure through Swimming

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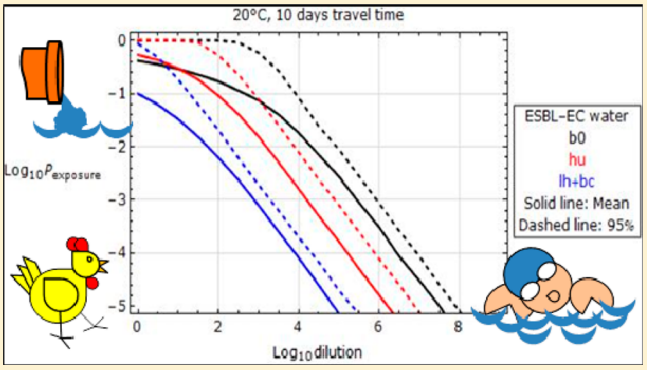
ABSTRACT: The goal of this study was to determine the fate of ESBL-producing *Escherichia coli* (ESBL-EC) emitted from faecal sources in surface water, and the probability of human exposure through swimming. Concentrations of ESBL-EC were measured in recreational waters and in source waters, being water in ditches surrounding poultry farms and municipal wastewater. Additionally, the potential of ESBL-EC in source waters to reach recreational waters located downstream of these sources was modeled. Modeled ESBL-EC concentrations in recreational waters appeared to be mostly determined by the concentrations in the source waters and by subsequent dilution in surface water, and less by inactivation. The mean (95%) risk of human exposure to ESBL-EC per person per swimming event, as assessed from measured ESBL-EC concentrations in recreational waters, was 0.16 (0.89) for men, 0.13 (0.72) for women and 0.20 (0.95) for children. Similar exposure risks were estimated for hypothetical recreational waters containing 100- or 1000-times diluted source water, located 10 days water travel time downstream of the sources. Human exposure to ESBL-EC through swimming is likely, if recreational waters are located downstream of poultry farms and municipal wastewater discharge points.



INTRODUCTION

The World Health Organization (WHO) has estimated that each year 25 000 deaths in the European Union (EU) are due to infections with antibiotic-resistant bacteria,[1] emphasizing the urgency of the antibiotic resistance issue. The increase of antimicrobial resistance caused by bacteria producing extended spectrum beta-lactamase (ESBL) is particularly of concern because of their resistance to third- and fourth-generation cephalosporins, which have been considered critically important to human medicine.[2] Extended-spectrum beta-lactamases (ESBL) are plasmid-encoded enzymes found in the bacterial family *Enterobacteriaceae*, commonly in *Escherichia coli* and *Klebsiella pneumonia*. ESBL confer resistance to a variety of beta-lactam antibiotics, including penicillins; second-, third-, and fourth-generation cephalosporins, and monobactams. Infections caused by ESBL-producing bacteria are increasing worldwide.[3,4] Moreover, ESBL resistance may contribute to an increase in the use of carbapenems as a last resort.[3] The use of this class of drugs is associated with an increased emergence of carbapenem- resistance,[5] which enables bacteria to hydrolyze all the beta-lactams. The options to treat infections caused by carbapenem-resistant bacteria are very limited, and the increase of carbapenem-resistance is therefore of great concern.

Multiple sources and reservoirs of ESBL-producing bacteria have been identified: humans (either or not travel-related), animals, food and the environment. Although the exact role of the environment in the dissemination of ESBL-producing bacteria is unclear, ESBL-producing bacteria appear to be omniprevalent in the environment. Among others, agricultural environments and recreational waters have been shown to be contaminated with ESBL-producing bacteria.[6−10] Bacteria in aquatic environments may originate from humans and/or animals that excrete high loads in their faeces.[11] Human waste contaminates surface waters directly, through sewage overflows or by discharge from wastewater treatment plants (WWTP). Animals, such as waterfowl, may defecate onto surface waters directly, or animal faeces may reach surface waters by runoff of droppings from wildlife, of animal manure from fertilized grounds, or from farm premises. Although ESBL-producing bacteria are omniprevalent in the environment, the relative contribution of this to the antimicrobial resistance burden in humans has yet to be determined. Exposure of humans to

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ESBL-producing bacteria in the aquatic environment may occur through accidental ingestion of faecally contaminated water through recreation, through consumption of contaminated drinking water, or through consumption of fresh produce irrigated with contaminated surface water. Estimation of the magnitude of exposure to and risk of infection with particular hazards in water and food can be achieved by quantitative microbial risk assessment (QMRA).[12,13] A recent consultation into possible approaches to assess human health risk from antimicrobial resistance specifically related to the role of the environment also identified QMRA as a possible approach.[14] However, the application of QMRA in assessing risk from antimicrobial resistance requires dose−response approaches to address doses of antibiotic resistant bacteria for various health outcomes (e.g., difficult-to-treat infection, or carriage of resistant commensal bacteria with the risk of horizontal transfer of resistance genes to pathogens) and exposure pathways. These data are currently not available and, as a consequence, full QMRA assessing health risks associated with antibiotic resistance is currently not possible.

The goal of this study was to estimate the probability of human exposure to ESBL-producing *E. coli* (ESBL-EC) in recreational waters through swimming, which is an important first step in QMRA. Additionally, the fate of *E. coli* (EC) and ESBL-EC emitted by municipal WWTPs (mWWTPs) and poultry farms was assessed. Finally, the relation between EC and ESBL-EC concentrations in surface waters and contamination sources was established, to explore the possibility of using EC measurement data as a proxy for ESBL-EC in surface water for risk management purposes.

■ MATERIALS AND METHODS

Description of Samples Used in Exposure Probability Assessments. Two types of probability of exposure assessments were made: one based on measured ESBL-EC concentrations in recreational waters (28 samples), and one based on modeled ESBL-EC concentrations in hypothetical recreational waters, based on concentrations in "source waters" (i.e., wastewater and surface water at the site of emission), taking into consideration dilution and die-off during transport from the site of emission to hypothetical recreational water. The source waters consisted of mWWTP effluents (13 samples) and surface water at the mWWTP discharge site (10 samples), and ditches around poultry farms (40 samples).

Description of Poultry Farms. During 2011 and 2012, poultry manure and farm environments were sampled at three broiler farms (Location (L) 01 to L03) and five laying hen farms (L04 to L08). Six of the eight farms were bordered by surface water, in the form of ditches (L01 to L03, L04, L07) or a stream (L06). The vicinity of ditches near (poultry) farms that are part of larger water systems is a common situation in The Netherlands. At the broiler farms, also runoff gullies were present containing runoff water from farm premises. Manure, surface water, and if applicable wastewater collected during cleaning, were sampled at one occasion on laying hen farms, and on at least two occasions at broiler farms: once while broilers were present, and once just after the same broilers were removed and stables had just been cleaned or were being cleaned. The reason for the different sampling strategies at the two types of farms is the high turnover of flocks at broiler farms (every 6 to 7 weeks) and the low turnover at laying hen farms (approximately once in every 1.5 year). Farm L01 was sampled during three different production rounds, two in 2011 and one in 2012 (i.e., in total six sampling occasions). All broiler farms used conventional farming, with capacities of 38 000 (L02), 87 000 (L01), and 150 000 (L03) broilers. Two of the laying hen farms were conventional farms with a capacity of 78 000 (L08) and 80 000 (L04) chickens, and three were free range farms with capacities of 30 000 (L05, L06) and 43 000 (L07) chickens. Sampled manure consisted of fresh (i.e., still soft and warm) and semifresh (i.e., generally still identifiable as individual droppings, but no longer soft and warm) poultry faeces sampled from barn and free-range areas. Surface water was sampled from water bodies bordering on, or within 50 m distance of farm premises. Wastewater was sampled during or shortly after cleaning at broiler farms from drains, collection pits, and storage basins. This wastewater consisted of barn rinsewater (either or not diluted with rainwater), and was therefore considered to reflect poultry manure contents. This was confirmed by high homology between isolates from manure and wastewater sampled during the same production rounds at the same farm.[15] Only one of the five laying hen farms (L08) had a wastewater pit filled with wastewater at the moment of sampling, and this was sampled as well.

Description of Recreational Waters and Wastewater Treatment Plants. Four recreational waters appointed under European Bathing Water Directive 2006/7/EC ("official"), classified as "good",[16] and one not appointed ("unofficial") recreational water were sampled during the bathing seasons of 2011 and/or 2012 which, in The Netherlands, lasts from May first until September 30th. The recreational waters (L13−17) were situated in different regions in The Netherlands, and each was located 1 to 2 km (as the crow flies) from a mWWTP that did not disinfect treated effluents before discharge (L09-L12).[6] L16 and L17 were located downstream of WWTP discharge points; in the waters between L13−L15 and the WWTP discharge points, the direction of the current is variable, depending on operational schedules of pumping engines and/or sluices. All recreational waters are presumed to be under influence of WWTP, although to what extent, and whether they were under influence of other faecal sources as well, was not known. Three of the official recreational waters (L13−L15) were situated in freshwater lakes; the fourth (L16) was located in the North Sea. The fifth and unofficial recreational water (L17) was a small river that is frequently used for canoeing. In 2011, recreational waters L13−L16 were sampled one (L14), four (L15) or five (L13, L16) times during the bathing season. During the 2012 bathing season, waters were sampled three (L15−L17) or four (L13) times. At each sampling time-point, also effluents and surface water at discharge points were sampled at the nearby situated mWWTPs (L09−L12).

Sampling. Manure samples each consisted of five hand-picked individual droppings, collected in sterile containers. Surface water and poultry farm wastewater was sampled by submerging sterile glass bottles, according to NEN-EN-ISO 19458.[17] WWTP effluents were collected by WWTP staff prior to the day of sampling of recreational waters, using automated systems that continuously sample effluent, rendering 24h flow-proportional, homogeneous samples that are collected in mixing vessels. One liter of effluent sample was taken from these mixing vessels. All samples were transported and stored at 5 ± 3 °C, and analyzed within 24 h after sampling.

Microbiological Detection and Enumeration. Samples were obtained as part of different projects, because of which slightly different procedures were used for the isolation of ESBL-EC and EC from samples from recreational waters,

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WWTP effluents and surface water at WWTP discharge points on one hand,[6] and poultry manure, wastewater from poultry farms and surface water adjacent to poultry farms on the other.[7] Manure samples were prehomogenized by addition of buffered peptone water (BPW) in a 1:1 or 1:2 ratio and using a sterile spoon. Equivalents of 10 g of faeces were suspended in 90 mL of BPW and homogenized using a Pulsifier (Microgen Bioproducts, Camberley, UK). From the homogenate 10-fold serial dilutions were prepared in peptone-saline (PS) and 100 μL of different dilutions was streaked in 2-fold onto tryptone bile X-glucuronide (TBX) agar for the isolation of EC,[18] and on ChromID ESBL agar (Biomerieux, Boxtel, The Netherlands), for the isolation of ESBL-EC. Of water samples, multiple volumes were filtered through 0.45 μm pore size membrane filters (Millipore, Amsterdam, The Netherlands), ranging from 0.01 to 200 mL, depending on the target-organism (EC or ESBL-EC) and type of water (surface water, rinsewater, WWTP effluents). EC enumeration in WWTP effluents, discharge points and recreational waters was performed and described by Blaak et al.[6] using tryptone soy agar (TSA) and tryptone bile agar (TBA), according to ISO 9308−1 "Rapid test",[19] tryptone bile x-glucuronide agar (TBX) was used for enumeration in water samples from poultry farms.[7] For isolation of ESBL-EC, filters were placed onto ChromID ESBL agar. Plates were incubated 18−24 h at 36 ± 2 °C (ESBL-EC from water from WWTP and recreational waters),[6] or for 4−5 h at 36 ± 2 °C, followed by 19−20 h at 44 ± 0.5 °C (all ESBL-EC and EC isolations from water and manure at poultry farms).[7] Suspected ESBL-EC were confirmed to produce ESBL by disk diffusion following CLSI guidelines,[20] using Sensi-Discs (BD, Breda, The Netherlands) according to the manufacturer's instructions. Zone diameters were determined for cefotaxime (30 μg) ± clavulanic acid (10 μg), ceftazidime (30 μg) ± clavulanic acid (10 μg), and cefoxitin (30 μg). ESBL-producing isolates were defined as strains resistant to cefotaxime (zone diameter ≤22 mm) and/or ceftazidime (zone diameter ≤17 mm), and an increase in zone diameter of ≥5 mm with the disks containing clavulanic acid.[20]

Data Analysis. *Concentration Distributions of ESBL-EC.* As part of the fate and exposure assessments, the concentration distributions of ESBL-EC in recreational water and source waters were characterized. In this regard, the concentrations of ESBL-EC, including in samples in which no ESBL-EC were detected (i.e., nondetects), were assumed to vary according to a Gamma distribution. If microbial particles in water are homogeneously distributed then the counts n within each sample of volume V are Poisson distributed. Because it is assumed that the concentration is Gamma distributed among samples, the counts $n_1 ... n_N$ of N samples with samples volumes $V_1 ... V_N$ have a Negative Binomial distribution with parameters r and $1/(1+\lambda V_i)$.[21]

Parameters r (shape) and λ (scale) were estimated by minimizing the following deviance function:

$$L[r, 1/(1+\lambda V_i)] = -2\ln \prod_{i=1}^{n} [f(r)\mathrm{NegBin}(n_i, V_i | r, s/(s+\lambda V_i))] \quad (1)$$

where $f(r) = 1 - \phi(x - 20)/20$ is a prior function for the shape factor r, with $\phi(x - 20)/20$ the cumulative normal distribution with a mean and variance of 20. This function can be interpreted as a very flat prior that prevents extremely small values of r from occurring thereby facilitating robust parameter estimation without strongly affecting the estimates. s is a scaling factor. If the mean sample volume is less than one liter then s = 0.001. This scaling avoids computational underflows.[22]

By means of likelihood ratio testing,[23] it was investigated whether the Gamma distributed concentrations differed between recreational water locations, between ditches adjacent to broiler and laying hen farms, and also between the bacterial concentration data of mWWTP effluents and surface water at mWWTP discharge points.

Fate and Transport of ESBL-EC. When ESBL-EC from human and animal faecal sources are discharged into surface water, they will be subject to dilution, advection and dispersion, inactivation (die-off) and sedimentation.[24] For our purposes, solely the effects of dilution and inactivation on the concentrations of ESBL-EC were considered, dispersion was considered to be part of dilution. Sedimentation certainly plays a role in stagnant and slowly flowing waters, but is very difficult to quantify, moreover, also resuspension occurs. For simplicity, these processes were not considered. Additionally, plasmid loss may add to the decrease in concentrations of EC-ESBL in water. Because currently data for plasmid loss rate are lacking, the effect of this process was not considered either.

Inactivation of ESBL-EC was assumed to be the same as for EC in general. This inactivation is temperature dependent according to the multivariate regression analyses of Franz et al.[25] for all water types. The formula for calculation ESBL-EC and EC concentrations in hypothetical recreational water taking into account inactivation and dilution is as follows:

$$C_{t,T} = C_0 \exp[-10^{-1.04+0.017T+\varepsilon} t] Q \quad (2)$$

where $C_{t,T}$ is the time and temperature dependent concentration [N/L], C_0 the initial (=source water) concentration [N/L], T the water temperature [°C], t the time [day], ε = 0.50 the prediction uncertainty [per day] and Q the dilution factor. The calculations were conducted for a water temperature of 20 °C (which is considered to be a comfortable water temperature for swimming), a travel time of up to 10 days from the source to a potential site of exposure through swimming, and a dilution factor Q of up to 10^9 times.

Probability of Exposure Assessment. The probability of exposure, P_{exp}, to ESBL-EC by swallowing of water during swimming was assessed by applying the following governing equation.[26]

$$P_{exp} = 1 - e^{-CV} \quad (3)$$

where C is the concentration of ESBL-EC (N/L) and V (L) the swallowed volume of water per person per swimming event. V is Gamma distributed with parameters r and λ of the Gamma distribution for men (r = 0.45 and λ = 60), women (r = 0.51 and λ = 35) and children (r = 0.64 and λ = 58) who are swimming in fresh water.[27] Using these parameter values, the volume is in ml and should be converted to liters.

Multivariate Regression Analysis. Multivariate regression analysis was conducted in order to determine which factors affect the concentration of ESBL-EC and the fraction ESBL-EC/EC in water and manure.

Multivariate regression analysis was conducted by means of linear model fitting using the statistical package R (version 2.14.0). In these analyses, log-transformation of the response variable was applied. The effects of the factors (i.e., matrix, 'most likely ESBL-EC source' and EC concentration) and all two-way interactions were considered. By means of stepwise

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Table 1. Descriptive Statistics of Sample Analysis for EC and ESBL-EC

matrix[b]		most likely ESBL-EC source[a]		location (L)[a]		parameter	EC[b]	ESBL-EC[b]
manure	55	poultry	55	L01	16	no. of samples	55	55
		bc: broiler farm	28	L02	8	no. of nondetects	0	18
		lh: laying hen farm	27	L03	4	mean	4.8×10^{10}	2.6×10^{8}
				L04	4	median	2.0×10^{10}	1.8×10^{6}
				L05	6	95-percentile	1.9×10^{11}	8.5×10^{8}
				L06	5	minimum	1.4×10^{9}	0
				L07	6	maximum	2.2×10^{11}	8.3×10^{9}
				L08	6			
wastewater	12	poultry	12	L01	7	no. of samples	12	12
		bc: broiler farm	1	L02	3	no. of nondetects	4	4
		b0: cleaned broiler farm	10	L03	1	mean	8.9×10^{8}	1.1×10^{7}
		lh: laying hen farm	1	L08	1	median	1.7×10^{7}	2.7×10^{4}
						95-percentile	4.6×10^{9}	8.3×10^{7}
						minimum	0	0
						maximum	4.6×10^{9}	8.3×10^{9}
surface water	40	poultry	40	l01	18	no. of samples	36	40
		bc: broiler farm	19	l02	9	no. of nondetects	1	15
		b0: cleaned broiler farm	13	l03	5	mean	1.2×10^{6}	1.9×10^{4}
		lh: laying hen farm	8	l04	4	median	3.8×10^{3}	6.5×10^{0}
				l06	2	95-percentile	6.0×10^{6}	1.7×10^{4}
				l07	2	minimum	0	0
						maximum	3.2×10^{7}	5.0×10^{5}
wastewater	23	human	23	l09	6	no. of samples[d]	23	23
		hu: mwwtp effluent +	13	l10	6	no. of nondetects	0	0
		surface water at discharge	10	l11	5	mean	3.1×10^{5}	2.6×10^{3}
		point[c]		l12	6	median	1.8×10^{5}	1.9×10^{3}
						95-percentile	1.2×10^{6}	9.3×10^{3}
						minimum	3.7×10^{3}	9.7×10^{1}
						maximum	1.5×10^{6}	1.2×10^{4}
recreational water	28	human+animal mix	28	L13	8	no. of samples	28	28
				L14	7	no. of nondetects	0	9
				L15	9	mean	5.0×10^{3}	1.6×10^{1}
				L16	1	median	1.9×10^{3}	1.6×10^{0}
				L17	3	95-percentile	1.8×10^{4}	8.1×10^{1}
						minimum	2.3×10^{2}	0
						maximum	5.2×10^{4}	1.5×10^{2}

[a]The number of samples are indicated. [b]Concentrations in manure are expressed as colony forming units (cfu) per kg, and in water as cfu per liter. [c]Because the (distributions of) concentrations in mWWTP effluent (3.2×10^{5} cfu/L) and surface water at discharge points of the same mWWTP (2.7×10^{5} cfu/L) were very similar, statistics description of samples were pooled; [d]13 effluent samples and 10 samples from surface water at the discharge points of these mWWTP.

model selection by Akaike's Information Criterion (AIC) with k, the multiple of the number of degrees of freedom used for the penalty in AIC set to 3.84, the best model was selected. A k value of 3.84 corresponds to the Chi-square with 95% confidence and one degree of freedom. The best model selected in R was transferred to Mathematica 9.0.1.0 (Wolfram Inc., Champaign Illinois), where it was implemented in a function for abstracting the linear equations for combinations of values of the categorical and numerical factors. Model predictions included 95% confidence intervals for the mean as well as 95% prediction intervals.

A log−log scale was needed to visualize the concentration data properly, because concentrations stretched over about 12 orders of magnitude. Log-transformation of the concentrations was also justified by assuming that microbial counts are Poisson-distributed and by the application of 10-fold dilutions for enumeration; for such log-transformed concentration values, it is commonly observed that they are reasonably normally distributed. This log-transformation implied that the total data set of 158 samples was reduced to 112 samples, omitting 46 samples with nondetects. It is plausible that the nondetects represent low concentrations below detection limit for which the same relation between the concentration of ESBL-EC and EC on log−log scale applies.

RESULTS

Description of Manure and Water Data. In total, 55 manure samples and 103 water samples were analyzed for the prevalence of EC and ESBL-EC. Table 1 provides the descriptive statistics of all sample analyses according to the

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matrix (manure, and types of water) and the most likely source of the ESBL-EC present in the matrices (animal or human). Bacterial concentrations in manure were included as a reference; all further analyses concern the different water types.

Of all matrices and parameters, the EC concentrations in poultry manure were the highest at a mean of more than 10^{10} colony forming units (cfu)/kg. In wastewater derived from poultry farms, EC concentrations were on average about two orders in magnitude lower than those in manure from the same farms, at approximately 10^9 cfu/L. The EC concentrations in surface water surrounding the poultry farms (mean 1.2×10^6 cfu/L) were three orders in magnitude lower than that in poultry farm wastewater. Other sources of EC may have contributed to the EC in these surface waters, but given the vicinity of the farms, poultry was the most likely source. The EC concentrations in effluent from mWWTP and in surface waters at discharge points (mean 3.1×10^5 cfu/L) were one order in magnitude lower than surface water adjacent to poultry farms. Finally, the EC concentrations in recreational waters (mean 5.0×10^3 cfu/L) were two orders in magnitude lower than those in WWTP effluents/at discharge points, and 3 orders of magnitude lower than those in surface waters surrounding poultry farms. Overall, mean ESBL-EC concentrations were about two orders in magnitude lower than the EC concentrations in the same matrix (Table 1). ESBL-EC concentrations ranged from 0 to 1.5×10^2 cfu/L in recreational waters.

Gamma Distributed ESBL-EC Concentrations in Water. From likelihood-ratio testing, it appeared that the Gamma distributed concentrations of ESBL-EC in the surface waters surrounding the poultry farms did not differ between laying hen and broiler farms, therefore these data were pooled for further analyses; this also applied for data from the five recreational waters. Similarly, likelihood-ratio testing demonstrated the same distribution of ESBL-EC concentrations in mWWTP effluents and the surface water at the sites of discharge. Apparently, at the site where the effluent flows into the water body, mixture with and dilution by the surface water has not yet occurred. For this reason (and because the discharge points and effluents were sampled at the same mWWTP), these water samples were considered to represent the same "source water" and were described with one gamma-distribution. Figure 1 summarizes the concentration distributions in surface waters around laying hen and broiler farms, either or not during or shortly after cleaning of poultry houses, in municipal wastewater (mWWTP effluents and surface waters at discharge), and in recreational waters. The ESBL-EC concentrations in the surface waters adjacent to broiler farms during or shortly after cleaning (mean 4.7×10^4 cfu/L) were significantly higher than those in the presence of flocks, namely about two orders in magnitude (mean 1.2×10^2 cfu/L). The concentrations in the municipal wastewaters were intermediate to the former two. The Gamma distributed ESBL-EC concentrations in recreational waters (mean 1.6×10^1 cfu/L) were about two orders in magnitude lower than those in the mWWTP effluents and in the surface waters at discharge points upstream of the recreational waters.

Fate in Surface Water and Human Exposure. Based on the measured ESBL-EC concentrations in recreational waters, the median, mean and 95-percentile values of the probability of exposure to ESBL-EC per person per swimming event were 0.017, 0.16, and 0.89 for men, 0.014, 0.13, and 0.72 for women, and 0.036, 0.20, and 0.95 for children, respectively.

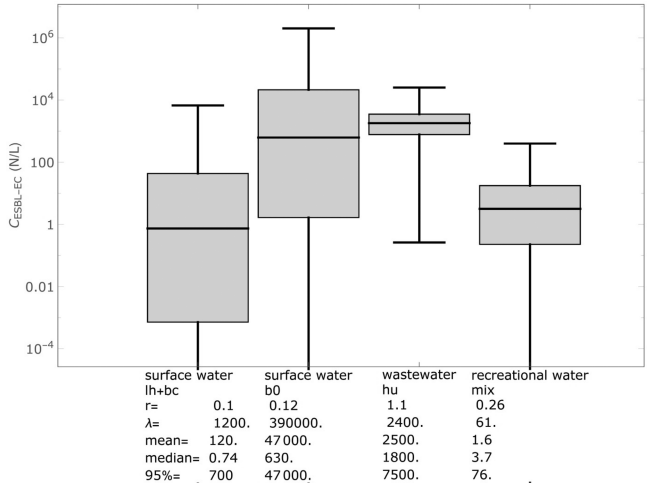

Figure 1. Concentration distributions of ESBL-EC in different waters. Surface waters surrounding poultry farms, municipal wastewater (effluents and surface water at discharge points), and recreational waters are shown, and the most likely ESBL-EC sources for these waters: lh = laying hen farms, bc = broiler farms, b0= broiler farms during or shortly after cleaning; hu = human; mix = human and animal. Values for parameters r and λ of the corresponding Gamma distribution are shown below the bars, as well as mean, median and 95 percentile values. The top whiskers represent maximum values and the boxes the quartiles.

Figure 2 shows the probabilities of exposure associated with swimming in hypothetical recreational water containing ESBL-EC concentrations based on 10 days of travel time at 20 °C from the different source waters (i.e., surface water surrounding poultry farms and mWWTP effluents and surface waters at discharge points) and for a range of dilution factors. The estimated ESBL-EC concentrations in surface water—based on the measured ESBL-EC concentrations in source waters, travel time, dilution, and inactivation—were found to be mostly determined by ESBL-EC concentrations in source waters and the dilution in surface water, and less by inactivation of the bacteria. After a water travel time of 10 days at 20 °C, ESBL-EC concentrations were calculated to have declined only about 0.6 $\log_{10}$-units (76%) by inactivation. The risks of exposure

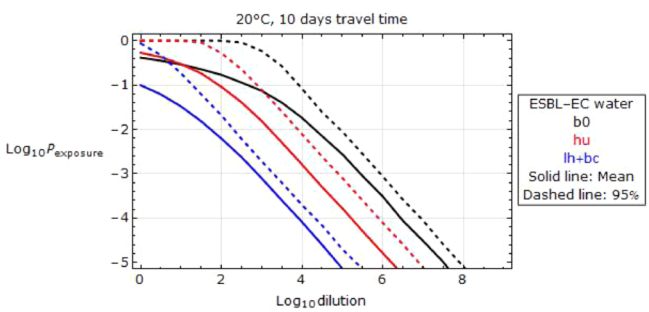

Figure 2. Probabilities of exposure based on fate modeling. Mean and 95-percentiles of probability of exposure of men to ESBL-EC concentrations at potential sites of exposure through swimming, estimated from different faecal sources as a function of dilution (log-scale) at 20 °C and after 10 days of travel time. The faecal sources used for the model were surface water surrounding poultry farms (lh = laying hens; bc = broilers), surface water surrounding broiler farms during or shortly after cleaning (b0 = cleaned broiler farms), and municipal wastewater (hu = human).

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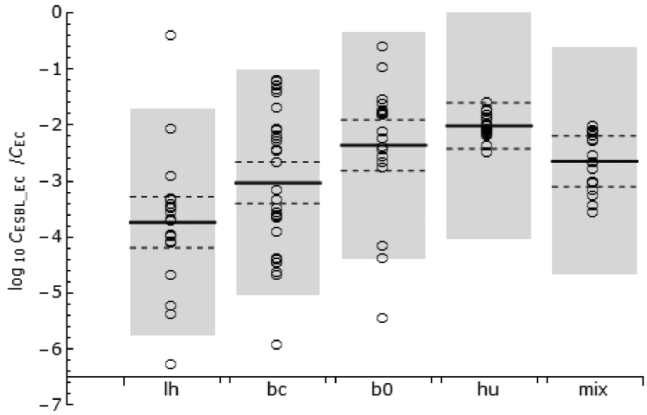

Source	Mean	95%–CI		95%–PI	
lh	−3.7	−4.2	−3.3	−5.8	−1.7
bc	−3.	−3.4	−2.7	−5.	−1.
b0	−2.4	−2.8	−1.9	−4.4	−0.33
hu	−2.	−2.4	−1.6	−4.	0.0093
mix	−2.6	−3.1	−2.2	−4.7	−0.61

lh: laying hen farm
bc: broiler farm
b0: cleansed broiler farm
hu: human
mix: human+animal

○ Observations
— Mean
-- 95%–Confidence Interval
▪ 95%–Prediction Interval

Figure 3. Fractions ESBL-EC/EC from different faecal sources. Shown are the logarithms of the fraction of ESCL-EC/EC ($\log_{10} C_{ESBL\text{-}EC}/C_{EC}$) in manure, wastewater and surface water for the sources "lh" (laying hen farms), "bc" (broiler farms) and "b0" (broiler farms during or shortly after cleaning), in wastewater for the source "hu" (human), and in surface water for the source "mix" (human and animal). R^2 = 26%.

through swimming, based on estimated ESBL-EC concentrations in waters that received either undiluted surface water surrounding poultry farms during the presence of flocks, 100-times diluted mWWTP effluents, or 1000-times diluted surface water surrounding poultry farms during or after cleaning (each after 10 days of travel), were comparable to the exposure risks based on the measured ESBL-EC concentrations in the recreational waters.

Relation between ESBL-EC and EC. The dependency of the fraction ESBL-EC/EC population in water and manure on the variables "most likely source", that is, poultry, human or mixed human and animal, "matrix" and "location" (see Table 1) was investigated. Best model selection showed that "most likely source" was the only explanatory variable. Figure 3 shows the logarithm of the mean fraction ESBL-EC/EC as well as the confidence and prediction intervals for the different sources. The mean fraction ESBL-EC/EC varied from 0.02% to 1%. Although the logarithm of this fraction was found to be significantly different between ESBL-EC sources, the uncertainty in the prediction spans four $\log_{10}$-units. Note that the variability of the fraction ESBL-EC/EC is very large for the source "poultry", spanning up to six orders in magnitude. In comparison, the variability of the fraction ESBL-EC/EC in municipal wastewater (source "human") spans only about one order in magnitude and that in recreational water (source "human and animal") about 1.5 orders in magnitude.

From best model selection, it was found that the log concentration of ESBL-EC depended linearly on the log concentration of EC (slope 0.87, Figure 4). The slope of 0.87 is significantly different from one (with a small standard error), which implies a power relation between the concentrations of ESBL-EC and EC meaning that at lower concentrations of EC the fraction ESBL-EC/EC is lower.

The abscissas in Figure 4 (equal to $\log_{10}$ $(((C_{ESCBL-EC})/(C_{EC}^{0.87})))$) differ significantly between sources as follows from the analysis of the fraction ESBL-EC/EC in which "most likely source" appeared to be a significant variable.

Variability of the fraction ESBL-EC/EC was determined by the large variability of the poultry farm data: the uncertainty of predicting this fraction spans four orders in magnitude. A regression analysis on the relation of ESBL-EC and EC concentrations for municipal wastewater data only, instead of the regression analysis of all data, gives the following relation: $\mathrm{Log}_{10}C_{ESBL\text{-}EC} = 0.81\mathrm{Log}_{10}C_{EC} - 1.0$ with an uncertainty interval of 0.8 $\log_{10}$-units only; similarly considering only the recreational water data, this relation is $\mathrm{Log}_{10}C_{ESBL\text{-}EC} = 0.87\mathrm{Log}_{10}C_{EC} - 2.2$ with an uncertainty interval of about two $\log_{10}$-units. When considering only these subsets of data, prediction intervals increase strongly when extrapolating to lower or higher concentrations.

■ DISCUSSION

Based on ESBL-EC concentrations measured in recreational waters, the probability of human exposure to ESBL-EC by swimming in recreational waters was high. All the recreational waters in this study are assumed to be under the influence of the (partially) upstream located mWWTPs.[6] Nevertheless, the most likely source of EC and ESBL-EC is unknown, because runoff of animal faeces from agricultural land and from farm premises cannot be excluded. According to the Bathing Water Directive[16] four of the recreational waters in this study were classified as "good", although our measurements would classify them as "poor". Bathing waters are classified as "good", when the 95-percentile of EC concentrations is larger than 5000 cfu/L and lower than or equal to 10 000 cfu/L. Based on these EC concentration limits and extrapolating the here observed ESBL-EC/EC ratio of 0.02 to 1%, ESBL-EC concentrations in "good quality"waters could vary from a few cfu/L to up to 100 cfu/L. This implies that exposure to ESBL-EC can also occur through swimming in "good" quality recreational waters. Estimation of ESBL-EC concentrations in recreational waters based on the concentrations in source waters and taking into account dilution and inactivation, showed that exposure to ESBL-EC in recreational waters, if downstream of farms and municipal wastewater discharge points is likely. Besides swimming, other relevant exposures to recreational water include, for example, diving, canoeing, or surfing, for which similarly high exposure risks may apply.[28]

Risks for public health associated with exposure could not yet be determined, due to a lacking dose−response relation for exposure to ESBL-EC. Although a dose−response relation for different EC variants has been derived for use in QMRA,[29] the risk involved in exposure to ESBL-EC is more complex and only in part determined by risk of infection or risk of illness upon exposure to pathogenic variants. It additionally involves possible colonization followed by asymptomatic carriage of opportunistic pathogens, which is dependent on their capacity to compete with strains already present in the gut, as well as the

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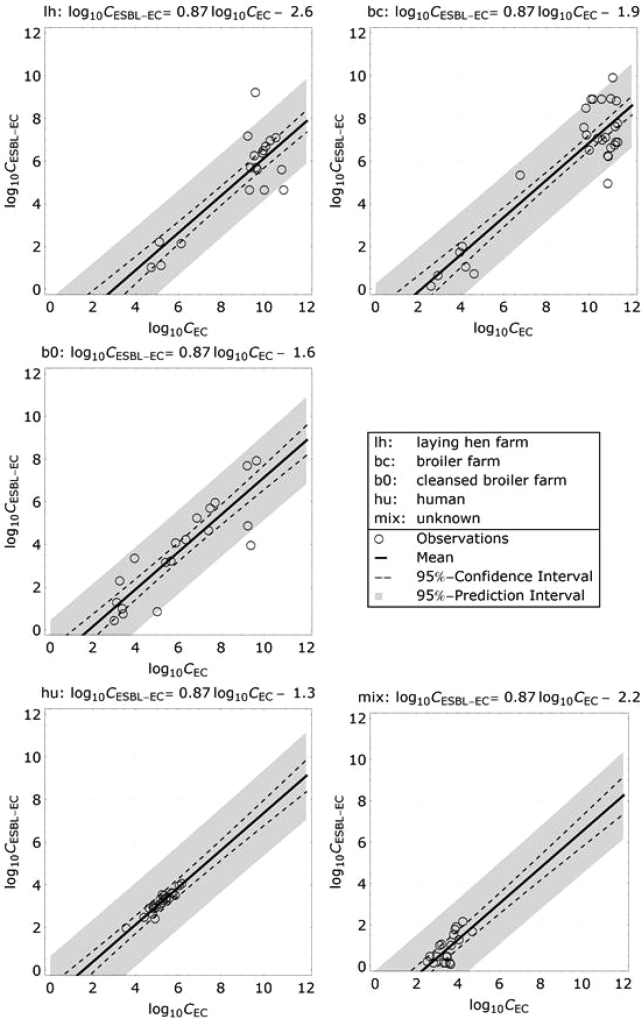

Figure 4. The log concentration of ESBL-EC as a function of the log concentration of EC and the most likely ESBL-EC source. Shown are for each source the logarithms of the ESBL-EC and EC concentrations in the different matrices: manure, wastewater and surface water for the sources "lh" (laying hen farms), "bc" (broiler farms) and "b0" (broiler farms during/shortly after cleaning), in wastewater for the source "hu" (human), and in surface water for the source "mix" (human and animal). Concentrations are in numbers per liter. R^2 = 88%. Regression equations are shown on top of each plot.

stability of the plasmids carrying ESBL-genes. Asymptomatic carriage may cause disease at later stages in cases of increased vulnerability, dissemination to more vulnerable individuals (e.g., the elderly or hospitalized individuals), and horizontal gene transfer within the gut resulting in acquisition of resistance to beta-lactams by other enteric pathogens.[30] Health impact of exposure to ESBL-EC and other antimicrobial resistant commensal bacteria is not only complex due to the different components of risk but also due to uncertainty about their relative contribution.

The concentrations ESBL-EC were 10 times higher in surface water surrounding broiler farms during or shortly after cleaning of stables compared to effluents from municipal WWTPs. The elevated concentrations of ESBL-EC in surface water surrounding broiler farms associated with cleaning are presumably due to runoff of manure with wastewater from the farm premises. Such emissions may occur on average every 6−7 weeks on broiler farms and approximately once every 1.5 years on laying hen farms when flocks are replaced. Still, it could be hypothesized that effluents from municipal WWTPs are a greater hazard for recreational water quality than poultry farms, because of the continuous emission of municipal wastewater, compared to intermittent emission associated with cleaning at poultry farms. However, if farms are clustered, subsequent emissions associated with cleaning activities at different farms could result in high continuous emission levels, therewith impacting recreational water downstream. Further research is needed to establish the relative contribution of human wastewater and agricultural sources (runoff from farms as well as usage of manure for field application) to the prevalence of ESBL-EC in surface water.

The fraction of ESBL-EC in EC in water and manure was found to be source specific. However, given the uncertainties in this relation, it is not possible to identify the faecal contamination source from a single surface water observation. This is further hampered by the fact that the ESBL-EC in surface waters may originate from multiple types of contamination sources (i.e., with different fractions ESBL-EC/EC). In our study, we have not tracked down, let alone quantified, the possible sources that contributed to the ESBL-EC concentration in the recreational waters. Identification of the source(s) of ESBL-EC in surface water additionally requires comparison of molecular characteristics of variants in the suspected source(s) and surface water. The quantitative data that were currently used for modeling the fate of ESBL-EC from poultry farms were gathered as part of a larger project in which ESBL-EC isolates were also obtained and characterized. Isolates from runoff water on poultry farms and surface water surrounding poultry farms had approximately 82% and 58% homology with respect to phylogenetic group, sequence type, ESBL-genotype, and antibiotic resistance profile, with ESBL-EC in poultry manure indicating the farms as major, but not the sole contamination sources for the adjacent surface waters.[15] Similarly, some ESBL-EC in recreational waters showed such homology with isolates in wastewater at WWTP discharging upstream of these locations.[6]

For the protection of public health from (among others) waterborne infectious diseases, regularly health based targets are set as maximum levels of EC. Therefore, EC (or faecal coliforms) are routinely monitored in surface waters such as those used for recreation[16] and as source water for the production of drinking water.[31] In our study, empirical formulas (Figure 4) were developed that enable the prediction of ESBL-EC concentrations from EC concentrations for recreational waters (fresh water), municipal wastewater and small surface waters under the influence of poultry farms, taking into account a given level of uncertainty. Such predictions are, currently, limited to the waters we have investigated, because extrapolation is hampered by the fact that the ESBL-EC/EC relation appeared to be source-specific and each surface water may be subject to different (combinations of) sources of ESBL-EC. The formulas describe a power relation between the concentration of ESBL-EC and EC. According to this relation, at lower concentrations of EC, the fraction ESBL-EC/EC is lower. Lower EC concentrations were specifically observed in recreational waters, that is, in waters at certain distance from faecal sources in which EC can be assumed to have been subjected to environmental conditions for a longer time. This would suggest that ESBL-EC is less persistent in the environment than the total population, or that the plasmids containing ESBL-genes are lost during prolonged stay in the

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environment. Another explanation may be that recovery of ESBL-EC is less efficient at lower concentrations.

In conclusion, our data show that exposure to ESBL-EC by swimming is likely, if recreational waters are located downstream of mWWTPs or livestock farms. Further research is warranted into public health effects, such as colonization, infection, or horizontal gene transfer, upon exposure.

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Notes

The authors declare no competing financial interest.

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