Journal of Applied Microbiology, 2024, 135, lxae144
https://doi.org/10.1093/jambio/lxae144
Advance access publication date: 21 June 2024
Research Article

JOURNAL OF APPLIED MICROBIOLOGY
OXFORD UNIVERSITY PRESS

Exploring the presence, genomic traits, and pathogenic potential of extended-spectrum β-lactamase *Escherichia coli* in freshwater, wastewater, and hospital effluents

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Abstract

Aims: The purpose of this work was to study extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* (ESBL-EC) in freshwaters, hospital effluents, and wastewaters during two sampling campaigns in 2021.

Methods and results: Water sampling was performed at 24 stations in the Ourthe watershed in Belgium. A total of 644 ESBL ($n = 642$) and AmpC ($n = 2$) *E. coli* strains were isolated. Disk-diffusion assays were performed following the EUCAST's recommendations. All strains were tested for the presence of $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-2}}$, and $bla_{\text{CTX-M-9}}$ gene groups by PCR. Genes belonging to $bla_{\text{CTX-M-1}}$ and $bla_{\text{CTX-M-9}}$ groups were detected, respectively, in 73.6% and 14.9% of the strains. No $bla_{\text{CTX-M-2}}$ group's gene was found. A subset of strains ($n = 40$) was selected for whole genome sequencing. *Escherichia coli* serotype O18: H7 ST 1463 was predominant ($n = 14$) in the sequenced strains and showed pathogenicity in the *Galleria mellonella* larvae model. β-lactamase genes identified were $bla_{\text{CTX-M}}$ ($n = 21$), with $bla_{\text{CTX-M-15}}$ mostly represented ($n = 15$), as well as bla_{TEM} ($n = 11$), bla_{OXA} ($n = 7$), bla_{SHV} ($n = 9$), and carbapenemase (CP) genes were observed in several strains—$bla_{\text{KPC-3}}$ ($n = 19$), $bla_{\text{NDM-1}}$ ($n = 1$), $bla_{\text{VIM-1}}$ ($n = 2$), and $bla_{\text{OXA-244}}$ ($n = 2$)—even from freshwaters.

Conclusions: ESBL-EC are widely distributed in the aquatic environment in Belgium and contain a variety of ESBL and CP genes.

Impact Statement

This study highlights the role of hospital effluents and wastewater treatment plants in the dissemination of antimicrobial resistance through the dissemination of several extended-spectrum β-lactamase clones in the environment.

Keywords: *Escherichia coli*; antimicrobial resistance; extended-spectrum β-lactamase; carbapenemase; *Galleria mellonella*; freshwaters

Introduction

The World Health Organization (WHO) lists antimicrobial resistance (AMR) as one of the top ten threats to global health (WHO 2019a). Without intervention, AMR could cause 10 million deaths each year by 2050 and force up to 24 million people into extreme poverty by 2030 (WHO 2019b).

Fighting against AMR implies a "One Health" approach with an integrated and holistic multisectoral collaboration, in particular the need for better integration of environmental, aquatic, and wildlife issues into current approaches (White and Hughes 2019).

Escherichia coli, an intestinal bacterium of human and warm-blooded animals, is used as fecal contamination indicator of aquatic systems and foodstuffs (Ghaderpour et al. 2015). The release of antimicrobial-resistant (AR) *E. coli* into the environment from anthropogenic sources, as well as the overuse of antibiotics in human or veterinary medicine and agriculture, are driving AMR (Berendonk et al. 2015). AR *E. coli* can, among others, enter aquatic systems through discharge from hospital or municipal wastewaters and from intensive livestock production facilities (Pruden et al. 2006, Pereira et al. 2013).

Extended-spectrum β-lactamases (ESBLs) are one of the most important and widely spread mechanism of resistance among *E. coli* (Lenart-Boroń et al. 2020), and extended-spectrum β-lactamases-*E. coli* (ESBL-EC) have been declared by the WHO as a key indicator for the surveillance of AMR worldwide (WHO 2021). Since the ESBLs were first reported in 1979 (Sanders and Sanders 1979) and the first carbapenemase in 1993 (Naas et Nordmann 1994), the prevalence of ESBL-producing bacteria has been frequently detected worldwide from clinical strains due to the increasing use of β-lactam antibiotics. The mortality, length of hospital stays, and hospital costs associated with infections due to ESBL-producing *Enterobacteriaceae* were generally higher compared to those with non-ESBL infections, as ESBL-producing bacteria often cause more severe infections and require more complex

Received 16 November 2023; **revised** 30 May 2024; **accepted** 20 June 2024

treatments (Zhen et al. 2019, Shamsrizi et al. 2020). They are therefore responsible of a serious global health issue.

ESBLs are mostly plasmid-encoded, which facilitates horizontal gene transfer between bacterial groups (Reinthaler et al. 2010). They hydrolyze β-lactam antibiotics, resulting in resistance to penicillins, cephalosporins, and aztreonam. In many European countries, CTX-M-type enzymes are the predominant ESBLs and replaced TEM and SHV mutants, with *E. coli* joining *K. pneumoniae* as a major host, increasingly isolated from community patients (Livermore et al. 2006). ESBL of the CTX-M-15 type has increased over time in most countries and is now dominant in most regions except for Asia, where gene's group 9 (especially CTX-M-14) is dominant, and South America, where $bla_{\text{CTX-M-2}}$ is still significant (Bevan et al. 2017).

Aquatic environments, in particular, have attracted attention as a hotspot for the accumulation, emergence, and dissemination of clinically important AMR, including ESBLs. The freshwaters, including rivers and lakes, are constantly influenced by anthropogenic activities, including the discharge of wastewater treatment plants (WWTPs) that reduce the bacterial concentration in water, but not appreciably reduce the proportion of AR bacteria (Blaak et al. 2015) and pollute receiving waters with high levels of fecal indicator bacteria, including *E. coli* and antimicrobial resistance genes (ARGs) (Reynolds et al. 2020). This not only increases the background level of ESBLs in the aquatic environment but also provides an ideal setting for the horizontal exchange of ESBL genes with other pathogenic or indigenous bacteria present in the water. The impact on human health of ESBL-producing Enterobacteriaceae present in aquatic environments is considerable, and it is therefore important to understand the role of aquatic environments as reservoirs and transmission routes for ESBL-producing *Enterobacteriaceae* to humans (Cho et al. 2023).

Bathing waters are also risk areas for humans; exposure to ESBL-EC by swimming is likely if recreational waters are located downstream of WWTPs or livestock farms (Schijven et al. 2015). In Belgium, ESBL-EC, found in nine bathing areas, represents 3% of the isolated *E. coli* strains (Crettels et al. 2023).

Despite the high levels of virulence present in many ESBL-EC, however, few studies have comprehensively assessed the pathogenicity of ESBL-EC. In order to study the pathogenicity of some ESBL-EC strains and speculate on their origin, the *Galleria mellonella* larvae model was used. This invertebrate model enables large-scale studies due to its short life cycle, constituting a preliminary model to the use of vertebrate animals such as mice or rats, for which there are ethical, budgetary, and logistical obstacles (Rossoni et al. 2019). These larvae are used as model organisms to study many human pathogens, including bacteria, fungi, and protozoa (Chen et al. 2016). Zhao et al. (2022) tested the pathogenicity of ESBL-EC isolated from ovine origin to investigate the presence of virulence genes and to assess their pathogenicity and zoonotic potential. They found ESBLs-producing *E. coli* isolates with numerous virulence-related genes were able to cause multiple infectious diseases in animal models (mice, neonatal rats, and *G. mellonella*).

The aims of this study were therefore: (i) to assess the ESBL-EC prevalence in freshwaters in Belgium; (ii) to assess the role of hospital effluents and WWTPs on the AMR dissemination; (iii) to characterize the ESBL-EC genes by PCR and whole genome sequencing; (iv) to assess the genetic heterogeneity among and within the populations of isolated *E. coli*; and (v) to assess the pathogenicity of a selection of ESBL-EC in the *G. mellonella* larvae model.

Materials and methods

Water sampling

In this study, ESBL-EC were studied in the Ourthe river but also in a continuum hospital-WWTP-freshwater, allowing to directly observe the bacterial dispersion and the main sources of contamination. The Ourthe river flows for 180 kilometers in southeast Belgium to the Meuse river. Its sources are located in a rural and forest areas, and then it is submitted to demographic pressure and urbanization. In the Ourthe watershed, 24 sampling stations were sampled, including 13 freshwaters, two hospital effluents, one mixed effluent from a veterinary faculty and surrounding facility wastewaters, and the input/output and upstream/downstream of two WWTPs that each receive hospital effluents (Fig. 1). Effluents from hospital A are discharged into the WWTP's Tilff, and effluents from hospital B into the WWTP's Angleur.

Two sampling campaigns were performed in January (1st campaign) and August 2021 (2nd campaign) to assess the seasonal effect, linked to antibiotic use and climatic variations, on the presence of ESBL-EC. In July 2021, there were major floods in Belgium, which led to the non-operation of the WWTPs in the Ourthe and Vesdre watersheds (Cornwall 2021). Sampling at the input/output of Tilff and Angleur WWTPs (sampling stations with the codes N, O, R, and S) could only be carried out in August 2022 instead of August 2021, when the WWTPs were no longer damaged.

Water samples were collected in 1-l sterile polyethylene bottles without any preservative, transported at 4°C, stored at laboratory at 5 ± 3°C and analyzed within 24 h.

Escherichia coli and ESBL-EC enumeration

From each sample, different volumes (ranging from 1 to 500 ml) and 10-fold dilutions of untreated water were membrane filtered through 0.45-µm pore size filters (Millipore Corporation, USA), which were placed on Tryptone Bile X-glucuronide (TBX) (Bio-Rad, Marnes-la-Coquette, France) for *E. coli* enumeration and on Brilliance ESBL agar (Oxoid, Basingstoke, UK) for ESBL-EC enumeration. The Petri dishes were incubated overnight at 37°C to select the optimal two dilutions for colony-forming unit (CFU) counts, according to ISO 8199:2018 (International Organization for Standardization, Switzerland). All the ESBL-EC from a selected dilution was picked up from Brilliance ESBL agar and re-spread on Brilliance ESBL agar to check the purity. Tryptophanase activity tests with Kovac's reagent were applied to confirm the species of presumptive ESBL-EC strains. Only indole-positive strains were considered for further analysis and stored at −80°C. A code was given to each isolate: the letter indicates the sampling point (Table 1), and the number indicates the isolate number.

Antimicrobial susceptibility testing

A susceptibility test was performed on all the strains on Mueller-Hinton (MH) agar (Bio-Rad, Marnes-la-Coquette, France) using the disk diffusion assay, and plates were incubated for 18 ± 2 h at 35 ± 1°C according to the European Committee on Antimicrobials (Matuschek et al. 2014). The inhibition diameters were measured using the Adagio

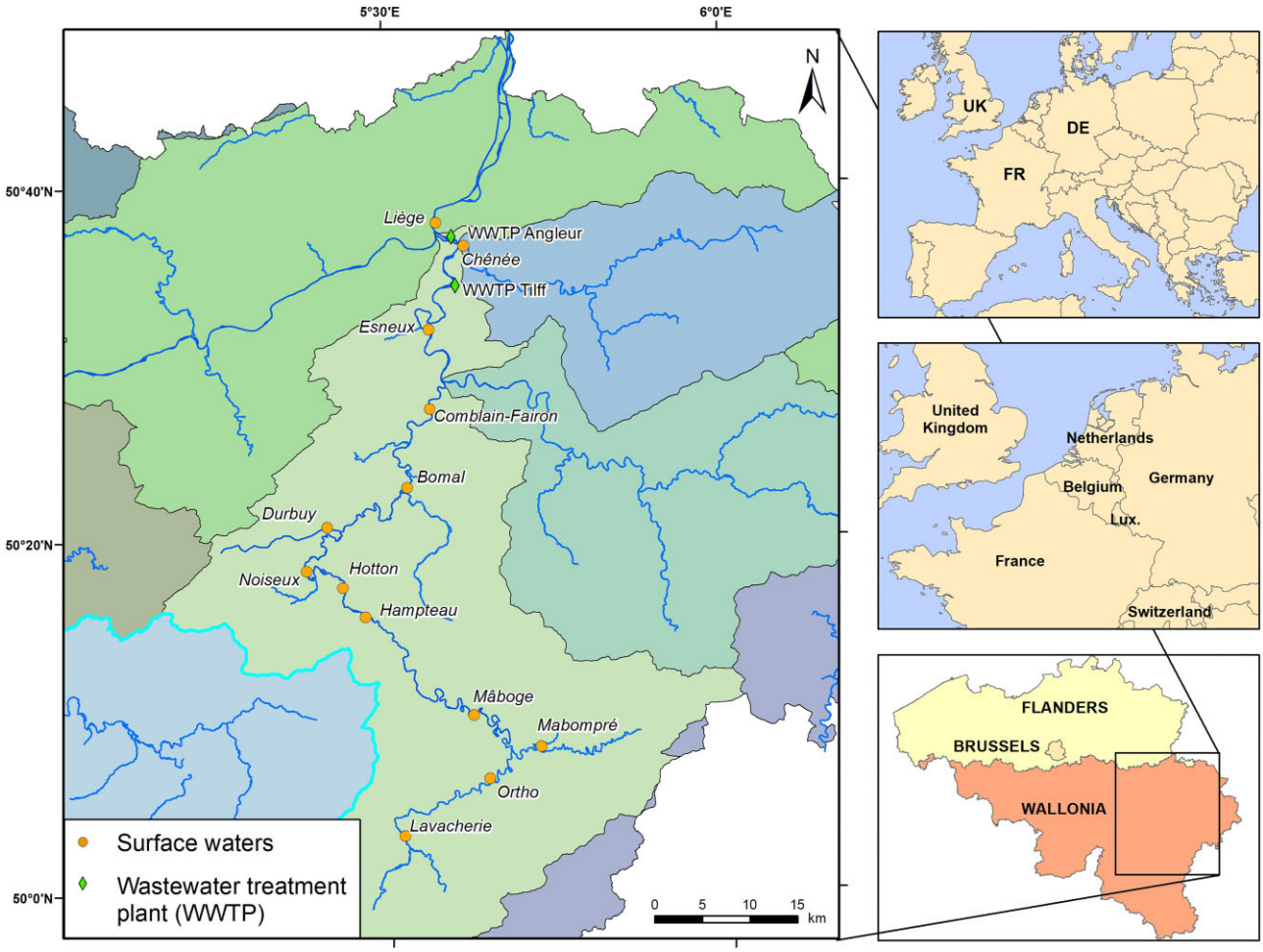

Figure 1. Wastewater and surface water sampling stations in the Ourthe watershed (for confidentiality, hospitals and the veterinary faculty are not shown on the map).

system (Bio-Rad, Marnes-la-Coquette, France) and compared with the clinical breakpoints 2020 V.10.0 provided by EUCAST to determine whether a strain is sensitive or resistant to a given antibiotic. Resistance rates, to determine the proportion of resistant strains in relation to the wild population, were also calculated using the ECOFFs given by EUCAST for *E.* coli (consulted on 20 July 2022). *Escherichia coli* ATCC 25922 (American Type Culture Collection, Manassas, VA, USA) was included in each assay as a negative control and *K. pneumoniae* ATCC 700603 as ESBL-producer positive control.

The antimicrobial disks (Bio-Rad, Marnes-la-Coquette, France) were placed on MH agar with automatic disk dispenser (16 disks per plate) (Bio-Rad, Marnes-la-Coquette, France).

A total of 16 antibiotics were tested: ampicillin (AMP, 10 μg), amoxicillin/clavulanic acid (AMC, 20/10 μg), azithromycin (AZM, 15 µg), cefepime (FEP, 30 µg), cefotaxim (COX, 5 µg), cefotaxim/clavulanic acid (CCO, 5/10 μg), ceftazidim (CZD, 10 μg), ceftazidim/clavulanic acid (CCZ, 10/10 μg), cefuroxime (CXM, 30 μg), cefoxitin (FOX, 30 µg), ciprofloxacin (CIP, 5 μg), colistin (COL, 10 µg), ertapenem (ETP, 10 μg), meropenem (MEM, 10 μg), piperacillin/tazobactam (PTZ, 30/6 μg), temocillin (TEM, 30 µg).

Of these 16 antibiotics, cephalosporin discs without COX, CZD, and with clavulanic acid (CCO, CCZ) were used for phenotypic confirmation of ESBL production by the double disk synergy test according to the EUCAST technical guide version 2.0. 2017. The resistance rate is determined as the ratio between the number of strains resistant to an antibiotic and the total number of strains tested by antibiogram. Multidrug resistance is defined as resistance to at least one agent from at least three antimicrobial classes (Magiorakos et al. 2012).

ESBL genotyping

All the strains were tested for the presence of $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-2}}$, and $bla_{\text{CTX-M-9}}$ gene groups with specific primer (Table 2). Total DNA was extracted by the boiling method (Dashti et al. 2009). PCR was then performed on 1 µl of DNA extract mixed with 20 pmol of each primer and 12.5 µl Fastgene 2x (Nippon Genetics, Filter Service, Eupen, Belgium) in a final volume of 25 µl on a CFX96 thermocycler (Bio-Rad, Marnes-la-Coquette, France) with the following protocol: initial denaturation at 94°C for 10 min, followed by 30 cycles of denaturation for 30 s, annealing for 35 s at 62.5°C, extension at 72°C for 1 min, and a final extension at 72°C for 9 min. PCR products were visualized by UV light after electrophoresis at 100 V for 55 min in 1xTAE, 1.5% agarose gel.

Table 1. *Escherichia coli* and ESBL-EC concentrations measured by locations for the 1st and 2nd campaigns.

			1st campaign (january 2021)			2nd campaign (august 2021)		
Code	Locations	Origin	*Escherichia coli* (CFU) x (100 ml)$^{-1}$	BLSE-EC (CFU) x (100 ml)$^{-1}$	% BLSE-EC	*Escherichia coli* (CFU) x (100 ml)$^{-1}$	BLSE-EC (CFU) x (100 ml)$^{-1}$	% BLSE-EC
A	Ourthe at Lavacherie	Freshwater	2.0×10^3	94	4.7	3.2×10^3	15	0.5
B	Ourthe at Ortho	Freshwater	1.1×10^3	25	2.3	6.1×10^2	5	0.8
C	Ourthe at Mabompré	Freshwater	9.7×10^2	11	1.1	8.3×10^2	5	0.6
D	Ourthe at Maboge	Freshwater/bathing localization	6.1×10^2	8	1.4	3.2×10^2	4	1.3
E	Ourthe at Hampteau	Freshwater	6.5×10^2	6	0.9	2.8×10^2	2	0.6
F	Ourthe at Hotton	Freshwater/bathing localization	4.7×10^2	5	1.1	4.3×10^2	2	0.5
G	Ourthe at Noiseux	Freshwater/bathing localization	6.5×10^2	8	1.3	6.2×10^2	11	1.7
H	Petite Somme at Durbuy	Freshwater	1.5×10^4	52	0.3	2.1×10^3	12	0.6
I	Ourthe at Bomal	Freshwater	1.3×10^4	86	0.6	9.8×10^2	5	0.5
J	Ourthe at Comblain-Fairon	Freshwater	9.9×10^3	1.5×10^2	1.5	1.5×10^3	17	1.2
K	Ourthe at Esneux	Freshwater	1.2×10^4	1.3×10^2	1.1	1.5×10^3	20	1.3
L	Upstream of WWTP Tilff in the Ourthe	Freshwater	2.6×10^3	33	1.3	1.0×10^4	95	0.9
M	Downstream of WWTP Tilff in the Ourthe	Freshwater	1.3×10^3	60	4.7	7.2×10^3	1.4×10^2	1.9
N	Input WWTP Tilff	Hospital/community effluent	2.5×10^6	2.3×10^5	9.2	5.4×10^6	1.1×10^6	20.5
O	Output WWTP Tilff	Treated effluent	1.2×10^5	3.1×10^3	2.6	3.2×10^4	1.5×10^3	4.6
P	Upstream of WWTP Angleur in the Ourthe	Freshwater	2.8×10^3	42	1.5	1.2×10^4	1.4×10^2	1.1
Q	Downstream of WWTP Angleur in the Ourthe	Freshwater	3.6×10^3	43	1.2	1.5×10^4	1.9×10^2	1.3
R	Input WWTP Angleur	Hospital/community effluent	7.3×10^6	1.6×10^4	0.02	1.0×10^7	2.0×10^5	2
S	Output WWTP Angleur	Treated effluent	1.2×10^5	7.6×10^2	0.6	1.9×10^4	2.4×10^2	1.2
T	Ourthe at Chênée	Freshwater	8.6×10^3	2.1×10^2	2.5	8.5×10^3	1.1×10^2	1.3
U	Meuse at Liège	Freshwater	1.3×10^4	2.4×10^2	1.9	9.2×10^3	1.7×10^2	1.9
V	Hospital A*	Hospital effluent	7.3×10^5	1.1×10^5	15.6	4.3×10^5	1.1×10^5	25.7
W	Hospital B*	Hospital effluent	4.0×10^6	6.3×10^3	0.2	1.0×10^4	2.0×10^2	2
X	Veterinary faculty and surrounding facilities*	Hospital/community effluent	1.1×10^6	6.7×10^4	6.3	3.2×10^6	4.1×10^3	0.1

*Effluents of Hospital A and the Veterinary faculty and surrounding facilities are transferred to the Tilff WWTP, while Hospital B effluent to the Angleur WWTP

Table 2. Target genes, primer sequences, and amplified fragment lengths of the CTX-M 1, 2, and 9 groups PCR.

Targeted groups	Primer sequences	Amplicon size (bp)	Reference
$bla_{\text{CTX-M-1}}$	CTX-M-1-F: 5′- TTAGGAAGTGTGCCGCTGTA-3′ CTX-M-1-R: 5′-CGGTTTTATCCCCCACAAC-3′	655 bp	Ogutu et al. 2015
$bla_{\text{CTX-M-2}}$	CTX-M-2-F: 5′-GCGACCTGGTTAACTACAATCC-3′ CTX-M-2-R: 5′-CGGTAGTATTGCCCTTAAGCC-3′	351 bp	Pitout et al. 2004
$bla_{\text{CTX-M-9}}$	CTX-M-9-F: 5′-GGTGATGAACGCTTTCCAAT-3′ CTX-M-9-R: 5′-TTATCACCTGCAGTCCACGA-3′	518 bp	Ogutu et al. 2015

Whole-genome analysis

Based on the susceptibility tests (highest number of antibiotic resistances) and the PCR results as well as their origin (diversity of sampling points), 40 *E. coli* strains (20 from the 1st and 20 from the 2nd campaign) were confirmed by API 20E and selected for whole genome sequencing.

Total DNA extraction was performed on a single colony using the Dneasy® Blood and Tissue Kit (Qiagen, Venlo, Netherlands).

The sequencing libraries were prepared using the Nextera XT library preparation kit (Illumina). Sequencing was carried out using the Illumina MiSeq platform to generate 300 bp paired-end reads by the GIGA Institute (ULiège). Raw read sequences obtained in this study were deposited to GenBank/SRA under the BioProject PRJNA1012687.

Assembly of the Illumina sequence reads was performed using the SPAdes (v3.13.0) assembler. The assembled *E. coli* genomes were uploaded to the *Escherichia* database on PubMLST to confirm the species (https://pubmlst.org/organisms/escherichia-spp/). Bioinformatic analysis was carried out using the Center for Genomic Epidemiology (CGE) pipelines (Serotype Finder v2.0, MLST v2.0, cgMLST Finder v1.2, VirulenceFinder v2.0, ResFinder v4.1, MobileElement Finder v1.0.3, PathogenFinder 1.1). PATRIC and CARD

(https://www.bv-brc.org/) were also used to compare the results obtained.

The CGE pipelines were used to identify acquired antibiotic resistance genes as well as *E. coli* virulence genes.

Phylogenetic tree

The phylogenetic relationships of the sequenced *E. coli* strains were investigated, the concatenated sequences of 7 housekeeping genes (*adk*, *fumC*, *icd*, *gyrB*, *mdh*, *purA*, *recA*) were aligned with the sequences of the related reference genes in GenBank (Fakih et al. 2017) with a length of 3424 nucleotides using Muscle in MEGA11 software. Phylogenetic relationships of all sequences were analyzed with MEGA11 software using Maximum Likelihood based on Tamura-Nei model (Tamura et al. 2013). The statistical confidence of the tree was set by bootstrapping 1000 replicates.

A SNP analysis was performed with *Escherichia/Shigella* Enterobase V 1.1.5 (https://enterobase.warwick.ac.uk/species/index/ecoli) (Zhou et al. 2020). Briefly, the Enterobase RefMasker identifies in the assembly unwanted DNA regions (i.e. tandem repeat, CRISPR regions, dispersed repeat). The Enterobase RefMapper aligns the rest of the assembly regions to the reference assembly and computes a single SNP matrix. A final maximum likelihood tree using RAxML is generated.

Galleria mellonella larvae infection assays

The pathogenicity of three sequenced strains (Q22 and N8, and K9 strains isolated from the first and second campaigns, respectively) was assessed in *G. mellonella* larvae (Nusect, Deerlijk, Belgium). Randomly chosen *G. mellonella* larvae ($n = 2 \times 10$ per group) were inoculated by injecting 10 µl of bacteria at 10^4, 10^5, and 10^6 CFU/10 µl into the last proleg with an automatic injector (Cole Parmer, Vernon Hills, IL, USA). Injections were performed using BD Plastipak™ 1 ml sterile syringes (Becton-Dickinson, Franklin Lakes, NJ, USA) and sterile 30-gauge needles (Terumo Corporation, Tokyo, Japan). Equal volumes of PBS served as a negative control.

Post-inoculation, the larvae were put into an incubator in the dark at 37°C, and survival was monitored daily for 4 days. Kaplan–Meier survival curves were drawn using Prism software and log-rank tests were performed to highlight any significant difference in survival rates between the groups (P-value ≤ 0.05).

Statistical analysis

Chi-square tests were performed to compare the resistance rates per antibiotic during the two campaigns and to compare the resistance rates per antibiotic between origins with the procedure freq of SAS software 9.4 program (SAS Institute, Cary, NC, USA). A P-value < 0.05 was defined as a statistically significant difference between the resistance rates.

Results

Escherichia coli and ESBL-EC prevalence

Escherichia coli concentrations in freshwaters ranged from 2.8×10^2 (Hampteau) to 1.5×10^4 (Durbuy, Angleur downstream) (CFU) x $(100\,ml)^{-1}$, while ESBL-EC concentrations ranged from 2.0×10^0 (Hampteau, Hotton) to 2.4×10^2 (Liège) (CFU) x $(100\,ml)^{-1}$ for the two sampling campaigns (Table 1). In freshwaters, the average concentrations of *E. coli* were 4.5×10^3(CFU) x $(100\,ml)^{-1}$ and 6.1×10^1(CFU) x $(100\,ml)^{-1}$ for ESBL-EC. In freshwaters, the proportion of ESBL-EC compared to the total number of *E. coli* per sampling point varied from 0.3% (Durbuy) to 4.7% (Lavacherie, Tilff downstream) and was 1.4% in average. In the hospital effluents, the *E. coli* concentrations have reached (maximum) 4.0×10^6 (CFU) x $(100\,ml)^{-1}$ (Hospital B) and 1.1×10^5 (CFU) x $(100\,ml)^{-1}$ (Hospital A) for ESBL-EC. In hospital A, ESBL-EC represented up to 25.7% of the total *E. coli* flora.

At the WWTP's output, *E. coli* concentrations are of the order of log4-log5, while ESBL-EC concentrations are log2-log3. The log removal values for *E. coli* were 1.3–2.2 log for the Tilff WWTP and 1.8–2.7 log for the Angleur WWTP for 1st–2nd campaigns, respectively, while those for ESBL-EC were 1.9–2.9 log for the Tilff WWTP and 1.3–2.9 log for the Angleur WWTP.

Escherichia coli and ESBL-EC concentrations measured in freshwater during the second campaign in August 2021 were not higher than those measured during the first campaign in January 2021, even though the WWTPs were no longer in operation at that time following the floods that occurred in July 2021.

Antibiotic resistance

Since the strains were selected directly on the Brilliance ESBL medium, 642 out of the 644 strains had the ESBL phenotype, and the two others had AmpC phenotype. Five percent of them had a carbapenemase producer profile.

A total of 372 and 272 strains were isolated, respectively, from the 1st to 2nd campaigns. Calculated resistance rates for each campaign are compared with clinical breakpoints (Fig. 2a). The Chi-square test did not show any significant difference (P-value > 0.05 for each antibiotic) between the two campaigns for each AMR rate by antibiotic except for COX.

In Fig. 2a, the highest rates of resistance were found for AMP (100%–100%), COX (98.1%–89.3%), and CXM (99.5%–97.8%) and the lowest for ETP (4.0%–5.5%) and MEM (0.3%–0.4%) for the 1st–2nd campaigns.

A total of 630 of the 644 ESBL-EC and AmpC strains (97.8%) were multidrug resistant (MDR), i.e. resistant to 3 different antibiotic classes. The most frequent combinations (68.3%) were resistance to penicillin/2nd generation cephalosporin/3rd–4th generation cephalosporin.

Based on clinical breakpoints, 100% of the strains are resistant to penicillin (AMP) and 3rd–4th generation cephalosporins (COX, CZD, and FEP). A total of 23.3% strains are resistant to penicillin + β-lactamase inhibitors (AMC); 61.5% are resistant to 2nd generation cephalosporins (CXM); and 48.8% are resistant to fluoroquinolones (CIP).

Resistance rates were calculated by origin and plotted in a histogram (Fig. 2b). Rates measured in freshwaters for AMC, COX, FOX, CXM, PTZ, and TEM were lower than those measured at the input/output of WWTPs and hospitals. The Chi-square test measured a difference (P-value < 0.05) by origin for 7 antibiotics (AMC, FEP, FOX, CZD, ETP, PTZ, and TEM).

In Fig. 2c, some resistance rates calculated with the ECOFFs are higher (FEP, COX, CZD, CIP, MEM, PTZ, and TEM) than those obtained with clinical breakpoints, while others are lower (AMC) but still close (FOX, CXM, and ETP).

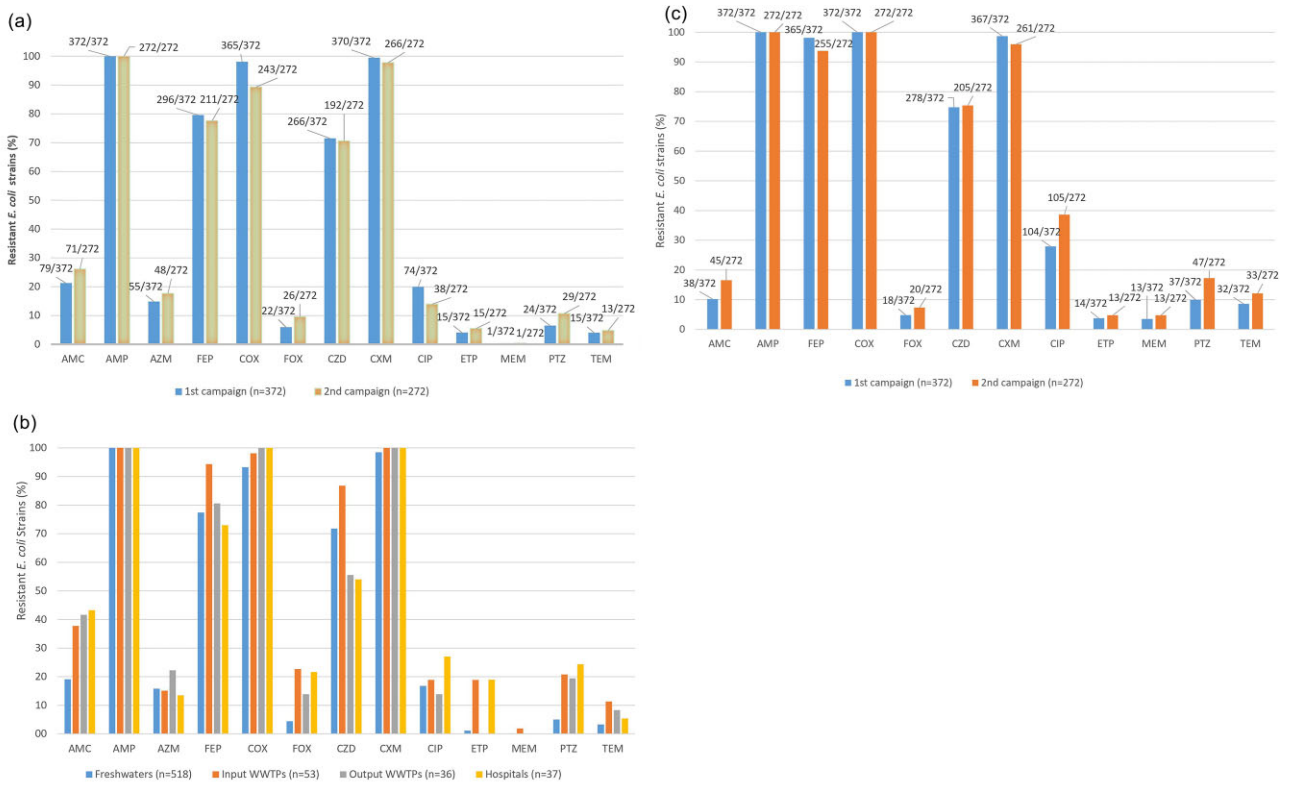

Figure 2. (a) Resistance rates of the ESBL ($n = 642$) and AmpC ($n = 2$) *E. coli* strains isolated, respectively, from the 1st ($n = 372$) and 2nd ($n = 272$) campaigns using the clinical breakpoints given by EUCAST (b) Resistance rates of the 644 strains sorted by water sources using the clinical breakpoints given by EUCAST (c) Resistance rates of the 644 strains isolated, respectively, from the 1st to 2nd campaigns using the ECOFF given by EUCAST (ECOFF for AZM does not exist). (AMC: amoxicillin/clavulanic acid, AMP: ampicillin, AZM: azithromycin, FEP: cefepime, COX: cefotaxim, FOX: cefoxitin, CZD: ceftazidim, CXM: cefuroxime, CIP: ciprofloxacin, ETP: ertapenem, MEM: meropenem, PTZ: piperacillin/tazobactam, TEM: temocillin, WWTP: wastewater treatment plant).

Genotyping

A total of 474 strains out of 644 (73.6%) had a CTX-M-1 group's gene, 96 (14.9%) had a CTX-M-9 group's gene, and none had CTX-M-2 group's gene. No strain had a combination of two CTX-M groups.

Whole-genome analysis

Serotyping and MLST

Several serotypes were detected for the 40 sequenced genomes (Table 3). Serotype O18:H7 belonging to ST1463 was predominant with 14 strains isolated from hospital effluents or treated effluents (output of WWTP). No O antigen was detected in one strain (T2), while no H antigen was detected in four others (T3, T7, V1, and V2). Three O86 serogroup strains belonging to ST38, three O128ac:H12 serotype strains belonging to ST11028, and four O16:H5 serogroup strains belonging to ST131 were found for the first campaign. For the second campaign, two strains were detected as O102:H6, belonging to ST405; and two strains belonged to serogroup O8 (ST88 and ST1642) (Table 3).

Resistance genes

All strains had genes for resistance against AMP, FEP, and CIP. All strains except one (strain U9) had COX resistance genes. The most frequently detected ESBL gene's group was $bla_{\text{CTX-M-1}}$ ($n = 21$) with $bla_{\text{CTX-M-15}}$ the most represented ($n = 15$). bla_{TEM} variants ($n = 11$), bla_{OXA} variants ($n = 7$), and bla_{SHV} ($n = 9$) variants were also detected (Table 3). However, the variants $bla_{\text{TEM-1}}$, $bla_{\text{TEM-35}}$, $bla_{\text{TEM-84}}$, and $bla_{\text{SHV-12}}$ are listed as broad-spectrum but not extended-spectrum in the beta-lactamase database (Naas et al. 2017), and the function of $bla_{\text{SHV-182}}$ is not listed in this database.

Four different carbapenemase-producing *E. coli* genes were identified among the sequenced genomes: the $bla_{\text{KPC-3}}$ gene in 19 strains, the $bla_{\text{NDM-1}}$ gene in one strain, the $bla_{\text{VIM-1}}$ gene in two strains, and the $bla_{\text{OXA-244}}$ in two strains.

With few exceptions, all strains belonging to the same ST clustered together, while a second basis for clustering was the resistance gene profiles with the presence of $bla_{\text{CTX-M-1}}$ (blue), $bla_{\text{CTX-M-9}}$ (red), or $bla_{\text{KPC-3}}$ carbapenemase-coding genes (green) (Fig. 3). Some reference strains are included beside the isolated strains in this study.

SNP analysis

Serotype O18:H7 belonging to ST1463 was predominant in 14 isolated strains. SNP analysis showed that these 14 strains appeared to originate from the same clone (Fig. 4a), ranging between 0 and 53 SNPs in the matrix and with only a few differences identified in plasmid replicons (Fig. 4b). These 14 strains belong to the B1 Clermont phylotype.

Plasmid

The most frequently detected plasmid replicons were IncFIB ($n = 37$), IncFII ($n = 27$), and col156 ($n = 18$) (Table 3).

Table 3. Serotype, sequence type, plasmid replicons, virulence genes, β-lactamase genes with their location on mobile genetic elements, and other resistance genes of the 40 sequenced strains classified by sample type (1st campaign, 2nd campaign).

Sample type	Code (strain)	Serotype	ST type	Plasmid replicons	Virulence genes	β-lactamase genes(mobile element)	Other resistance genes
Freshwaters	H1*	O33:H4	ST117	IncFII	*chuA, fyuA, irp2, iss, terC, traT, astA, cea, ireA, lpfA, ompT, pic, vat*	$bla_{CTX-M-14}$, bla_{TEM-84}	*dfrA1, aadA1, qacE, mph(A), aac(6′)Ib, aac(6′)-Ib-cr, sul1, catA1, erm(B)*
	K26*	O86:H18	ST38	/	*afaD, air, chuA, eilA, fyuA, gad, hra, irp2, iss, kpsE, kpsMII_K5, terC*	$bla_{CTX-M-14\ (ISEc9)}$	*sul2, mph(A), aph(6)-Id, aph(3′′)-Ib, qacE, sul1*
	Q22*	O25:H4	ST131	Col156, IncFIA, IncFIB, IncFII	*chuA, fyuA, gad, irp2, iss, kpsE, kpsMII_K5, terC, traT, cea, ompT, sitA, usp, yfcV, senB, sat, mcbA, iha*	$bla_{CTX-M-27}$	*tet(A), sul2, dfrA17, qacE, sitABCD*
	T2*	H:20	ST2040	IncC, IncFIA, IncFIB, IncFIC, IncHI2, IncHI2A	*gad, terC, traT, lpfA, sitA*	bla_{TEM-1B}, bla_{OXA-1}, $bla_{CTX-M-15}$, $bla_{SHV-182}$, $bla_{CMY-6\ (Tn6196–IS682-ISAeme15)}$, $bla_{DHA-1/7/24\ (ISKpn14)}$, $bla_{NDM-1\ (ISKpn14)}$	*aph(6)-Id, aadA1, catB3, qnrB9, tet(A), qnrB1, aac(3)-IId, aac(3)-Iia, catA1, aac(6′)-Ib3, aac(6′)-Ib-cr, sitABCD, dfrA14*
	T3*	O13/O129/ O135	ST176	Col(IMGS31), Col(Ye449), Col156, Col440I, IncFIB, IncFII, IncX3	*gad, iss, terC, traT, celB*	$bla_{SHV-182}$, $bla_{KPC-3\ (Tn4401)}$	*qnrS1*
	T7*	O13/O129/ O135		Col(IMGS31), Col(pHAD28), Col156, Col440I, IncFII, IncX3	*gad, iss, terC, traT, celB*	$bla_{SHV-182}$, $bla_{KPC-3(Tn4401)}$	*qnrS1*
	T8*	O128ac:H12	ST11028	IncFIB, IncFII, IncHI2, IncHI2A, IncY	*gad, terC, traT, lpfA*	bla_{OXA-1}, $bla_{CTX-M-15}$	*sul1, ant(2′′)-Ia, catB3, tet(D), catA2, aph(3′)-Ia, tet(A), mph(A), dfrA14, qnrB1, mcr-9, aac(3)-Iia*
	U3*	O25:H12	ST607	IncFII, IncFIB, FII, IncHI2, IncHI2A, IncM1, IncX3	*gad, terC, lpfA*	$bla_{CTX-M-9}$, $bla_{SHV-182}$, $bla_{KPC-3\ (Tn4401)}$	*qacE, sul1, catA1, qnrA1, ant(2′′)-Ia, aadA2b, dfrA16, mcr-9*
	C1**	O153:H9	ST3268	IncFIB	*air, chuA, eilA, fyuA, gad, irp2, kpsE, kpsMII_K5, terC, cea, sitA,*	$bla_{CTX-M-15\ (ISKpn19-ISEc9)}$, bla_{TEM-35}	*sul2, aph(6)-Id, mph(A), qnrS1, sitABCD*
	H8**	O8:H17	ST88	IncFIA, IncFIB, IncFII	*iutA, iucC, afaC, papA_F11, papC, afaA, afaB, lpfA, mcmA*	bla_{OXA-1}, bla_{TEM-1B}	*aadA1, qacE, sul2, tet(B), aph(6)-Id, aph(3′′)-Ib, aph(3′)-Ia, floR, sitABCD*
	I5**	O102:H6	ST405	IncB/O/K/Z, IncFIA, IncFIB, IncFII, p0111	*air, chuA, eilA, fyuA, gad, irp2, kpsE, kpsMII_K5, terC, traT, iutA*	$bla_{CTX-M-15\ (IsEc9)}$, bla_{TEM-1C}	*tet(B), sul2, aph(6)-Id, floR*
	K9**	O86:H2	ST349	IncFIB, IncFII, IncI1-I	*ORF3, ORF4, afaD, aggA, aggC, aggD, aggR, air, chuA, eilA, fyuA, irp2, iss, kpsE, kpsMII_K5, terC, traT, cea, celB, aap, aar, aatA*	$bla_{CTX-M-15\ (ISEc9)}$, bla_{TEM-1B}, $bla_{DHA-1\ (IS6100)}$	*qnrB4, qacE, sul2, tet(A), dfrA17*

Table 3. Continued

Sample type	Code (strain)	Serotype	ST type	Plasmid replicons	Virulence genes	β-lactamase genes(mobile element)	Other resistance genes
	L9**	O102:H6	ST405	Col156, IncB/O/K/Z, IncFIA, IncFIB, IncII, p0111	*air, chuA, eilA, fyuA, gad, irp2, kpsE kpsMII_K5, terC, traT, lpfA, iutA*	$bla_{\text{CTX-M-15 (IsEc9)}}$, $bla_{\text{TEM-1C}}$	*tet(B), sul2, aph(6)-Id, florR*
	U9**	O1:H15	ST38	Col(BS512), IncFIA, IncFIB, IncFII, IncX4	*afaD, air, chuA, eilA, fyuA, gad, hra, irp2, kpsE, kpsMII_K5, terC, traT, sitA, sat, iha, papA_F43, iutA, iucC, afaC, nfaE, afaA*	$bla_{\text{OXA-1}}$	*tet(B), qacE, sitABCD*
Hospital/community effluent (Input WWTP)	N17*	O128ac:H12	ST11028	IncFIB, IncFII, IncHI2, IncHI2A, IncY	*gad, terC, traT, lpfA*	$bla_{\text{OXA-1}}$, $bla_{\text{CTX-M-15 (ISEcl1)}}$, $bla_{\text{VIM-1}}$	*catB3, tet(A), ant(2'')-Ia, aadA2b, qnrB1, aac(3)-Iia, catA2, aph(3')-Ia, sul1, qacE, aac(6')-Ib3, aac(6')-Ib-cr, aac(6')-29b, tet(D), mph(A), dfrA14, mcr-9*
	R12*	O86:H30	ST38	IncFIB, IncFII	*ORF3, ORF4, afaD, agg3D, agg5A, air, capU, chuA, eilA, fyuA, hra, irp2, iss, kpsE, kpsMII_K5, terC, traT*	$bla_{\text{CTX-M-15 (ISEc9)}}$	*aadA1, dfrA1*
	N6**	O18:H7	ST1463	IncFIB, IncHI1A, IncHI1B, IncX3, IncX6, pKPC-CAV1193	*fyuA, gad, irp2, terC*	$bla_{\text{SHV-182}}$, $bla_{\text{KPC-3 (Tn4401)}}$	*ant(2'')-Ia, qacE*
	N8**	O18:H7		Col156, IncFIB, IncHI1A, IncHI1B, IncX3, IncX6, pKPC-CAV1193	*fyuA, gad, irp2, terC, lpfA, celB*	$bla_{\text{SHV-182}}$,$bla_{\text{KPC-3 (Tn4401)}}$	*ant(2'')-Ia, qacE*
	N9**	O18:H7		Col156, IncFIB, IncFII, IncHI1A, IncHI1B, IncX3, IncX6, pKPC-CAV1193	*fyuA, gad, irp2, terC, lpfA, celB*	$bla_{\text{SHV-182}}$,$bla_{\text{KPC-3 (Tn4401)}}$	-
	N10**	O18:H7		Col156, IncFIB, IncHI1A, IncHI1B, IncX3, IncX6, pKPC-CAV1193	*fyuA, gad, irp2, terC, lpfA, celB*	$bla_{\text{SHV-182}}$, $bla_{\text{KPC-3 (Tn4401)}}$	*ant(2'')-Ia, qacE*
Treated effluent (Output WWTP)	O3*	O128ac:H12	ST11028	Col(pHAD28), IncFIB, IncFII, IncHI2, IncHI2A, IncY	*gad, terC, traT, lpfA*	$bla_{\text{TEM-1B (ISEc9)}}$, $bla_{\text{OXA-1}}$, $bla_{\text{CTX-M-15 (ISEc9)}}$, $bla_{\text{VIM-1}}$	*catB3, tet(A), ant(2'')-Ia, aadA2b, qnrB1, aac(3)-Iia, catA2, aph(3')-Ia, sul1, qacE, aac(6')-Ib3, aac(6')-Ib-cr, aac(6')-29b, tet(D), mph(A), dfrA14, mcr-9*

Table 3. Continued

Sample type	Code (strain)	Serotype	ST type	Plasmid replicons	Virulence genes	β-lactamase genes(mobile element)	Other resistance genes
	O5*	O18:H7	ST1463	IncFIB, IncFII, IncHI1A, IncHI1B, IncX3, IncX6, pKPC-CAV1193	*fyuA, gad, irp2, terC, lpfA*	bla_{SHV-12}, $bla_{KPC-3\ (Tn4401)}$	*ant(2′′)-Ia, qacE*
	S5*	O16:H5	ST131	Col156, IncFIA, IncFIB, IncFII, IncY	*chuA, fyuA, gad, irp2, kpsE, kpsMII_K5, terC, traT, ompT, vat, usp, yfcV, sat, iha, papa_F43, iutA, iucC*	$bla_{CTX-M-15\ (IS630)}$, $bla_{OXA-244}$	*erm(B), dfrA17, qacE, sitABCD*
	S9*	O16:H5		Col156, IncFIA, IncFIB, IncFII, IncY	*chuA, fyuA, gad, irp2, kpsE, kpsMII_K5, terC, traT, ompT, vat, usp, yfcV, sat, iha, papa_F43, iutA, iucC*	$bla_{CTX-M-15\ (IS630)}$, $bla_{OXA-244}$	*erm(B), dfrA17, qacE, sitABCD*
	O1**	O18:H7	ST1463	IncFIB, IncHI1A, IncHI1B, IncX3, IncX6, pKPC-CAV1193	*fyuA, gad, irp2, terC, lpfA, celB*	$bla_{SHV-182}$, $bla_{KPC-3\ (Tn4401)}$	*ant(2′′)-Ia, qacE*
	O4**	O18:H7		Col156, IncFIB, IncFII, IncHI1A, IncHI1B, IncX3, IncX6, pKPC-CAV1193	*fyuA, gad, irp2, terC, lpfA, celB*	$bla_{SHV-182}$, $bla_{KPC-3\ (Tn4401)}$	*ant(2′′)-Ia, qacE*
	O5**	O18:H7		Col156, IncFIB, IncFII, IncHI1A, IncHI1B, IncX3, IncX6, pKPC-CAV1193	*fyuA, gad, irp2, terC, lpfA, celB*	$bla_{SHV-182,}$ $bla_{KPC-3\ (Tn4401)}$	*ant(2′′)-Ia, qacE*
	O6**	O18:H7		Col156, IncFIB, IncFII, IncHI1A, IncHI1B, IncX3, IncX6, pKPC-CAV1193	*fyuA, gad, irp2, terC, lpfA, celB*	$bla_{SHV-182}$, bla_{KPC-3}	*ant(2′′)-Ia, qacE*
Freshwaters/bathing site	F19*	O86:H30	ST38	IncFIB, IncII	*ORF3, ORF4, afaD, agg3C, agg3D, agg5A, air, capU, chuA, eilA, fyuA, gad, hra, irp2, iss, kpsE, kpsMII_K5, terC, traT*	$bla_{CTX-M-15\ (ISEc9)}$	*aadA1, dfrA1*
	D12**	O8:H7	ST1642	IncFIA, IncFIB, IncFII	*gad, hra, terC, traT, cea, ireA, lpfA, sitA, iutA, iucC*	$bla_{CTX-M-1,}$ bla_{TEM-1B}	*qacE, ant(2′′)-Ia, aph(3′′)-Ib, aph(3′′)-Ib, aph(3′)-Ia, aac(3)-Iid, mph(A), floR, dfrA36, sul2, tet(B), qnrS1, sitABCD, aadA2b, lnu(F)*
	G5**	O148:H30	ST2967	Col(IMGS31), IncFIB, IncR	*gad, terC*	$bla_{CTX-M-15\ (ISEc9)}$, bla_{OXA-1}	*catB3, tet(A), dfrA14, qnrB1, aac(3)-Iia*

Table 3. Continued

Sample type	Code (strain)	Serotype	ST type	Plasmid replicons	Virulence genes	β-lactamase genes(mobile element)	Other resistance genes
Hospital effluent	V5*	O16:H5	ST131	Col156, IncFIA, IncFIB, IncFII	*afaD, chuA, fyuA, irp2, kpsE, kpsMII_K5, terC, traT, ompT, sitA, usp, yfcV, senB, sat, iha, papA_F43, iutA, iucC, afaC, nfaE*	$bla_{\text{TEM-1B}}$, $bla_{\text{CTX-M-15 (ISEc9)}}$	*aph(6)-Id, sul2, aac(3)-Iid, mph(A), qacE, sitABCD*
	V7*	O18:H7	ST1463	IncFIB, IncHI1A, IncHI1B, IncX3, IncX6, pKPC-CAV1193	*fyuA, gad, irp2, terC, lpfA*	$bla_{\text{SHV-182}}$, $bla_{\text{KPC-3 (IncX3, Tn4401)}}$	*ant(2'')-Ia, qacE*
	V12*	O18:H7		Col156, IncFIB, IncHI1A, IncHI1B, IncX3, IncX6, pKPC-CAV1193	*fyuA, gad, irp2, terC, lpfA, celB*	$bla_{\text{SHV-182}}$, $bla_{\text{KPC-3 (IncX3)}}$	*ant(2'')-Ia, qacE*
	V13*	O16:H5	ST131	Col156, IncFIA, IncFIB, IncFII	*afaD, chuA, fyuA, irp2? kpsE, kpsMII_K5, terC, traT, ompT, sitA, usp, yfcV, senB, sat, iha, papA_F43, iuA, afaD, chuA, fyuA, irp2, kpsE, kpsMII_K5, terC, traT, ompT, sitA, usp, yfcV, senB, sat, iha, papA_F43, iuA, iucC, afaC, nfaE*	$bla_{\text{TEM-1B}}$, $bla_{\text{CTX-M-15 (ISEc9)}}$	*tet(A), sul2, aac(3)-Iid, dfrA17, qacE, sitABCD*
	V14*	O18:H7	ST1463	Col156, IncFIB, IncFII, IncHI1A, IncHI1B, IncX3, IncX6, pKPC-CAV1193	*fyuA, gad, irp2, terC, lpfA, celB*	$bla_{\text{SHV-12}}$, $bla_{\text{KPC-3 (Tn4401)}}$	*ant(2'')-Ia, qacE*
	V1**	O13/O129/O135	ST176	Col(IMGS31), Col(Ye4449), Col4401, IncFIB, IncFII, IncHI2, IncHI2A, IncX3	*gad, iss, terC, traT, celB*	$bla_{\text{KPC-3 (Tn4401)}}$, $bla_{\text{CTX-M-9}}$, $bla_{\text{SHV-182}}$	*qnrS1, qacE, sul1, qnrA1, aadA2b, ant(2'')-Ia, dfrA16, catA1, mcr-9*
	V2**	O13/O129/ O135		Col(IMGS31), Col156, Col4401, IncFIB, IncFII, IncX3	*gad, iss, terC, traT, celB*	$bla_{\text{KPC-3 (Tn4401)}}$, $bla_{\text{SHV-182}}$	*qnrS1*
	V9**	O18:H7	ST1463	IncFIB, IncFII, IncHI1A, IncHI1B, IncX3, IncX6, pKPC-CAV1193	*fyuA, gad, irp2, terC, lpfA*	$bla_{\text{KPC-3 (Tn4401)}}$, $bla_{\text{SHV-11}}$	*ant(2'')-Ia, qacE*
	V10**	O18:H7		Col156, IncFIB, IncHI1A, IncHI1B, IncX3, IncX6, pKPC-CAV1193	*fyuA, gad, irp2, terC, traT, lpfA, celB*	$bla_{\text{KPC-3 (Tn4401)}}$, $bla_{\text{SHV-182}}$	*ant(2'')-Ia, qacE*

*1st campaign

**2nd campaign

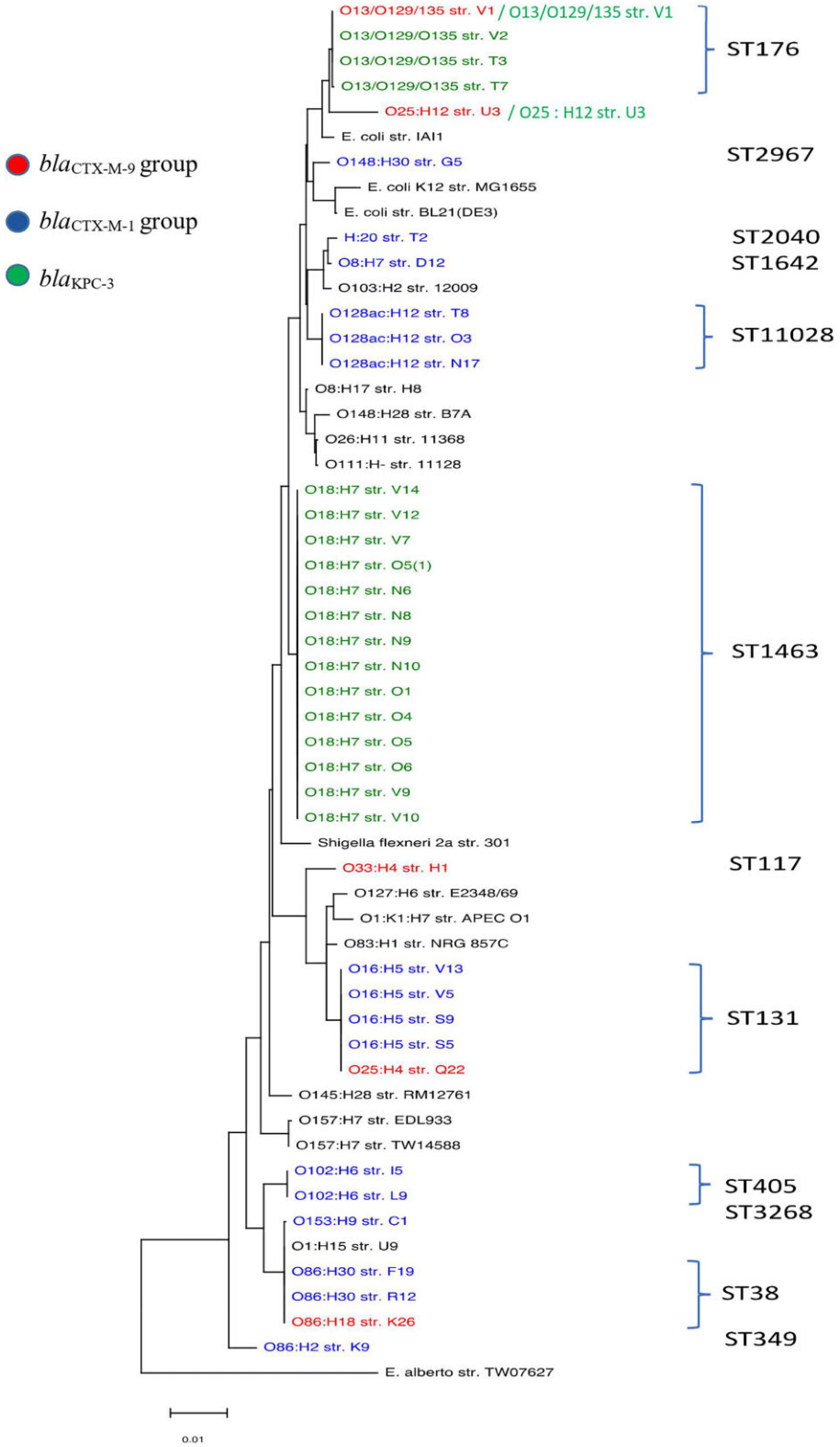

Figure 3. Phylogenetic relationships and sequence types of the sequenced *E. coli* genomes based on the concatenated sequences of the 7 housekeeping genes (*adk, fumC, icd, gyrB, mdh, purA, recA*) with a length of 3424 nucleotides using Muscle in MEGA11 software (maximum likelihood based on Tamura–Nei model and a bootstrap of 1000 replicates) (Tamura et al. 2013).

Virulence genes

The VirulenceFinder program identified the presence of several virulence genes in the 40 strains sequenced (Table 3). The *ter*C gene, which codes for tellurite resistance, was the most abundant in each category of samples. Other genes were also detected in high proportion such as *fyuA* (siderophore), *gad* (glutamate decarboxylase), and *lpfa* (polar fimbriae). The detected virulence genes were quite similar within each

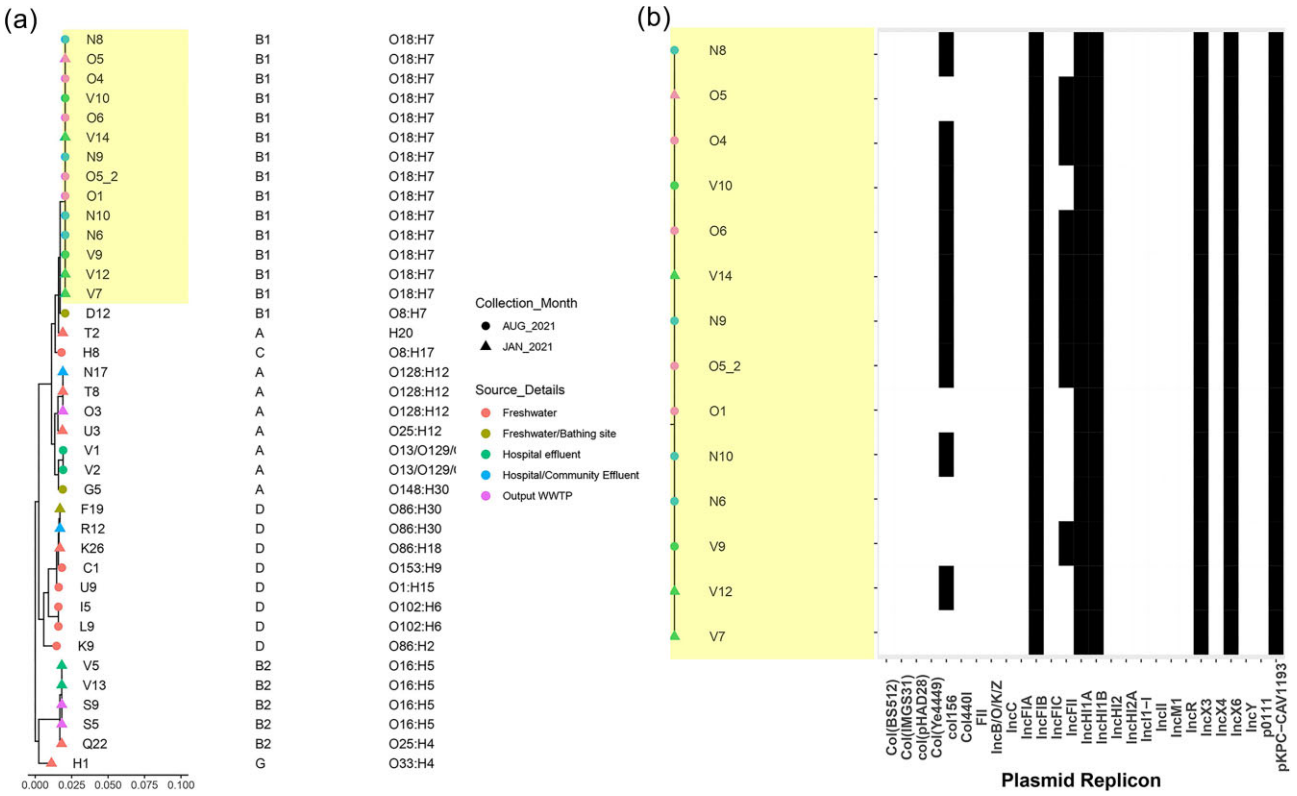

Figure 4. (a) SNP tree of the sequenced *E. coli* genomes with phylotype, serotype, and sampling origin (b) Comparison of plasmid replicon of the 14 strains O18:H7. Plot with the following R packages: ggplot2, treeio, ggtree, ggpubr, and aplot.

type of sample (hospital effluents, freshwaters, input/output WWTP).

A total of 13 other strains from different water categories and different serogroups (O16, O25, and O86) possessed the genes *kpsE* and *kpsMII_K5* coding for a possible K5 capsule, an essential virulence factor. Only six strains had the *iha* gene coding for an adhesin (Table 3).

Mobile genetic elements

The MobileElementFinder program detected several mobile elements associated with ESBL genes (Table 3). Many $bla_{\text{CTX-M}}$ genes are associated with insertion sequences (ISEc9), and the bla_{KPC-3} genes are generally located in a Tn4401 transposon, sometimes on the IncX3 plasmid.

Galleria mellonella larvae infection assays

The Q22 and K9 strains were virulent in the *G. mellonella* model at 10^5 CFU/10 µl, while the N8 strain was virulent at 10^6 CFU/10 µl. The Kaplan–Meier survival curves are shown in Fig. 5.

Discussion

This study reports, for the first time, the presence of ESBL-EC in non-recreational aquatic environments in Belgium. Several studies have been realized in different countries to date (Blaak et al. 2015, Johnson et al. 2020, Hooban et al. 2021, Kimera et al. 2021, Cho et al. 2023). ESBL-EC was enumerated in each of the 24 sampled sites. Surface water is highly impacted by anthropogenic activities as it receives contaminants from variants sources, and the detection of ESBL-EC has become common in surface water worldwide (Cho et al. 2023). Their concentrations were higher than those found by Blaak et al. (2014) in four recreational waters in the Netherlands, where average ESBL-EC concentrations of 1.3 (CFU) x $(100\ \text{ml})^{-1}$ were measured, and their presence was found in 62% of all samples. In 2021, in nine recreational (freshwater) areas in Belgium, ESBL-EC had been found at least once in each bathing site and in 33% of all samples and represented 3% of the *E. coli* isolated (Crettels et al. 2023). However, this value of 3% is certainly overestimated, as it is calculated from isolates obtained on TBX and TBX + AMX media.

In this study, the proportion of ESBL-EC in the total *E. coli* flora ranged from 0.3% to 4.7% per sampling point in freshwaters, with an average of 1.4%.

This is lower than what was found in two rivers in southern Poland, where ESBL-EC constituted 15.30% of the *E. coli* isolated from these two rivers (Lenart-Boroń 2017). In Norway, the ratio of ESBL-EC to total *E. coli* CFU ranged from 0% to 3.8% in recreational water samples (Jørgensen et al. 2017).

In hospital effluents, the *E. coli* concentrations have reached (maximum) 4.0×10^6 (CFU) x $(100\ \text{ml})^{-1}$ (Hospital B) and 1.1×10^5 (CFU) x $(100\ \text{ml})^{-1}$ (Hospital A) for ESBL-EC. Servais et Passerat (2009) had found similar concentrations of *E. coli* in samples from 17 hospitals in France. Therefore, concentrations of ESBL-EC seem high in hospital effluents, which are directed to WWTPs. The MAR index, which reflects the level of contamination by antibiotic-resistant *E. coli* in a sample, of these hospital effluents had been measured in 2019 in the same watershed and reached 0.16–0.22 on TBX medium (Crettels et al. 2022). A MAR index >0.2 means that the high-risk source of contamination is where antibiotics are frequently used (Davis et Brown 2016). In hospital A, ESBL-EC represents up to 25.7% of the total *E. coli* flora. This proportion

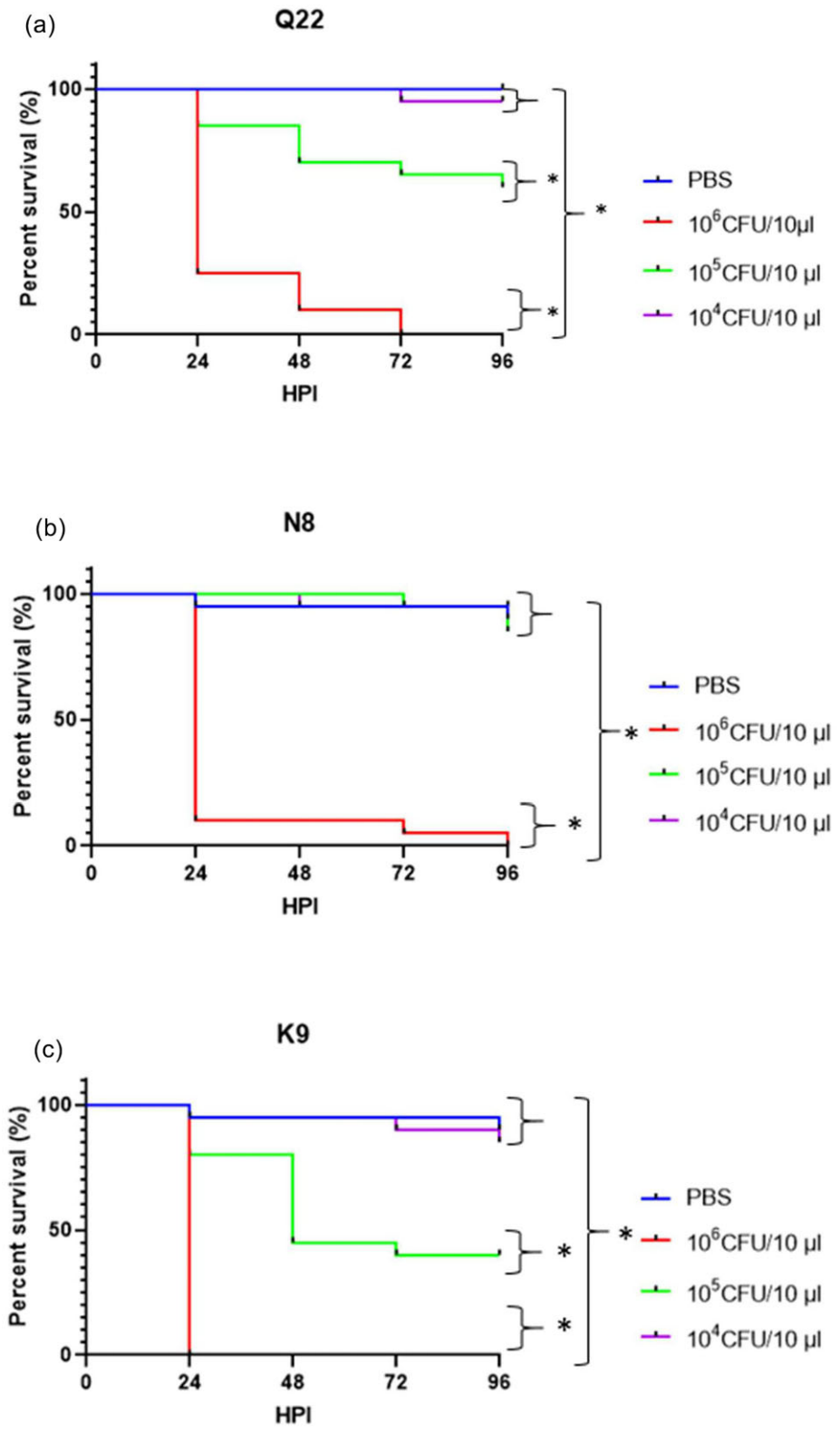

Figure 5. Kaplan–Meier survival curves of the experiments with *G. mellonella* larvae inoculated with *E. coli* Q22 (a), *E. coli* N8 (b), and *E. coli* K9 (c). Each group contained 20 larvae separated in two subgroups of 10 larvae. HPI: hours post-inoculation; *P*-value () < 0.05 (log-rank tests).

has a large variability from one sample to another, explained by the fact that it is a direct sampling in the pipes with a presume variability between samplings dependent, among others, on the number of hospital beds (635 beds for hospital A and 226 beds for hospital B).

The log removals calculated for the WWTPs are within the expected ranges of removal—i.e. log reduction of heterotrophic bacteria up to 3 log (Munir et al. 2011, Kwak et al. 2015) and ESBL-EC with removals of 1.5 log (Anses 2020). Despite this, ESBL-EC concentrations remain high at

the WWTP output and are discharged into the Ourthe River. This corroborates with different studies that have shown that WWTPs are not able to stop the release of ESBL-EC and MDR bacteria into receiving rivers (Reinthaler et al. 2010, Blaak et al. 2015, Lenart-Boroń et al. 2020).

PCR genotyping has shown that 474 of the 644 ESBL-EC and AmpC strains (73.6%) contain genes belonging to the CTX-M-1 group. No strain contained CTX-M-2 group's genes. Herrig et al. (2020), on the river Lahn in Germany, showed that $bla_{\text{CTX-M}}$ concentrations are largely explained by fecal pollution. In animal health, the prevalence of pathogenic and non-pathogenic ESBL-EC isolated from diarrhea and septicaemia in young calves over three calving seasons showed that out of 394 isolates studied, the majority contain CTX-M-1 (243, 61.7%), M-9 (74, 18.8%), and CTX-M-2 gene groups (64, 16.2%) (Guérin et al. 2022). In a nationwide survey in Belgian hospitals in 2006–2008, CTX-M-1, M-2, and M-9 gene groups were found in 79%, 12%, and 9% of the ESBL-EC isolates, respectively. DNA sequencing revealed that blaCTX-M-15 was the most prevalent CTX-M-encoding gene, representing 85% of all ESBLs within the CTX-M-1 group (Rodriguez-Villalobos et al. 2011).

A panel of 40 strains selected on the basis of a large number of antibiotic resistances were sequenced. In Ireland, WGS analysis revealed many different sequence types (ST) circulating in water and wastewater, including *E. coli* with ST131 ($n = 15$), ST38 ($n = 8$), and ST10 ($n = 4$) (Hooban et al. 2021). In New Zealand, four ESBL-EC strains from freshwater were sequenced, three strains were ST 131 (CTX-M-27 positive) and the other ST69 (CTX-M-15-positive) (Burgess et al. 2022). In this study, *E. coli* ST131 ($n = 5$) and ST38 ($n = 4$) were identified, but neither ST10 nor ST69. *Escherichia coli* ST131 has been recognized as one of the top contributors to urinary tract infections in humans (Kudinha et al. 2013). The intercontinental dissemination of this sequence type 131 has highly contributed to the worldwide emergence of fluoroquinolone-resistant and CTX-M-producing *E. coli* (Peirano et al. 2014). Recent surveillance studies have shown that its overall prevalence ranges from 12.5% to 30% of all *E. coli* clinical strains, from 70% to 80% of fluoroquinolone-resistant strains, and from 50% to 60% of ESBL-producing strains (Peirano et al. 2014).

β-lactamase genes identified were $bla_{\text{CTX-M}}$ ($n = 21$), with $bla_{\text{CTX-M-15}}$ the most represented ($n = 15$), as well as bla_{TEM} ($n = 11$), bla_{OXA} ($n = 7$), and bla_{SHV} ($n = 9$). Epidemiologically, CTX-M-15 is the most prevalent ESBL in *E. coli* clones worldwide in both community and hospital settings (Merida-Vieyra et al. 2016, Robin et al. 2017). CTX-M-15 is typically associated with IncF-type plasmids, a predominant group of large conjugative plasmids that carry and mobilize multiple resistance and virulence determinants (Mahérault et al. 2019). CTX-M-15 was the most prevalent ESBL in surface water in Europe, whereas CTX-M-1 was most prevalent in Italy and CTX-M-14 was most prevalent in Spain (Cho et al. 2023). While CTX-M-1, CTX-M-14, CTX-M-15, and CTX-M-27 were commonly detected in Europe, less frequently detected CTX-M variants included CTX-M-2, CTX-M-3, CTX-M-9, CTX-M-24, CTX-M-28, CTX-M-32, CTX-M-55, and CTX-M-79 (Cho et al. 2023). Other ESBLs, including TEM-3, TEM-52, SHV-2, and GES-1 were also detected, and especially SHV-12 widely distributed in surface water (Cho et al. 2023). In this study, many plasmids such as IncFIB, IncFII, and col156 were found in the strains. Baron et al. (2020) showed the genetic and plasmid diversity of *E. coli* AR isolated from French rivers, probably related to the various animal and human origins of the isolated bacteria. In this study, the $bla_{\text{CTX-M}}$ *genes* were often associated with ISEc9 (synonym: ISEcp1 or ISEcp1B) (Table 3). These insertion sequences (IS), which belong to the IS1380 family, are known to be associated with three of the five CTX-M groups (CTXM-1, -M-2, and -M-9 groups). They enable to mobilize the CTX-M genes by a transpositional mechanism in *E. coli* by recognizing a variety of DNA sequences as right inverted repeats (Poirel et al. 2005).

The pathogenicity of three ESBL-EC (Q22, N8, and K9 strains) was tested in the *G. mellonella* model like it was the case with Zhao et al. (2022), who tested the pathogenicity of ESBL-EC from ovine origin in this model. The Q22 strain belonging to serotype O25:H4 and ST131 was selected as it is a rapidly spreading lineage and an emerging problem in community and hospital infections (Peirano et Pitout 2010). It possesses numerous virulence factors (Table 3), including an adhesin (*iha*). In the *G. mellonella* larvae model, Q22 strain showed pathogenicity at 10^5 CFU/10 µl, confirming its pathogenicity. Strain N8 was chosen because it is one of the 14 strains of serotype O18:H7 ST 1463, and possesses the $bla_{KPC\text{-}3}$ gene encoding a carbapenemase. Its virulence factors are *fyuA*, *gad, irp2*, *terC*, *LpfA*, and *celB*. Strain N8 showed pathogenicity in the *G. mellonella* model from a concentration of 10^6 CFU/10 μl. The K9 strain, with serotypes O86:H2 and ST349, was also tested as it possesses numerous virulence factors, including the *lpfa* gene, which codes for long polar fimbriae important for adhesion of pathogenic strains, and the *agg* genes characteristic of enteroaggregative *E. coli*. This K9 strain is predicted as human pathogen with a probability of 92.6%, according to Pathogenfinder. K9 is pathogenic in the *G. mellonella* model from a concentration of 10^5 CFU/10 μl, like the Q22 strain, showing that these two strains have a higher pathogenicity than the N8 strain. A human origin can be hypothesized for strains Q22 and K9, both isolated from freshwater, in the Ourthe at Esneux (K9) and in the Ourthe downstream of the Angleur WWTP (Q22).

One of the most observed concerns was the large number of strains containing carbapenemase genes—$bla_{\text{KPC-3}}$ ($n = 19$), $bla_{\text{NDM-1}}$ ($n = 1$), $bla_{\text{VIM-1}}$ ($n = 2$), and $bla_{\text{OXA-244}}$ ($n = 2$)—even in freshwaters. In an American survey, the most commonly detected carbapenemase gene in wastewater was bla_{VIM} ($n = 36$), followed by bla_{KPC} ($n = 2$) (Hoelle et al. 2019).

SNP analysis showed that the 14 strains with serotype O18:H7 ST1463 appeared to originate from the same clone. These 14 strains belong to the B1 Clermont phylotype. The current classification of *E. coli* extends to eight phylotypes (A, B1, B2, C, D, E, F, and G) (Clermont et al. 2013). These phylotypes are associated with certain hosts, specific environments, or lifestyles. For example, strains from phylotypes B2 and D are involved in extra-intestinal infections (Picard et al. 1999), while water-adapted strains belong to phylogroup B1 (Berthe et al. 2013). The majority of the strains that contained $bla_{KPC\text{-}3}$ had this gene located on transposon Tn4401, which is a Tn3-based transposon known to be involved in the mobilization of the bla_{KPC} gene (Cuzon et al. 2011). The $bla_{KPC\text{-}3}$ genes of 2 strains (V7 and V12) were located on an IncX3 plasmid known to carry carbapenemase genes in carbapenemase-producing *Enterobacteriaceae* (CPE) worldwide (Mouftah et al. 2019).

One of these strains (strain O5) was found at the output of the Tilff's WWTP demonstrating that hospitals may

represent a source of environmental contamination as suggested by Cahill et al. (2019) but none of them was found in freshwaters.

Each of these 14 strains showed resistance to ertapenem and susceptibility under conditions of high-dose exposure to meropenem (intermediate). Extra-intestinal pathogenic *E. coli* (ExPEC) strains of serotype O18:K1:H7 are primarily responsible for neonatal meningitis and sepsis in humans, and this serotype is also frequently isolated from extra-intestinal lesions of colibacillosis in poultry (Moulin-Schouleur et al. 2007). However, the O18:H7 strains in this study do not possess the *Neu* gene encoding the K1 capsule. Despite this, Kaplan–Meier survival curves for serotype O18:H7 strain N8 injected in *G. mellonella* larvae showed mortality from 10^6 CFU/10 µl with a larvae mortality of 80% after 24 HPI. This result could be correlated to the ones obtained by Antoine et al. (2021) with avian pathogenic *E. coli* O18:K1 (APEC), which presented a similar larvae mortality (70%–90%) after 24 HPI with the same concentrations (10^6 CFU/10 µl) of APEC. In addition, the N8 strain has virulent properties in comparison with a *E. coli* K12-DH5α strain selected on its avirulent background and which showed a larvae mortality < 10% for a range of concentrations between 10^3 and 10^6 CFU/10 µl (Habets et al. 2022).

The bla_{KPC-3} gene is generally associated with ST1463 and thus with serotype O18:H7, in contrast to other studies where it is generally associated with ST167 or even ST3948 (Peirano et al. 2014, Mani et al. 2017). KPC-producing *E. coli* strains, although distributed worldwide, are rare and only described in countries where the prevalence of this carbapenemase in *K. pneumoniae* is high, Italy is characterized by the highest rates in Europe (Peirano et al. 2014). Carbapenemase-producing bacteria represent a global health problem because of their multidrug resistance and limited therapy options (Bonomo et al. 2018).

This study shows that ESBL-EC are widespread in the region of Belgium studied and found at all sampling points. Sampling along the hospital-WWTP-freshwater continuum supposed that hospital effluents represent one of the sources of AR bacteria in the aquatic environment. More worryingly, some of the ESBL-EC isolated were also resistant to carbapenems and possessed genes for carbapenemase production, which represents a threat to public health. Further studies are now needed to determine the impact of each sector and the risk associated to the environmental dissemination of AR bacteria by the aquatic environment.

Conflict of interest: There is no conflict of interest to declare.

Funding

The authors thank the ISSEP for the funding of this study ("EBLSE" project).

Author contributions

Leslie Crettels (Conceptualization, Methodology, Project administration, Writing – original draft), Nadine Burlion (Supervision), Audrey Habets (Formal analysis, Writing – review & editing), Bernard Taminiau (Formal analysis, Writing – review & editing), Georges Daube (Writing – review & editing), Elisa Delrée (Formal analysis), Anne-Françoise Mouchette (Formal analysis), and Damien Thiry (Supervision, Writing – review & editing)

Data availability

The data underlying this article are available in the article.

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Received 16 November 2023; **revised** 30 May 2024; **accepted** 20 June 2024