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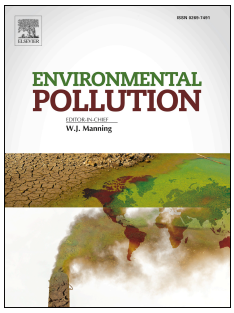

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Credit Author Statement

Roger Stephan: Conceptualization, Supervision; Reviewing. **Marc J.A. Stevens:** Data curation; Software; Visualization; Writing – review & editing. **Magdalena Nüesch-Inderbinen:** Formal analysis; Supervision; Writing – original draft; **Stephanie Bleichenbacher:** Investigation; Methodology; Visualization. **Katrin Zurfluh:** Methodology; Project administration; **Vincent Perreten:** Project administration; Resources; Writing – review & editing. **Andrea Endimiani:** Project administration; Resources; Writing – review & editing.

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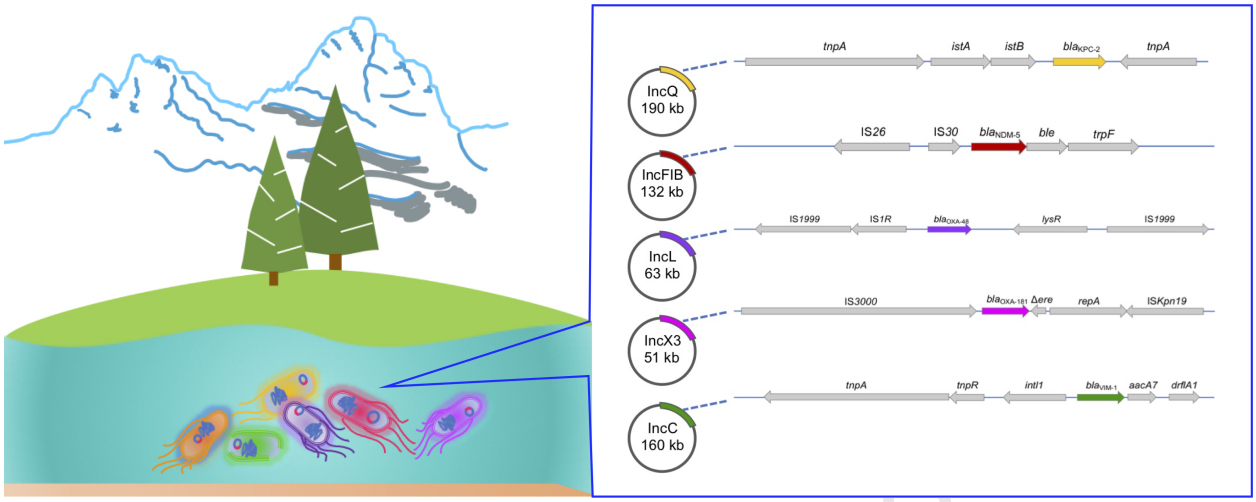

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Environmental dissemination of carbapenemase-producing Enterobacteriaceae in rivers in Switzerland

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Abstract

The aquatic environment takes on a key role in the dissemination of antimicrobial-resistant Enterobacteriaceae. This study assesses the occurrence of carbapenemase-producing Enterobacteriaceae (CPE) in freshwater samples from rivers, inland canals, and streams throughout Switzerland, and characterizes the isolated strains using phenotypic and NGS-based genotypic methods. CPE producing KPC-2 (n=2), KPC-3 (n=1), NDM-5 (n=3), OXA-48 (n=3), OXA-181 (n=6), and VIM-1 (n=2) were detected in 17/164 of the water samples. Seven *Escherichia coli* had sequence types (STs) that belonged to extra-intestinal pathogenic clonal lineages ST38, ST73, ST167, ST410, and ST648. The majority (16/17) of the carbapenemase genes were located on plasmids, including the widespread IncC (n=1), IncFIIA (n=1), and IncFIIB plasmids (n=4), the epidemic IncL (n=1) and IncX3 (n=5) plasmids, a rare Col156 plasmid (n=1), and the mosaic IncFIB, IncR, and IncQ plasmids (n=3). Plasmids were composed of elements that were identical to those of resistance plasmids retrieved from clinical and veterinary isolates locally and worldwide. Our data show environmental dissemination of high-risk CPE clones in Switzerland. Epidemic and mosaic-like plasmids carrying clinically relevant carbapenemase genes are replicating and evolving pollutants of river ecosystems, representing a threat to public health and environmental integrity.

Main finding

Carbapenem-resistant Enterobacteriaceae and their genetic mechanisms of resistance are replicating and evolving pollutants of river ecosystems, representing a threat to public health.

Keywords: carbapenems; antibiotic resistance; plasmids; aquatic environment; pollution

Introduction

Beta-lactam antibiotics including penicillins, cephalosporins, monobactams, and carbapenems are the most frequently consumed antibiotics worldwide (WHO, 2018). Carbapenem antibiotics, for example ertapenem, imipenem, and meropenem are classified by the World Health Organization WHO as critically important for human health and are currently considered last resort antimicrobials to treat severe infections by multidrug resistant (MDR) Gram-negative nosocomial pathogens (WHO, 2017, van Duin and Doi, 2017). Carbapenem resistance therefore represents a significant public health concern of global dimensions. One of the most significant mechanisms of carbapenem resistance among Enterobacteriaceae involves the synthesis of carbapenemases, enzymes that inactivate carbapenems and other β-lactam antibiotics (Queenan and Bush, 2007). Since the first isolation of carbapenemase producing Enterobacteriaceae (CPE) harboring the bla_{KPC} gene in 1996, (Yigit et al., 2001) clinical CPE carrying chromosomal or plasmid-mediated carbapenemase-genes such as bla_{NDM}, $bla_{\mathrm{OXA\text{-}48}}$, bla_{VIM} and bla_{IMP} have been found worldwide (Kopotsa et al., 2019, Nordmann et al., 2011). In recent years, clinically relevant CPE have been detected in non-human sources including companion and food-producing animals, the food chain, wildlife, and the environment, giving rise to health and ecological issues at the human-animal-environmental interface (Mills and Lee, 2019). Addressing these issues necessitates a holistic and multidisciplinary approach known as the One Health Concept (Hernando-Amado et al., 2019). Of the One Health antibiotic resistance triad, the environment is the most dynamic, but also the least understood sector (Essack, 2018). Within this sector, the aquatic environment is of particular importance because it represents a most basic resource, and the role that it plays in the spread of antimicrobial resistance (AMR) is critical, with the genetic context of the AMR genes remaining largely unexplored (Kraemer

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et al., 2019, Mills and Lee, 2019, Furness et al., 2017, Williams et al., 2016, Alonso et al., 2001).

CPE producing clinically relevant carbapenemases including KPC, NDM, IMP, OXA-48-like, and VIM have been reported in European rivers since 2010 (Poirel et al., 2012), mostly rivers associated with effluent such as hospital or urban wastewater (Falgenhauer et al., 2019, Jelić et al., 2019, Lepuschitz et al., 2019, Khan et al., 2018, Mahon et al., 2017, Zurfluh et al., 2017). Furthermore, CPE in wastewater and in surface water may include intestinal pathogenic *E. coli* and extra-intestinal *E. coli* (ExPEC), which give rise to diseases in humans and animals by virtue of specific virulence factors (Mahon et al., 2017; Zurfluh et al., 2017; Kaper et al., 2004). Virulence traits include adhesins, capsular antigens, siderophores, and toxins that enable pathogenic *E. coli* to avoid host defense systems, colonize host surfaces and invade host tissues (Kaper et al., 2004). While anthropogenic influences are well recognized as major contributors of CPE to waterways, the possible pathways of transmission of CPE between humans, animals including wildlife, and the freshwater ecosystem are not well documented and potential human and animal health impacts caused by exposure to environmental CPE remain unclear (ECDC, 2019, Mills and Lee, 2019).

The aquatic environment provides ideal settings for carbapenemase harboring mobile genetic elements (MGEs) including plasmids, insertion sequences, and transposons, to be retained and to disseminate via horizontal gene transfer (Gillings et al., 2018, Marti et al., 2014, Pruden, 2014). Such MGEs contribute to what is becoming increasingly recognized as xenogenetic pollution of the aquatic ecosystem, with potentially adverse impact on human welfare and environmental integrity (Gillings et al., 2018).

This study was designed to evaluate the occurrence of CPE in different water bodies throughout Switzerland and to characterize the isolated strains using phenotypic and genotypic methods, including whole genome analyses. We also aimed to identify any genetic

relatedness of CPE present in the aquatic environment to CPE associated with documented human and animal infections in order to assess their relevance to public and environmental health. Particular emphasis was placed on identifying antimicrobial resistance genes (ARGs) and MGEs.

Material and Methods

Sampling. Between May and August 2019, a total of 164 surface water samples were taken from different water bodies including rivers (n=113), streams (n=42) and inland canals (n=9) located between 300 and 3000 m above sea level (Table S1). Water was collected from each site in sterile 500 mL containers and transferred to the laboratory in a cool box.

Microbiological analysis. CPE were isolated from the water samples using selective media as previously described (Zurfluh et al., 2013). For more details see Supplementary Material. Isolates were subjected to antimicrobial susceptibility testing (AST) according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (CLSI., 2018), as detailed in Supplementary Material. For each isolate the minimal inhibitory concentration (MIC) of the carbapenem antibiotics ertapenem, imipenem, and meropenem were determined, and each isolate was tested against a panel of further 16 antimicrobials using the disk diffusion method as described in Supplementary Material.

Isolates displaying resistance to three or more classes of antimicrobials (counting β-lactams as one class) were defined as multidrug-resistant (MDR) (Magiorakos et al., 2012).

Whole genome sequencing (WGS) and analysis of genomic content. Prior to WGS, each isolate was tested for carbapenemase production using the β-CARBA™ colorimetric test (Bio-Rad, Cressier, Switzerland). Isolates were screened by PCR for the presence of bla_{KPC},

bla$_{NDM}$, *bla*$_{OXA-48-like}$, or *bla*$_{VIM}$ genes, as described previously (Poirel et al., 2011, Ellington et al., 2007), and as outlined in Supplementary Material.

Genomic sequences were obtained using both Illumina MiniSeq (Illumina, San Diego, CA, USA) and MinION sequencer on a R9.4 Spot On flow cell (Oxford Nanopore Technologies, Oxford, United Kingdom). For specifics see Supplementary Material.

Reads were assembled as described previously (Stevens et al., 2019), and as further described in Supplementary Material. In silico analyses were carried out as detailed in Supplementary Material, to determine *Escherichia coli* core genome multilocus sequence types (cgMLST) and serotypes, as described by (Wirth et al., 2006) and by (Joensen et al., 2015), respectively. Core genome alignments were performed as described earlier (Treangen et al., 2014) to detect related strains available in public genome databases. All genomes were further screened in silico in order to identify virulence markers, antimicrobial resistance genes and plasmids as described previously (Xie et al., 2018, Jia et al., 2017, Carattoli et al., 2014, McArthur et al., 2013), using databases detailed in Supplementary Material.

Plasmid sequences were compared to reference sequences using the bacterial plasmid database PLSDB, available at https://ccb-microbe.cs.uni-saarland.de/plsdb/ (Galata et al., 2019), as outlined in Supplementary Material.

Geographical map. Geospatial visualization was performed by plotting GPS coordinates of the sampling sites onto a geographical map using the open source geographic information system (GIS) software QGIS (https://qgis.org).

Accession numbers. Genome assemblies and sequence reads are deposited at Sequence Read Archive (SRA) and GenBank hosted by the NCBI database under the BioProject ID PRJNA604100.

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Results

Occurrence of CPE in Swiss water bodies. CPE were detected in 17 (10%) of the 164 water samples, including 13 (12%) of the 113 river water samples, three (33%) of the 9 samples taken from inland canals, and one (2%) of the water samples from a stream (Figure 1). CPE were not detected in water bodies more than 1000 m above sea level (Figure S1/Table S1). Overall, the 17 CPE included 12 *E. coli*, one *Citrobacter freundii*, one *Enterobacter kobe*i, one *Klebsiella aerogenes*, one *Klebsiella variicola* and one *Raoultella ornithinolytica* strain, respectively (Figure 2).

The results of the initial PCR screening, combined with the results of WGS, identified the carbapenemase genes $bla_{\text{KPC-2}}$, $bla_{\text{KPC-3}}$ $bla_{\text{NDM-5}}$, $bla_{\text{OXA-48}}$, $bla_{\text{OXA-181}}$, and $bla_{\text{VIM-1}}$ (Figure 2).

Antimicrobial resistance phenotypes and genotypes of the CPE. Application of the β-CARBA™ test indicated the presence of carbapenem-hydrolyzing enzymes in all 17 isolates. Phenotypic AST revealed that eight strains exhibited MIC values above the carbapenem susceptibility breakpoints, with ertapenem non-susceptibility defined as an MIC of ≥1 mg/L, and meropenem and imipenem non-susceptibility defined as an MIC of ≥2 mg/L (CLSI., 2018) (Figure 2/Table S1). Resistance to other β-lactam antibiotics was common, with 14 (82%) and 10 (59%) of the strains exhibiting resistance to the 3rd and 4th generation cephalosporins cefotaxime and cefepime, respectively. Moreover, 15 (88%) of the strains were MDR (Figure 2).

Phylogenetic analysis and virulence gene profiles of the carbapenemase producing *E. coli*. To determine a possible clinical relevance of CPE cultured from the aquatic environment, the 12 *E. coli* strains were subjected to detailed analysis. cgMLST classified the strains according to nine different *E. coli* STs. ST167, ST410 and ST940 accounted for two

strains each (2/12), respectively, while ST38, ST73, ST205, ST648, ST656, and ST1284 occurred in one strain each (Figure 2).

E. coli ST38 (strain CF065) is an international AMR high risk clone responsible for the spread of OXA-48 producing *E. coli* (Pitout et al., 2019). Notably, in contrast to the other strains described in this study, CF065 lacked plasmid elements but contained a chromosomally located $bla_{\text{OXA-48}}$ in a genetic environment consisting of a ΔTn1999.2-like structure as described in *E. coli* ST38 clones from the UK (data not shown) (Turton et al., 2016, Pitout et al., 2019).

E. coli ST410 strains (strain CF038 and CF124, respectively) were assigned in silico to serotype O8:H9, suggesting that these strains belong to clade C which is an *E. coli* ST410 clade associated with humans, companion animals, and farm environments (Falgenhauer et al., 2016). Comparison showed that NDM-5 producing CF038 was very closely related with only 51 chromosomal SNPs to NDM-5 producing *E. coli* ST410 (strain ECS9) isolated from a patient with bloodstream infection in China in 2017 (Huang et al., 2019) (GenBank accession no. VBQE00000000) (Figure S2).

Moreover, there was genetic similarity (<100 different alleles in cgMLST) of NDM-5 producing *E. coli* ST410 (strain CF038) and ST167 (strains CF163 and CF164, respectively), and of OXA-181 producing *E. coli* ST1294 (strain CF032), to isolates associated with documented cases of human infection in Canada and India (Mataseje et al., 2018) (BioProject ID PRJNA390933) (Figure S3). Further, cgMLST comparison showed that the two OXA-181 producing *E. coli* ST940 (strains CF061 and CF064, respectively), although retrieved from geographically distinct sites, were clonal with identical cgMLST patterns (Figure S2 and Figure S3). By contrast, strains CF163 and CF164 were not genetically related to an NDM-5 producing *E. coli* ST167 clone that infected dogs and colonized veterinary employees at a Swiss veterinary clinic in 2018 (data not shown) (Endimiani et al., 2020). Further, OXA-181

producing *E. coli* ST410 (strain CF124) had no phylogenetic link to a clone that caused an outbreak involving companion animals at one Swiss veterinary hospital in 2018 (data not shown) (Nigg et al., 2019). An overview of the phylogenetic relatedness of *E. coli* strains from this study is shown in Figure S2.

At least one virulence gene associated with pathogenicity in intestinal and extra-intestinal *E. coli* (ExPEC) diseases was detected in 11 of the 12 carbapenemase-producing *E. coli* (Table 1 and Figure 2). Seventeen different virulence factors were identified, whereby the most frequent ones were *gad* (glutamate decarboxylase gene involved in gastric acid resistance), *lpfA*, (long polar fimbriae gene associated with the colonization of the intestine), and *capU*, (hexosyltransferase homolog gene associated with adhesion), which were identified in seven, six, and five isolates, respectively (Table 1).

Plasmid analysis. To investigate the host range, epidemiology, and possible relatedness, the carbapenemase-encoding plasmids were fully sequenced and compared to already published clinically relevant plasmids. Overall, 16 plasmids ranging in size from 7.8 kb to 244 kb were analyzed (Table 2). With the exception of two plasmids carrying $bla_{\text{OXA-48}}$ genes, all plasmids harbored at least one additional ARG (Table 2). Seven plasmids contained genes for type II toxin/antitoxins (T/As), which are genetic systems that play a role in plasmid maintenance and the dissemination of multidrug resistance in Gram-negative bacteria (Yang and Walsh, 2017) (Table 2). For further analysis, plasmids were categorized as KPC, NDM, OXA, or VIM-encoding plasmids, respectively (Table 2).

KPC-encoding plasmids. The three bla_{KPC} genes identified in *C. freundii*, *E. kobei*, and *K. variicola* were located on plasmids p062_B-KPC-2 determined to be IncQ1, p070_A-KPC-2 which was typed IncFIB_{K}, and p118_A-KPC-3 which belonged to IncFIIB, respectively (Table 2). On all three plasmids, the bla_{KPC} genes were located within the Tn*3*-like

transposon Tn*4401a*, which is the most common isoform of Tn*4401*, a genetic structure that typically surrounds *bla*$_{KPC}$ genes (Naas et al., 2012).

A sequence analysis of p062_B-KPC-2 presented a hybrid structure consisting of a 170 kb backbone which had a high degree of similarity with plasmid p1643_10 (GenBank accession no. KF056330) from the epidemic *Salmonella enterica* serovar Kentucky ST198 strain 1643/2010 isolated from a turkey in Poland in 2010 (Wasyl et al., 2015). Plasmid p062_B-KPC-2 further contained a 20 kb region carrying *bla*$_{KPC-2}$ identical to plasmid pKP1504-kpc (GenBank accession no. KF874496), which was purified from *K. pneumoniae* ST258 strain GR-1504 during the early phases of a hospital epidemic in Greece in 2008 (Papagiannitsis et al., 2016a, Giakkoupi et al., 2009) (Figure 3). The same *bla*$_{KPC-2}$ carrying structure was identified in p079_A-KPC-2, however, in p070_A-KPC-2, the region identical to plasmid pKP1504-kpc covered an ~35 kb region (data not shown). Plasmid p070_A-KPC-2 showed no further sequence homology to plasmids available in PLSDB.

Plasmid p118_A -KPC-3 was a mosaic plasmid that shared a common region (99% identity over a length of 120834 bp) with an unnamed 244 kb IncFIB$_K$ plasmid from *K. pneumoniae* ST323 strain KSB1_4E isolated from a rectal swab of a hospitalized patient in Australia in 2013 (Gorrie et al., 2018) (GenBank accession no. CP024500.1). Plasmid p118_A -KPC-3 further shared a 12 kb region carrying the *bla*$_{KPC-3}$ gene which was identical to an unnamed plasmid from *K. pneumoniae* strain AR438 registered in the culture collection of the Food and Drug Administration/ Centers for Disease Control and Prevention (FDA/CDC) Antimicrobial Resistant Isolate Bank, Atlanta, USA (GenBank accession no. NZ_CP029102.1) (Figure 3).

NDM-encoding plasmids. The three *bla*$_{NDM-5}$ genes from *E. coli* were located on 87 kb IncFIA, on a 132 kb IncFIB, and on a 10 kb pKPC-CAV1193-like plasmid, which was nontypeable by incompatibility group (Sheppard et al., 2016, Mathers et al., 2015).

Plasmid p038_A-NDM-5 shared 99.9% identity with pAMA1167-NDM-5, a multidrug resistance plasmid from a human clinical *E. coli* ST410 isolate from Denmark (Overballe-Petersen et al., 2018) (GenBank accession no. CP024805.1) (Figure 4). Furthermore, p164_A-NDM-5 and p163_C-NDM-5, both identified in *E.coli* ST167 in this study, were determined to be highly similar at the nucleotide level (99-100%), to plasmids pM309-NDM5 (Figure 4), and pM217_FII (data not shown), respectively. Both plasmids were detected in nosocomial *E. coli* ST167 strains from a hematology ward in Myanmar during 2015-2016 (Sugawara et al., 2019) (GenBank accession nos. AP018833.1 and AP018147.1, respectively). By contrast, the three NDM-5 plasmids from this study were not similar to previously reported NDM-5 plasmids from dogs and veterinary employees of a Swiss veterinary hospital (data not shown) (Endimiani et al., 2020, Peterhans et al., 2018).

OXA-48-encoding plasmids. Of the two plasmid-mediated $bla_{\text{OXA-48}}$ genes detected in *R. ornithinolytica* and *E. coli* ST205, the former was identified on a 63 kb IncL plasmid (p023_D-OXA-48) that shared >99% identity with an IncL plasmid p704SK10_2 identified in an *E. cloacae* isolated from wastewater in 2015 in Switzerland (Marti et al., 2017) (GenBank accession no. CP022150). Plasmid p023_D-OXA-48 was also highly identical to pEC745 identified in *E. coli* ST131 from Morocco (Stoesser et al., 2016) (GenBank accession no. CP015075.1), and to plasmid pOXA-48_4963 which was associated with a nosocomial outbreak of *K. pneumoniae* in 2015 in the Czech Republic (Skalova et al., 2017) (GenBank accession no. KX523900) (Figure 5). As is typical for IncL plasmids harboring $bla_{\text{OXA-48}}$, the $bla_{\text{OXA-48}}$ gene in p023_D-OXA-48 was located within the composite transposon Tn*1999.2* which is a Tn*1999* variant with an IS*1R* insertion upstream of $bla_{\text{OXA-48}}$ (Pitout et al., 2019).

In the second plasmid, the $bla_{\text{OXA-48}}$ gene was located on a 7.8kb Col156 plasmid (p053_E-OXA-48), that shared 99.8% nucleotide identity with pMTY17816_OXA48 identified in a

human *K. pneumoniae* isolate from a patient from Vietnam in 2017 (Honda et al., 2019) (GenBank accession no. AP019554.1) (Figure 5). The $bla_{\text{OXA-48}}$ gene was flanked by two copies of inverted insertion sequence IS*1R*, corresponding to the transposon variant Tn*1999.3*, which was described for the first time in an pOXA-48-like IncL plasmid in a clinical *E. coli* strain from Italy (Giani et al., 2012a). In p053_E-OXA-48 however, the two copies of IS*1999* and the *lysR* gene which are present in Tn*1999.3*, were missing (Figure S4).

OXA-181-encoding plasmids. The most prevalent carbapenemase gene was $bla_{\text{OXA-181}}$ which was located in five of six instances on 51 kb IncX3 plasmids (Table 2). All five were >99.9% identical to IncX3 plasmids from a human *K. pneumoniae* isolate from the Czech Republic (pOXA-181_29144) (Skalova et al., 2017), canine and human *E. coli* ST410 strains from a Swiss veterinary clinic (pAN-OXA-181) (Endimiani et al., 2020, Nigg et al., 2019), and *K. variicola* isolated from fresh vegetables imported from Asia to Switzerland (pKS22-OXA-181) (Zurfluh et al., 2015b) (Figure 6).

The remaining 155 kb plasmid, p142_A-OXA-181, was typed Inc FIB and had regions in common to an unnamed 136.4 kb plasmid from a human *E. coli* isolate from Australia (GenBank accession no. LR130556.1). Plasmid p142_A-OXA-181 also shared an ~15 kb region that contained the $bla_{\text{OXA-188}}$ gene with plasmid pABC260-OXA-181 from *K. pneumoniae* strain ABC260 isolated from a rectal swab in the United Arab Emirates (UAE) in 2014 (Mouftah et al., 2019) (GenBank accession no. MK412915.1) (Figure 6).

VIM-encoding plasmids. The $bla_{\text{VIM-1}}$ genes in this study were located on a 160 kb IncC plasmid (p009_A-VIM-1), and on an 89 kb IncR/IncY plasmid, respectively (Table 2). Plasmid p009_A-VIM-1 showed 99.9% nucleotide identity to pKP-Gr642, a $bla_{\text{VIM-19}}$-containing plasmid from a *K. pneumoniae* isolate recovered in 2011 from a patient hospitalized in Greece (GenBank accession no. KR559888.1) (Papagiannitsis et al., 2016b). The $bla_{\text{VIM-1}}$ gene was present on the In*416*-like integron In*4863*, comprising a bla_{VIM}–

aacA7–dfrA1– Δ*aadA*1–*smr2* cassette, as in pKP-Gr642 (Papagiannitsis et al., 2016b). Further, the presence of a bla_{CMY-4} carrying region consisting of bla_{CMY-4}–*blc–sugE–*Δ*ecnR* indicated that p009_A-VIM-1 belongs to a unique phylogenetic lineage of IncC plasmids that evolved from an ancestral pUMNK88_161-like plasmid that has spread among food-producing animals worldwide (Fernández-Alarcón et al., 2011).

Plasmid p035_A-VIM-1 was a mosaic plasmid that shared a common region over a length of ~22 kb with pENT-576 (GenBank accession no. NZ_CP008898) from a clinical *Enterobacter hormaechei* subsp. *hoffmannii* ECNIH3 isolated in 2011 in the USA (Conlan et al., 2014). A 15 kb region carrying bla_{VIM-1} was identical to a resistance region located on plasmid pMOS94 (GenBank accession no. MK671725.1) identified in clinical *Pseudomonas mosseli* isolate AM/94 in Italy in 1994 (Di Pilato et al., 2019) (Figure 7). As described for pMOS94, the bla_{VIM-1} gene was present on a bla_{VIM}–*aacA4–aphA15–aadA15* cassette as part of an In*70* integron (Di Pilato et al., 2019). Interestingly, *P. mosseli* AM/94 represents the earliest known VIM-1-producing strain and, as an opportunistic pathogen, is thought to have introduced bla_{VIM-1} from its natural soil reservoir into the clinical setting (Giani et al., 2012b). Finally, ten of the CPE isolates from this study contained one or more additional plasmids harboring genes conferring resistance to aminoglycosides, extended-spectrum beta-lactams, fluoroquinolones and macrolides (Table S2).

Discussion

Currently listed by the WHO as critical-priority bacteria (Tacconelli et al., 2018), CPE have spread globally within hospital and community settings, sewage environments and other environmental matrices (Mills and Lee, 2019). In this nationwide study, we detected CPE in surface water bodies in Switzerland, including rivers, inland canals and streams predominantly localized within urbanized areas, and none at high altitudes.

Among the isolates, several internationally disseminated clonal lineages harboring clinically relevant carbapenemase genes were identified. For example, *E. coli* ST38 is an international AMR ExPEC clone responsible for the spread of OXA-48 (Pitout et al., 2019). This particular clone has previously been identified among healthy carriers in Switzerland (Zurfluh et al., 2015a). Typically for *E. coli* ST38, strain CF065 chromosomally carried $bla_{\text{OXA-48}}$, one notable feature distinguishing it from the other strains in this study.

Further, *E. coli* ST410, detected in two water samples in this study, is an international high-risk ExPEC clone associated with MDR human and companion animal infections (Brilhante et al., 2020, Endimiani et al., 2020, Nigg et al., 2019, Roer et al., 2018, Timofte et al., 2016). Comparative genome analyses allowed us to disclose a epidemiologic link between a clinical NDM-5 producing *E. coli* ST410 strain from China (Huang et al., 2019), and a non-clinical strain isolated from surface water in Switzerland.

Likewise, *E. coli* ST167 is increasingly recognized as an MDR epidemic clone of significant public-health concern, predominantly in China (Zhu et al., 2016). *E. coli* ST167 harboring NDM-5 has been found previously among canine *E. coli* isolates, in fecal swabs of healthy humans employed at a veterinary clinic, and in wastewater in Switzerland (Endimiani et al., 2020, Peterhans et al., 2018, Zurfluh et al., 2017). Comparison of WGS data revealed genetic similarity of clinical NDM-5 producing *E. coli* ST167 from Canada and India with the isolates described in this study, providing further evidence for international dissemination of this particular NDM-5 producing ExPEC clone.

Other potentially pathogenic STs included *E. coli* ST73 which is a uropathogenic *E. coli* (UPEC) lineage associated with community acquired urinary tract infections (UTIs) (Gibreel et al., 2012), and *E. coli* ST648 which belongs to an emerging MDR, high-risk clonal lineage occurring frequently in various sources including wild bird populations, water fowl, companion animals, and humans (Schaufler et al., 2019, Hornsey et al., 2011). The

occurrence in surface water highlights the potential of these pathogenic lineages to be further disseminated into nature via watering systems affecting agriculture and food-producing animals, as well as to spread carbapenem resistance.

Virulence gene profiling revealed that the majority of the strains harbored genes associated with colonization of the host gut and pathogenicity in intestinal and extra-intestinal diseases, further underlining the virulence potential of the environmental CPE strains from this study. Taken together, these findings indicate a possible contribution of the aquatic environment to antibiotic-resistant infectious diseases in humans.

Plasmids are crucial for the horizontal spread of antimicrobial resistance genes (Carattoli, 2013). Tracking MGEs, especially plasmids, is an integral component required for a better understanding of the dissemination of clinically relevant carbapenemases. Comparative sequence analysis identified several plasmids that are considered epidemic plasmids, having been detected in other bacterial organisms, from locations worldwide, and from human and animal sources (Pitout et al., 2019, Carattoli, 2009). The IncL plasmid p023_D-OXA-48, and the five IncX3 plasmids carrying $bla_{\text{OXA-181}}$ described in this study confirm that these types of plasmids are major vehicles for dissemination of OXA-48-like carbapenemases and have become widespread in the ecosystem. The combination of the IncX3 plasmid and the *E. coli* ST410 clone, both acknowledged to possess epidemic potential (Endimiani et al., 2020, Pitout et al., 2019, Roer et al., 2018), is an especially worrisome finding in the aquatic environment.

Likewise, IncF plasmids spread bla_{KPC} and bla_{NDM} among Enterobacteriaceae (Kopotsa et al., 2019), and IncC plasmids like plasmid p009_A-VIM-1 from this study, have been described as vehicles for $bla_{\text{VIM-1}}$ with broad host range and interspecies, interclonal and international distribution (Matsumura et al., 2018). The association of an IncC plasmid harboring VIM-1

and UPEC ST73 in surface waters is of concern, since it may pose a direct risk to public health.

By contrast, Col156-type plasmids harboring *bla*$_{\text{OXA-48}}$ have only been reported in clinical isolates from Vietnam (Honda et al., 2019). Plasmids such as the Col156 plasmid p053_E-OXA-48 detected in this study therefore provide interesting epidemiological links to temporally and geographically segregated areas. To our knowledge, environmental *E. coli* harboring *bla*$_{\text{OXA-48}}$ on a Col156-type small plasmid has not been reported so far. Although rare, such plasmids may the source of resistance determinants for other epidemic plasmids.

In the set of plasmids analyzed in this study, three plasmids appeared to be composed of elements from various and distinct sources. Mosaic plasmids like p062_B-KPC-2, p118_A-KPC-3, and p035_A-VIM-1 may provide evidence for the possible rearrangement and evolution of plasmids in the aquatic environment. Although not uncommon, the impact of mosaic plasmids on public health is difficult to estimate (Pesesky et al., 2019). In these, as in many of the plasmids described in this study, the presence of toxin-antitoxin modules is likely to contribute to the maintenance of the plasmid within the strains and to the spread of carbapenem resistance genes in the environment.

This study has several limitations. First, due to low *in vitro* hydrolytic activity of many carbapenemases, the detection of CPE remains difficult (Bernabeu et al., 2017), thus, an underestimation of CPE cannot be excluded. Second, in this study, we did not perform conjugation experiments to establish transmissibility of the plasmids. Although the majority of the plasmids we analyzed shared >99% identity with known transmissible plasmids, further studies to assess the conjugal dynamics of all the plasmids described in this study are warranted. Third, the study was conceptualized as an observational study; periodic sampling at the same sites, and at additional locations would provide further information on the dynamics of dissemination of CPE and their resistomes. Given the severity of the risk of

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failing antimicrobial efficacy worldwide, future studies providing such data are urgently needed.

Conclusions

Our data point to the fact that many environmental CPE may represent anthropogenic contaminants of surface waters in Switzerland. The similarity of environmental and clinical isolates demonstrates their geospatial and temporal persistence locally and worldwide. This study demonstrates that clinically relevant carbapenemase genes are pollutants of river ecosystems and represent a significant challenge to public health and to technologies to minimize the entry into the water environment.

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CRediT authorship contribution statement

Roger Stephan: Conceptualization, Supervision; Reviewing. **Marc J.A. Stevens:** Data curation; Software; Visualization; Writing – review & editing. **Magdalena Nüesch-Inderbinen:** Formal analysis; Supervision; Writing – original draft; **Stephanie Bleichenbacher:** Investigation; Methodology; Visualization. **Katrin Zurfluh:** Methodology; Project administration; **Vincent Perreten:** Project administration; Resources; Writing – review & editing. **Andrea Endimiani:** Project administration; Resources; Writing – review & editing.

Declaration of Competing interests

The authors declare no conflict of interest.

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Figures and Tables

Figures 1-7

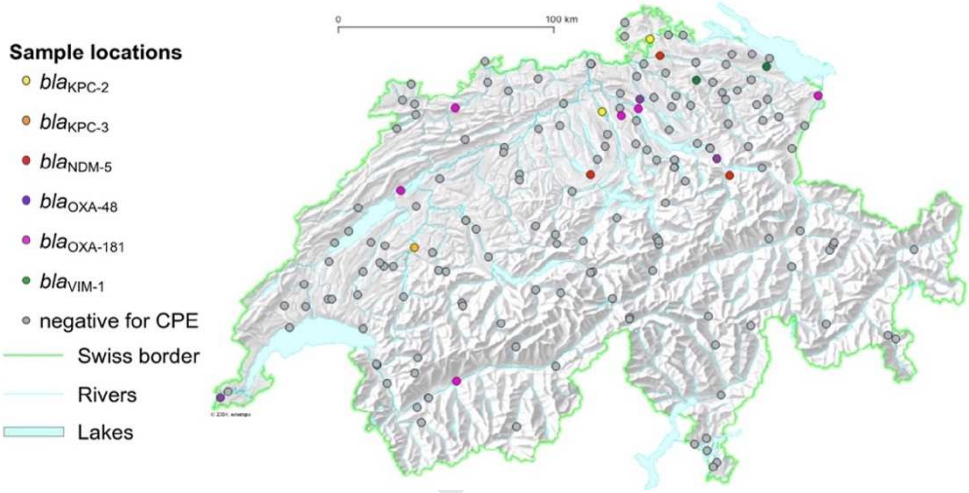

Figure 1. Map of Switzerland showing bodies of water, sample locations, and carbapenemase gene status.

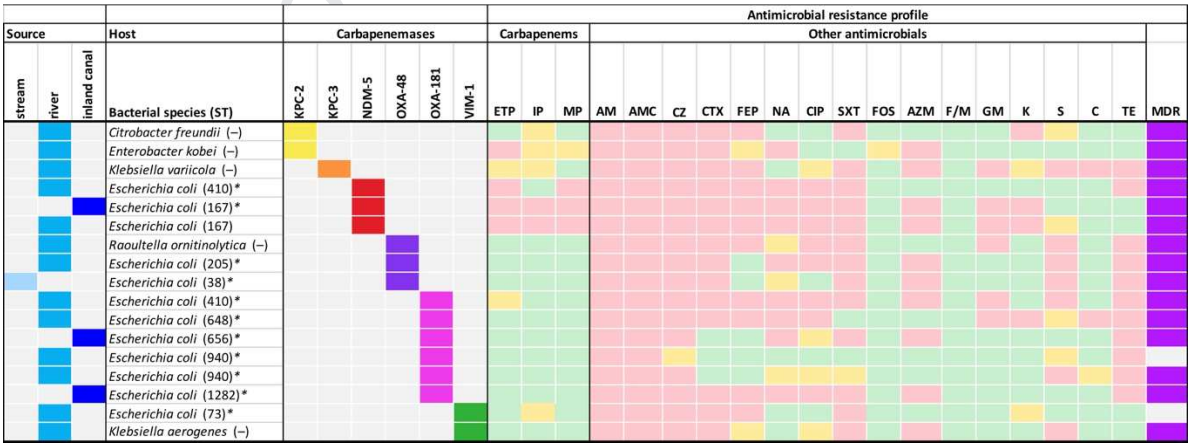

Figure 2. Source, species, carbapenemases, and antibiotic susceptibility profiles of carbapenemase producing Enterobacteriaceae isolated from surface water bodies in

Switzerland. Abbreviations: AM, ampicillin; AMC, amoxicillin-clavulanic acid; AZM, aztreonam; C, chloramphenicol; CIP, ciprofloxacin; CTX, cefotaxime; CZ, cefazolin; ETP, ertapenem; FEP, cefepime; F/M, nitrofurantoin; GM, gentamycin; IP, imipenem; K, kanamycin; MDR, multidrug resistance; MP, meropenem; NA, nalidixic acid; S, streptomycin; ST, sequence type; SXT, sulfamethoxazole//trimethoprim; TE, tetracycline; –, not applicable or not performed; *, *E. coli* with intestinal or extraintestnal pathogenic virulence genes. Colors of squares categorizing antibiotic resistance profiles: Pink, resistant; yellow, intermediate; green, susceptible, purple, multidrug resistant.

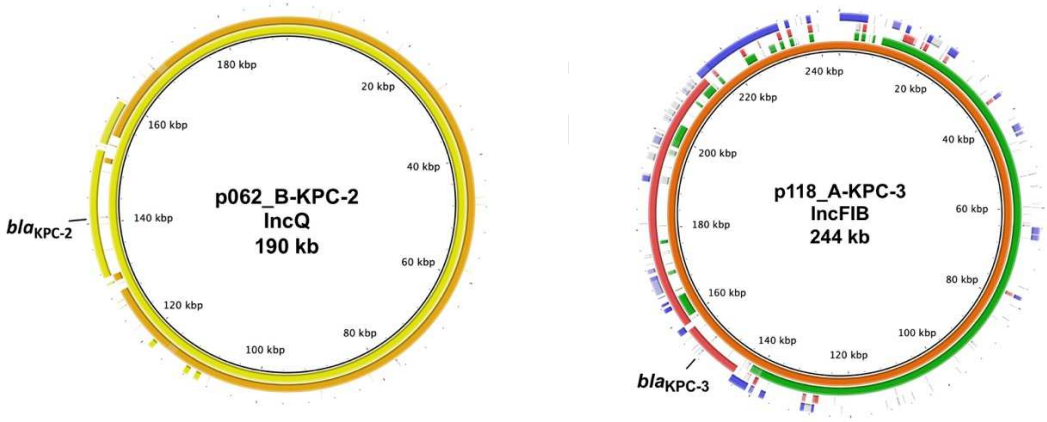

Figure 3. Comparative circular maps of bla_{KPC}-carrying plasmids generated using BRIG. The positions of the bla_{KPC} genes are indicated.

Left panel: p062_B-KPC-2 (GenBank acc. no. CP048384.1). The rings from the inner to the outer represent plasmids p062_B-KPC-2 from *C. freundii* from this study (yellow), p1643_10 (GenBank acc. no. KF056330) from poultry *Salmonella* Kentucky isolate 1643/2010 (orange), and pKP1504-kpc (GenBank acc. no. KF874496) from clinical *K. pneumoniae* isolate GR-1504 (yellow).

Right panel: Mosaic structure of p118_A-KPC-3 (GenBank acc. no. CP048380.1). The rings from the inner to the outer represent plasmids p118_A-KPC-3 from *K. variicola* from this study (orange), unnamed plasmid (GenBank acc. no. NZ_CP024500.1) from *K. pneumoniae* RJY9645 (green), unnamed plasmid (GenBank acc. no. NZ_CP029102.1) from *K. pneumoniae* strain AR438 (red), and pY9645-166 (GenBank acc. no. CP044029.1) from clinical *K. pneumoniae* isolate RJY9645 (blue).

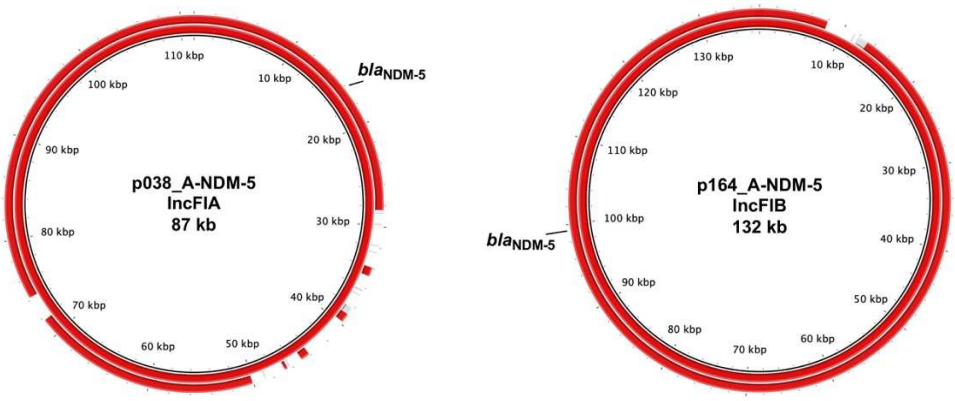

Figure 4. Comparative circular maps of *bla*$_{\text{NDM-5}}$-carrying plasmids generated using BRIG. The positions of the *bla*$_{\text{NDM-5}}$ genes are indicated.

Left panel: p038_A-NDM-5 (GenBank acc. no. CP048377.1). The rings from the inner to the outer represent plasmids pAMA1167-NDM-5 from *E. coli* ST410 (GenBank acc. no. NZ_CP024805.1), and p038_A-NDM-5 from *E. coli* ST410 from this study.

Right panel: p164_A-NDM-5 (GenBank acc. no. CP048368.1). The rings from the inner to the outer represent plasmids pM309-NDM5DNA from *E. coli* ST167 (GenBank acc. no. AP018833.1), and p164_A-NDM-5 from *E. coli* ST167 from this study.

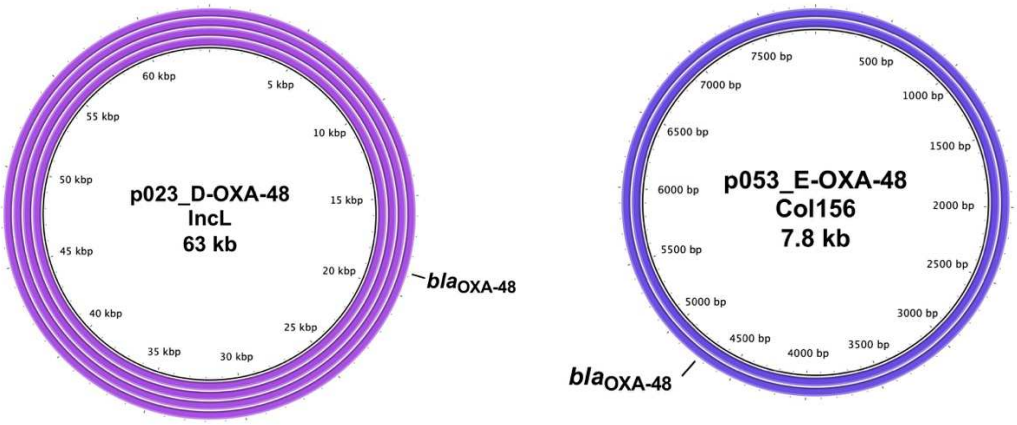

Figure 5. Comparative circular maps of *bla*$_{\text{OXA-48}}$-carrying plasmids generated using BRIG. The positions of the *bla*$_{\text{OXA-48}}$ genes are indicated.

Left panel: p023_D-OXA-48 (GenBank acc. no. CP048353.1). The rings from the inner to the outer represent plasmids p704SK10_2 from *E. cloacae* (GenBank acc. no. CP022150), pOXA-48_4963 from *K. pneumoniae* (GenBank acc. no. KX523900), p023_D-OXA-48 from *R. ornithinolytica* from this study, and pEC745 from *E. coli* ST131 (GenBank acc. no. CP015075.1).

Right panel: p053_E-OXA-48 (GenBank acc. no. CP048364.1). The rings from the inner to the outer represent plasmids p053_E-OXA-48 from *E. coli* ST205 from this study, and pMTY17816_OXA48 from *K. pneumoniae* isolate (GenBank acc. no. AP019554.1.

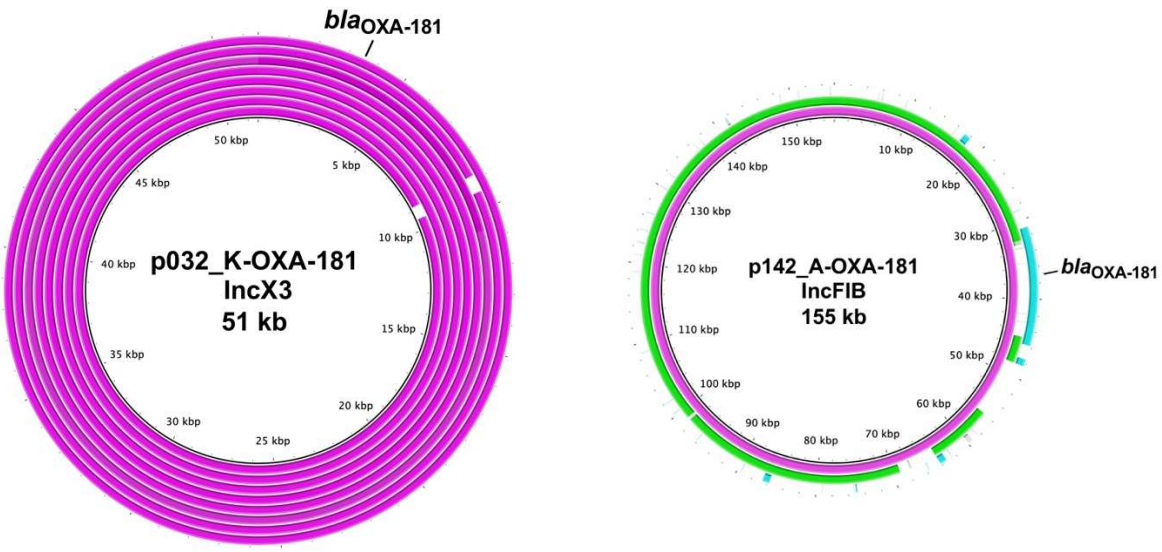

Figure 6. Comparative circular maps of *bla*$_{\text{OXA-181}}$-carrying plasmids generated using BRIG. The positions of the *bla*$_{\text{OXA-181}}$ genes are indicated.

Left panel: Plasmids containing *bla*$_{\text{OXA-181}}$ genes. The rings from the inner to the outer represent plasmids p032_K-OXA-181 from *E. coli* ST1284 (GenBank acc. no. CP048321.1, this study), pOXA-181_29144 from *K. pneumoniae* (GenBank acc. no.KX523903.1), p061_A-OXA-181 from *E. coli* ST940 (GenBank acc. no. CP048327.1, this study), pAN-OXA-181 from *E. coli* ST410 (GenBank acc. no. MK416154), p124_B-OXA-181 from *E. coli* ST410 (GenBank acc. no. CP048346.1, this study), p010_B-OXA-181 *E. coli* ST656 (GenBank acc. no.CP048332.1, this study), p064_C-OXA-181 *E. coli* ST940 (GenBank acc. no. CP048325.1, this study), and pKS22 from *K. variicola* (GenBank acc. no. KT005457).

Right panel: Mosaic structure of p142_A-OXA181 (GenBank acc. no. CP048338.1). The rings from the inner to the outer represent plasmids p142_A-OXA181 from this study (pink), unnamed plasmid (GenBank acc. no. NZ_LR130556.1) from *E. coli* (green), and pABC260-OXA-181(GenBank acc.no. MK412915.1) from *K. pneumoniae* (turquoise).

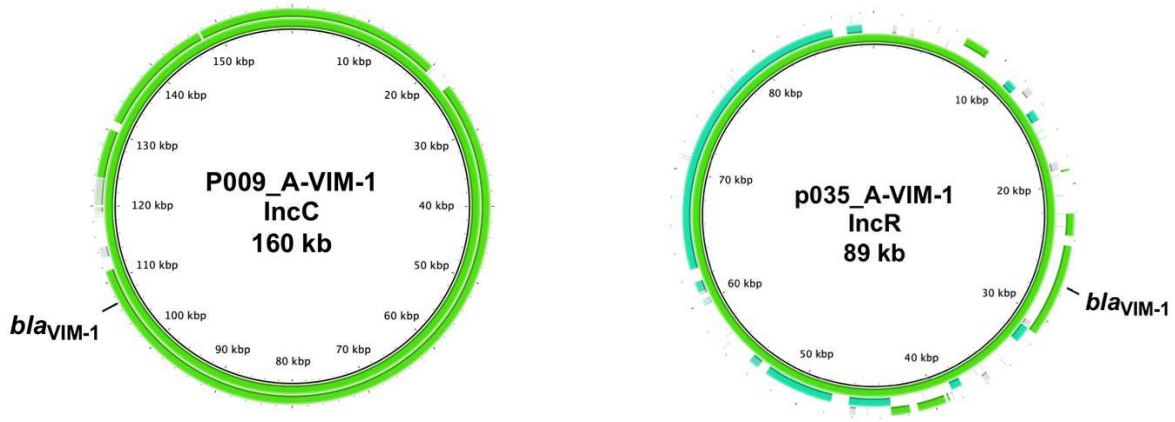

Figure 7. Comparative circular maps of *bla*$_{\text{VIM-1}}$-carrying plasmids generated using BRIG. The positions of the *bla*$_{\text{VIM-1}}$ genes are indicated.

Left panel: p009_A-VIM-1 (CP048305.1). The rings from the inner to the outer represent plasmids p009_A-VIM-1 from *K. aerogenes* from this study, and pKP-Gr642 from a *K. pneumoniae* isolate (GenBank acc. no. KR559888.1).

Right panel: Mosaic structure of p035_A-VIM-1 (GenBank acc. no. CP050069.1). The rings from the inner to the outer represent plasmids p035_A-VIM-1 from this study (green), plasmid pENT-576 (GenBank acc. no. NZ_CP008898) from *E. cloacae* (turquoise), and pMOS94 (GenBank acc. no. MK671725.1) identified in clinical *P. mosseli* (green).

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Tables 1-2

Table 1. Virulence factor profiles of 11 carbapenemase-producing *E. coli* strains cultured from water bodies in Switzerland

Strain ID	Carbapenemase	ST	Virulence factor (s)
CF038	NDM-5	410	*lpfA*
CF164	NDM-5	167	*capU, gad, iss*
CF053	OXA-48	205	*astA, gad, lpfA*
CF065	OXA-48	38	*air, eilA, iss*
CF124	OXA-181	410	*lpfA*
CF142	OXA-181	648	*air, eilA, gad, iha, lpfA, nfaE, sat*
CF010	OXA-181	656	*gad, iss*
CF061	OXA-181	940	*capU, gad, lpfA*
CF064	OXA-181	940	*capU, gad, lpfA*
CF032	OXA-181	1284	*astA, capU, gad, iss*
CF009	VIM-1	73	*capU, iha, iroN, iss, mchB, mchC, mchF, mcmA, pic, sat, vat*

air, enteroaggregative immunoglobulin repeat protein gene; *astA*, heat-stable toxin gene; *capU*, hexosyltransferase homolog gene; *eilA*, *Salmonella* invasion gene activator *hilA* homolog gene; *gad*, glutamate decarboxylase; *iha*, iron-regulated adhesin gene; *iroN*, enterobactin siderophore receptor gene; *lpfA*, long polar fimbriae gene; *mchB*, gene for microcin H47 part of colicin H; *mchC*, MchC protein gene; *mchF*, ABC transporter protein MchF gene; *mcmA*, gene for microcin M part of colicin H; *nfaE*, diffuse adherence fibrillar adhesin gene; *iss*, increased serum survival; *pic*, serine protease autotransporter gene of Enterobacteriaceae (SPATE); *sat*, secreted autotransporter toxin gene; *vat*, vacuolating autotransporter toxin gene.

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Table 2. Summary of the features associated with 16 carbapenemase-encoding plasmids from Enterobacteriaceae strains cultured from surface water bodies in Switzerland

Strain ID	Host species (ST)	Carbapenemase	Plasmid	Plasmid size	Inc group	Other AMR genes	T/A family	Accession no.
CF062	*C. freundii* (–)	KPC-2	p062_B-KPC-2	190 kb	IncQ	*aph(3')-Ia, aph(6)-Id, aph(3'')-Ib, sul2, ant(2'')-Ia, dfrA12, aadA, sul1,* bla_{OXA-9}, bla_{TEM-1}, $bla_{CTX-M-8}$	*yafQ/dinJ*	CP048384.1
CF070	*E. kobei* (–)	KPC-2	p070_A-KPC-2	110 kb	$IncFIB_K$	bla_{TEM-1}	–	CP050075.1
CF118	*K. variicola* (–)	KPC-3	p118_A-KPC-3	244 kb	IncFIB	*mphA, sul2, dfrA12, aadA, sul1, catII, tet(D), aac(3)-IId*	*relE/parE vapB/C*	CP048380.1
CF038	*E. coli* (410)	NDM-5	p038_A-NDM-5	87 kb	IncFIA	*tet(D), sul1, aadA5, dfrA32, aadA15, dfrA12,* $bla_{TEM-192}$, $bla_{TEM-118}$, *aac(6')-Ib-cr,* $bla_{OXA-140}$, *catB3,* $bla_{CTX-M-15}$	*pemI/K, ccdA/B, vapC, phd/yefM hok/sok*	CP048377.1
CF164	*E. coli* (167)	NDM-5	p164_A-NDM-5	132 kb	IncFIB	*aac(6')-Ib-cr,* $bla_{OXA-140}$, *catB3,* $bla_{CTX-M-15}$, *dfrA12, aadA, sul1, brp(mbl), rmtB,* bla_{TEM-1}, *mphA*	*pemI/K, vapB/C, ccdA/B, hok,*	CP048368.1
CF163	*E. coli* (167)	NDM-5	p163_C-NDM-5	10 kb	–	*dfrA12, aadA, sul1, brp(MBL)*	–	CP048374.1
CF023	*R. ornithinolytica* (–)	OXA-48	p023_D-OXA-48	63 kb	IncL	–	*pemI/K*	CP048353.1
CF053	*E. coli* (205)	OXA-48	p053_E-OXA-48	7.8 kb	Col156	–	–	CP048364.1
CF124	*E. coli* (410)	OXA-181	p124_B-OXA-181	51 kb	IncX3	*qnrS1*	–	CP048346.1
CF142	*E. coli* (648)	OXA-181	p142_A-OXA-181	155 kb	IncFIB	*qnrS1, aadA5, dfrA32, qnrB4, dha-1, sul1, mphA, catI, tet(D)*	*ccdA/B, parD, pemI/K, relE/parE hok/soc*	CP048338.1
CF010	*E. coli* (656)	OXA-181	p010_B-OXA-181	51 kb	IncX3	*qnrS1*	–	CP048332.1
CF061	*E. coli* (940)	OXA-181	p061_A-OXA-181	51 kb	IncX3	*qnrS1*	–	CP048327.1
CF064	*E. coli* (940)	OXA-181	p064_C-OXA-181	51 kb	IncX3	*qnrS1*	–	CP048325.1
CF032	*E. coli* (1284)	OXA-181	p032_K-OXA-181	51 kb	IncX3	*qnrS1*	–	CP048321.1
CF009	*E. coli* (73)	VIM-1	p009_A-VIM-1	160 kb	IncC	bla_{CMY-4}, *aac(6')-Il, dfrA15, aadA12, sul1, qnrA1,*	–	CP048305.1
CF035	*K. aerogenes* (–)	VIM-1	p035_A-VIM-1	89 kb	IncR/IncY	*catB, sul1, qnrS1*	*relB/dinJ, vapC*	CP050069.1

AMR, antimicrobial resistance; Inc, plasmid incompatibility; ST, sequence type determined for *E.coli*; T/A, toxin/antitoxin system; – feature not iden

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Highlights

- Carbapenemase producing Enterobacteriaceae (CPE) are present in surface waters
- Many environmental CPE are similar to clinical strains found worldwide
- Clinically relevant carbapenemase genes were identified on epidemic plasmids
- Carbapenemase genes are replicating and evolving pollutants of river ecosystems

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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