Dissemination of CTX-M-Producing Escherichia coli in Freshwater Fishes From a French Watershed (Burgundy)

Loic Bollache1,2, Emeline Bardet2, Géraldine Depret2, Sébastien Motreuil3, Catherine Neuwirth4, Jérôme Moreau3 and Alain Hartmann2*

1 UMR CNRS 6249, Laboratoire Chrono-Environnement, Besançon, France, 2 Agroécologie, AgroSup Dijon, INRA, Université de Bourgogne, Université de Bourgogne Franche-Comté, Dijon, France, 3 UMR CNRS 6282 Biogéosciences, Laboratoire Biogéosciences, Dijon, France, 4 Bacteriology Department, University Hospital, Université de Bourgogne Franche-Comté, Dijon, France

*Correspondence:
Alain Hartmann
alain.hartmann@inra.fr

OPEN ACCESS

Dissemination of extended-spectrum β-lactamases producing Escherichia coli (ESBL-Ec), has increased over several decades. Freshwater ecosystems are suspected to play an important ecological and evolutionary role in driving the dissemination of antimicrobial resistance. The aim of our study was to decipher the occurrence of ESBL-Ec in a small watershed (Ouche river, Burgundy, France), targeting environmental matrices and fishes. Among cefotaxime resistant E. coli (ctxR Ec) isolates, we detected and characterized 36 ESBL-Ec from water, biofilm and fish guts. ctxR Ec and ESBL-Ec were found in samples from sites near the first small town, located downstream from the watershed which was studied. Treatment of urban wastewater by waste water treatment plants (WWTP), might therefore be a major potential source of ctxR Ec and thus of ESBL-Ec. Prevalence of total E. coli and ctxR Ec in fish guts ranged between 0 to 92% and 0 to 85%; respectively, depending on the sampling site and the fish species. The diet of fish (predator or omnivore) seems to strongly influence the prevalence of total E. coli and ESBL-Ec. Extended spectrum beta-lactamases produced by the isolates from this study belonged to the CTX-M family (CTX-M group 1 and 9). Moreover, some environmental ESBL-Ec proved to share genotypic features (MLST types) with isolates which originated from 8 WWTP effluents discharged in the Ouche river and with the sequence type ST131, which is widely described in clinical isolates. Ninety-seven % (97%) of ESBL-Ec from the study harbored additional antibiotic resistances and can thus be considered as multi drug resistant (MDR) bacteria. Finally, 53% of the ESBL-Ec strains harbored class 1 integron-integrase (intI1). These results are discussed with the perspective of defining indicators of antibiotic resistance contamination in freshwater ecosystems.

Keywords: freshwater, ESBL producing Escherichia coli, blaCTX-M, class 1 integron-integrase, antibiotic resistance, fish, MLST E. coli

INTRODUCTION

Dissemination of extended-spectrum β-lactamases (ESBLs) among Enterobacteriaceae (especially by Escherichia coli) has dramatically increased among human populations (in community and hospital settings) over several decades and is recognized worldwide as a major public health concern (Woerther et al., 2010). With increasing numbers of ESBL-producing bacteria in healthcare-associated and community-acquired infections, ESBL-producers have become one of the most
important groups of multi-drug-resistant (MDR) bacteria involved in healthcare-associated infections caused by *E. coli* or other enterobacteria (Bonnet, 2004).

First described in 1989, the CTX-M type ESBL is nowadays the most prevalent clinical isolate worldwide (Livermore and Hawkey, 2005) but also the most prevalent in human and animal feces (Guenther et al., 2011), in sewage sludge (Reinhalter et al., 2010), in soils (Hartmann et al., 2012) and in aquatic environments (Zurfluh et al., 2013; Blaak et al., 2014; Maravic et al., 2015). Effluents discharged into aquatic ecosystems, by waste water treatment plants (WWTP) treating urban and hospital wastewater, have been clearly identified as a source of extended-spectrum β-lactamases producing *Escherichia coli* (ESBL-*Ec*) that are disseminated in aquatic environments (Brechtet et al., 2014; Blaak et al., 2015; Franz et al., 2015).

Whereas mechanisms conferring resistance to many antibiotic families in bacteria are well understood (Tenover, 2006; Stokes and Gillings, 2011), the processes facilitating persistence and dissemination of ESBL-producing bacteria in the environment have been less intensively studied.

Among ecological and environmental factors contributing to the environmental spread of antimicrobial resistant bacteria, the epidemiological role of wild fauna as reservoirs or transporters of resistant bacteria, has been repeatedly emphasized. Thus, ESBL-*Ec* strains have been reported in many wild species, particularly in wild bird populations living in areas inhabited by people (Bonnedahl et al., 2009, 2010; Literak et al., 2010; Poirel et al., 2012; Vittecoq et al., 2016; Mohsin et al., 2017; Carter et al., 2018) and mammals (Costa et al., 2006; Literak et al., 2009; Goncalves et al., 2012). Therefore, the role of the environment and especially aquatic ecosystems, has to be assessed with regards to the emergence of resistant bacteria like ESBL-*Ec* (Taylor et al., 2011; Marti et al., 2014). Marine and freshwater ecosystems are known to receive numerous chemical and biological contaminants, among those antibiotic residues and antibiotic resistant bacteria are released in aquatic ecosystems through discharge from livestock and WWTP. However, the spread of ESBL-*Ec* into freshwater ecosystems and the role of fishes in this process have received little attention (Tacao et al., 2012; Pereira et al., 2013). Indeed, fishes are mobile, have different feeding habits and use various microhabitats in the river (Popova and Sytina, 1977; Hyslop, 1982; Weatherley, 1987; Garner, 1996), thus freshwater fishes might also disseminate ESBL-*Ec* through their movements and might serve as integrators of a freshwater ecosystems bacterial contamination. Indeed, recently several authors have documented the contamination of wild fish in marine environments (Brahmi et al., 2018) and in lakes (Abgottsporn et al., 2014; Zurfluh et al., 2015; Moremi et al., 2016) as well as in farmed fish (Jiang et al., 2012; Hon et al., 2016), stressing that fishes could participate in the spread of multidrug resistant enterobacteria. However, such data is missing for fishes from rivers and streams.

Finally, freshwater might constitute an efficient vector for the spread of antibiotic resistance through various ways: i.e., irrigation of cultures or drinking water for livestock animals. Thus, freshwater ecosystems are suspected to play an important ecological and evolutionary role in driving the emergence, persistence and dissemination of antimicrobial resistance (Taylor et al., 2011), and to become a reservoir potentially leading to human contamination through water, food consumption or recreational activities (Leverstein-van Hall et al., 2011; Schijven et al., 2015). In this context, it is important to determine how these bacteria have spread to freshwater ecosystems and their associated fauna as well as to understand the role of human activities in the occurrence of contamination. Concerning freshwater fauna, this study targets fishes occurring naturally in river ecosystems.

The objectives of the study were to evaluate (i) the occurrence of ESBL-*Ec* strains in freshwater fishes and their natural environment (water and biofilm), collected at several sites of the Ouche watershed (Burgundy, France), (ii) the diversity of ESBL-*Ec* from fishes and their environment (MLST typing), their antibiotic susceptibility and the prevalence of *bla* genes as well as the occurrence of class 1 integrons, in the isolates. The genetic diversity of environmental ESBL-*Ec* strains was compared with that observed in a collection of ESBL-*Ec* strains isolated from effluents of eight WWTP, discharging into the Ouche watershed.

**MATERIALS AND METHODS**

### Sites Studied Along the Ouche River and Sampling Methods

In spring 2012, 14 sampling sites were defined along the river Ouche (Burgundy, Eastern France), from the spring to the Saône confluence (Figure 1, site 1: Lusigny-sur-Ouche 47°05′25.2″N 4°40′22.0″E, site 2: Bligny-sur-Ouche, site 3: Bligny-sur-Ouche 47°06′34.6″N 4°39′58.2″E, site 4: Pont d’Ouche 47°10′02.1″N 4°42′02.7″E, site 5: Pont de Pany 47°17′49.9″N 4°48′47.5″E, site 6: Mâlain 47°19′18.1″N 4°48′22.4″E, site 7: Plombières 47°19′40.8″N 4°59′16.8″E, site 8: Dijon 47°17′41.1″N 5°02′50.0″E, site 9: Dijon 47°17′11.761″N, 5°04′59.883″E, site 10: Varanges 47°13′59.4″N 5°11′58.2″E, site 11: Tart l’Abbaye 47°10′55.1″N 5°15′08.2″E, site 12: Trouhans 47°08′54.8″N 5°16′38.8″E, site 13: Saint-Jean-de-Losne (Saône river) 47°06′03.4″N 5°15′58.9″E, site 14: Saint-Jean-de-Losne (harbor) 47°06′08.2″N 5°15′35.0″E). For all sites, 500 mL of water were collected (0.5 m from the shore and between 20 to 50 cm depth). One to 5 g of epilithon or biofilm were collected directly from the stone surface and from aquatic plants, using a sterile single use blade to prevent cross contaminations between sites. Fishes were sampled (one campaign) only from the sites eight and nine using battery-powered portable electrofishing gear (Dream Électronique Society, France). All fishes were maintained in aerated 40 L plastic tanks until their arrival at the laboratory. Within 4 h after collection, fishes were identified to the species level according to Keith and Allardi (2001) and measured (fork length). A total of 14 water samples, 16 biofilm samples and 127 fish samples were collected and used for further microbiological analyses as described below. The locations of the eight WWTP discharging treated effluents into the Ouche river are available in Figure 1.
Detection, Enumeration and Isolation of Total E. coli and Cefotaxim Resistant E. coli (ctxR Ec) From Water, Biofilm Samples and From Fish Guts

Water sample processing: Four hundred milliliters of water samples were filtered on HAWP cellulose ester filters (porosity 0.45 µm) (Merck Millipore, Germany). Filters were then washed into 5 mL of buffered peptone water (BPW) (AES Chemunex, bioMérieux France) and mixed vigorously with a vortex to resuspend bacteria.

Biofilm sample processing: Epilithon and biofilm samples were resuspended in 5 mL of BPW and homogenized by vigorous shaking.

Fish sample processing: Fishes were anesthetized and gutted using aseptic techniques immediately upon arrival at the laboratory. The fish guts were then rinsed with sterile BPW. Guts were ground and homogenized in 1 mL of BPW by adding 0.1 g of 106 µm diameter glass beads (Sigma-Aldrich, United States) and vortexed for 2 min. The homogenized gut suspension was diluted using a 10-fold dilution series. The homogenized gut suspensions which originated from either water, biofilms and homogenized gut samples were spread onto Tryptone Bile X-glucuronide agar plates (AES Chemunex, bioMérieux France), supplemented or not with 4 mg/L of cefotaxime (Sigma-Aldrich, United States), in order to detect and enumerate ctxR Ec and the total E. coli, respectively. Plates were incubated at 37°C for 18 h. To maximize the sensitivity of the detection of ctxR Ec, a non-selective enrichment step was added: i.e., the remaining BPW suspensions were incubated at 37°C for 24 h and then 100 µL of this enrichment were plated as described above. After incubation, blue colonies (glucuronidase positive colonies) were counted. For enriched samples, results were expressed as positive (presence of blue colonies) or negative (absence of blue colonies).

For each (enriched or not) plate, 3 ctxR isolates were randomly picked up and purified twice on TBX, supplemented with cefotaxime (4 mg/L) plates. Isolates were stored at −80°C in a Luria Bertani medium (AES Chemunex, bioMérieux France) supplemented with 25% glycerol.

Phenotypic Characterization and Antibiotic Resistance Testing of ctxR Ec

Cefotaxim resistant isolates were confirmed to belong to the E. coli species, by testing their capacity to ferment lactose on Drigalski agar media (Conda, Spain) and by testing on an API 20E test kit (AES Chemunex, bioMérieux France). Some isolates were also identified by MALDI-TOF analysis (MALDI Biotyper, Brucker, France).

The antibiotic susceptibility tests were performed by the disk diffusion method. Susceptibility to 16 antibiotics, including penicillin (aminopenicillin with clavulanic acid), cephalosporins (cefotaxime and ceftazidime), cephapemycin (cefoxitin), carbapenems (imipenem and ertapenem), aminoglycosides (kanamycin, tobramycin, gentamycin, streptomycin, and spectinomycin), chloramphenicol, quinolones (nalidixic acid, ofloxacin), tetracycline (doxycycline), and cotrimoxazol was determined for all recovered E. coli isolates (CLSI, 2012a). The production of ESBL was assessed by the double-disk synergy test (Jarlier et al., 1988). Isolates were classified as susceptible or resistant to the drug (isolates showing intermediate susceptibility were considered as resistant) according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2012b).

Characterization of ESBL-Ec by MLST, Detection and Sequencing of bla Genes, as Well as Occurrence of Class 1 Integrons (Detection of intI1) Among These Isolates

DNA of each positive isolate for the production of ESBL was obtained using a rapid extraction method, i.e., boiling 500 µL of a dense culture suspension of each strain and recovering the supernatant after a 5 min centrifugation at 13000 g. ESBL-Ec were characterized by MLST according the recommendations of Wirth et al. (2006) by amplifying seven house-keeping genes. The PCR fragments were then sequenced according to the Sanger method by Genewiz (Takeley, United Kingdom) and the obtained sequences were analyzed with GeneDoc software (Nicholas and Nicholas, 1997). The alleles and sequence types (ST) were assigned according the E. coli MLST website1 (Alikhan et al., 2018). All E. coli MLST data was deposited at Enterobase.

A phylogenetic tree (Multiple Spanning tree, MS Tree) based on the different ST isolated from water, biofilm and fish gut was obtained with the package available at the Enterobase database.

1http://enterobase.warwick.ac.uk/species/index/ecoli
(Zhou et al., 2017). This phylogenetic tree was displayed and annotated with the online tool iTOL (Letunic and Bork, 2016). For comparison, ESBL-\textit{Ec} strains isolated from effluents of eight WWTP discharging into the Ouche river, over the same period of time, were included in the tree (Hartmann personal communication, ANSES PNREST CIREC grant 2013-1-254).

qPCR conditions, primers and probes used for the amplification of the \textit{bla}_{CTX-M} genes coding CTX-M group 1 and CTX-M group 9 have been previously described (Sabate et al., 2002; Hartmann et al., 2012). The occurrence of class 1 integrons among ESBL-\textit{Ec} strains was estimated by PCR using primers L2 and L3 as previously described (Maguire et al., 2001). Five PCR products were sequenced to confirm the identity with \textit{int}1 sequence. All PCR products were sequenced according to the Sanger method (Takeley, United Kingdom), and compared with those available in the GenBank database using the Blast algorithm\(^2\). Sequences were deposited in the GenBank database under accession numbers MF926412 through MF926442, MF977504 through MF977517, MF977541 through MF977549 and MG029088, MG029089 for \textit{bla}_{CTX-M} genes and MF769720, MF769721, MF769722, MF769723, MF769724 for \textit{int}1 gene.

### Statistical Analyses

Differences in the prevalence of \textit{ctxR} \textit{Ec} and total \textit{E. coli} between fish species and fish groups were tested using Fisher’s exact test, to compare the proportion of infected individuals between the two sites, or the proportion between the two groups consisting of omnivorous species and predator species, according to their diets as defined by Keith and Allardi (2001). All analyses were performed with JMP software v. 5.01 (SAS Institute Inc., Cary, NC, United States).

### RESULTS

#### Distribution of \textit{ctxR} \textit{Ec} in Environmental Samples (Water and Biofilm) Along the Ouche River

Twelve out of 14 sites were positive for the presence of \textit{ctxR} \textit{Ec} (yellow dots in Figure 1) in the water sample and nine sites were positive for the biofilm samples (Table 1). Only the two first sampling sites on the Ouche river, located upstream from the first small town, i.e., Bligny-sur-Ouche, didn’t present \textit{ctxR} \textit{Ec} in both water and biofilm samples. The water contamination with \textit{ctxR} \textit{Ec} ranged from presence (\textit{i.e.}, at least 0.5 bacteria \textit{\times} 100 ml\(^{-1}\) water) to \(9.5 \times 10^2\) CFU 100 ml\(^{-1}\) with the maximum observed at site 3# in Bligny-sur-Ouche. The same trend was observed for the biofilm with a range varying from below detection limit (<0.1 bacteria \textit{\times} 100 mg\(^{-1}\) biofilm) to \(6.6 \times 10^2\) CFU 100 mg\(^{-1}\) (site 6# in Mâlain). In water samples, the ratio \textit{ctxR} \textit{E. coli}/total \textit{E. coli} varied from less than 1 to 7%, while in biofilm samples, this ratio varied from less than 1 to 22%.

#### Patterns of Abundance and Distribution of \textit{ctxR} \textit{Ec} in Fish Guts in Two Sites of the Ouche River

One hundred twenty-seven fishes were captured; 20 chubs (\textit{Leuciscus cephalus}), 23 vairones (\textit{Telesstes souffia}), 12 loaches

---

\(^2\)http://blast.ncbi.nlm.nih.gov/Blast.cgi

| Site number# location\(^a\) | Water | | Biofilm |
| --- | --- | --- | --- |
| | Number of \textit{E. coli} | Number of \textit{ctxR} \textit{E. coli} | Occurrence of \textit{ctxR} \textit{E. coli}\(^b\) | Number of \textit{E. coli} | Number of \textit{ctxR} \textit{E. coli} | Occurrence of \textit{ctxR} \textit{E. coli}\(^b\) |
| 1# Lusigny-sur-Ouche | 0 | 0 | A | 16.6 | 0 | A |
| 2# Bligny-sur-Ouche | 500 | 0 | A | 58.1 | 0 | A |
| 3# Bligny-sur-Ouche | >3640 | 946.4 | NT | >830 | 498 | NT |
| 4# Pont d’Ouche | 75 | 0 | P | 66.4 | 8.3 | NT |
| 5# Pont de Pany | 300 | 0 | P | 41.5 | 0 | P |
| 6# Mâlain | >2500 | 37.5 | NT | >2490 | 664 | NT |
| 7# Plombières | 12.5 | 0 | P | 24.9 | 0 | A |
| 8# Dijon | >1250 | 0 | P | >830 | 116.2 | NT |
| 9# Dijon | >1250 | 62.5 | NT | 415 | 91.3 | NT |
| 10# Varanges | 175 | 12.5 | NT | 149.4 | 24.9 | NT |
| 11# Tart l’Abbeye | 250 | 0 | P | 41.5 | 8.3 | NT |
| 12# Trouhans | 375 | 12.5 | NT | 249 | 8.3 | NT |
| 13# Saint-Jean-de-Losne (Saône river) | 25 | 0 | P | 0 | 0 | A |
| 14# Saint-Jean-de-Losne (harbor) | 412.5 | 0 | P | >830 | 0 | A |

\(^a\)Sites are identified by a number and the name of the nearest city (location), \(^b\)enrichment cultures for ctxR \textit{E. coli} were analyzed only in case no or very few colonies were observed after direct plating of samples, NT not tested because colonies were obtained without enrichment.
TABLE 2 | Number and size range of fishes sampled in the Ouche river.

| Diet type and fish species | 8# Dijon N | Size range (cm) | 9# Dijon N | Size range (cm) |
|----------------------------|------------|----------------|------------|----------------|
| Omnivorous                 |            |                |            |                |
| Leuciscus cephalus         | 13         | 13.7–27.8      | 7          | 15.2–24.8      |
| Telestes souffia           | 6          | 13.2–17.9      | 17         | 14.7–19.5      |
| Phoxinus phoxinus          | 10         | 6.4–7.9        | 11         | 6.9–8.3        |
| Gobio gobio                | 9          | 7.8–11.2       | 4          | 8.8–9.5        |
| Barbus barbus              | 6          | 15.6–26.3      | 0          |                |
| Rutilus rutilus           | 6          | 11.5–17.6      | 0          |                |
| Predator                   |            |                |            |                |
| Barbatula barbatula        | 9          | 6.7–8.4        | 3          | 6.2–7.5        |
| Cottus gobio               | 9          | 8.7–12.2       | 14         | 9.8–12.3       |
| Rutilus fluviatilis        | 3          | 13.4–15.4      | 0          |                |

* N: number of individuals.

TABLE 3 | Prevalence of total E. coli and ctxR Ec among fish species in the Ouche river originated from 2 Dijon sites.

| Fish species                | 8# Dijon N | Number of fishes carrying E. coli (%) | Number of fishes carrying ctxR Ec (%) | 9# Dijon N | Number of fishes carrying E. coli (%) | Number of fishes carrying ctxR Ec (%) |
|-----------------------------|------------|-------------------------------------|-------------------------------------|------------|-------------------------------------|-------------------------------------|
| Omnivorous                  |            |                                     |                                     |            |                                     |                                     |
| Leuciscus cephalus          | 13         | 12 (92.3%)                          | 11 (84.6%)                          | 7          | 4 (57.1%)                           | 3 (42.8%)                           |
| Telestes souffia            | 6          | 3 (50%)                             | 3 (50%)                             | 17         | 6 (35.3%)                           | 5 (29.4%)                           |
| Phoxinus phoxinus           | 10         | 3 (30%)                             | 3 (30%)                             | 11         | 5 (45.5%)                           | 5 (45.5%)                           |
| Gobio gobio                 | 9          | 4 (44.4%)                           | 0 (0%)                              | 4          | 1 (25%)                             | 1 (25%)                             |
| Barbus barbus               | 6          | 4 (66.6%)                           | 3 (50%)                             | 0          |                                     |                                     |
| Rutilus rutilus            | 6          | 3 (50%)                             | 3 (50%)                             | 0          |                                     |                                     |
| Predator                    |            |                                     |                                     |            |                                     |                                     |
| Barbatula barbatula         | 9          | 1 (11.1%)                           | 0 (0%)                              | 3          | 1 (33.3%)                           | 0 (0%)                              |
| Cottus gobio               | 9          | 0 (0%)                              | 0 (0%)                              | 14         | 1 (7.1%)                            | 1 (7.1%)                            |
| Rutilus fluviatilis         | 3          | 0 (0%)                              | 0 (0%)                              | 0          |                                     |                                     |

* N: number of individuals.

(Barbatula barbatula), 21 minnows (Phoxinus phoxinus), 13 gudgeons (Gobio gobio), 23 bullheads (Cottus gobio), 6 barbells (Barbus barbus), 3 perchs (Perca fluviatilis), and 6 roaches (Rutilus rutilus). The characteristics of fishes sampled for this study are given in Table 2. Since the prevalence of total E. coli and ctxR Ec in fish guts was low, results were expressed as presence or absence of these bacteria after enrichment of the samples. The prevalence of total E. coli and ctxR Ec among fish species and from the two sampling sites is presented in Table 3. Grouping of fish species as omnivorous or predator species is given in Table 2.

No difference was found in fishes between the two sites concerning the prevalence of total E. coli (Fisher’s exact test \( p = 0.5493 \)) and the prevalence of ctxR Ec (Fisher’s exact test \( p = 0.5606 \)). However, in the two sites (Figure 2), prevalence of total E. coli and ctxR Ec between fish species were profoundly variable and seemed strongly influenced by their diet. That is, omnivorous species were significantly more colonized than predator species both for total E. coli (Site 8#: Fisher’s exact test \( p < 0.0001 \), Site 9#: Fisher’s exact test \( p = 0.0001 \)) and ctxR Ec (Site 8#: Fisher’s exact test \( p = 0.0351 \); Site 9#: Fisher’s exact test \( p = 0.0229 \)).
Phenotyping and Genotyping of ctxR Ec Isolated From Fish Guts and Environmental Samples

One hundred and twenty ctxR isolates originated from water, biofilm and fish guts were recovered from TBX plates supplemented with cefotaxime used for enumeration or detection of ctxR Ec. Water and biofilm isolates were recovered from the 14 sites and analyzed along the Ouche river, whereas fish isolates originated only from sites eight and nine. Seventy-three ctxR isolates were successfully purified and cryo-conserved. Among those, 52 isolates proved to belong to the E. coli species. These 52 isolates were screened for their antibiotic susceptibility and for their ESBL phenotype. Among these, 36 isolates were confirmed to produce ESBL (positive for the double-susceptibility and for their ESBL phenotype. Among these, 36 ESBL-Ec isolates were determined by PCR. The blaCTX-M genes and the intI1 gene in the 36 ESBL-Ec isolates were determined by PCR. The blaCTX-M gene type was determined for each strain by Sanger sequencing (Table 4). Four different blaCTX-M genes (blaCTX-M1, 14, 15, 27) were observed among the set of 36 isolates. These genes belonged to the two major blaCTX-M coding CTX-M group 1 and CTX-M group 9.

No significant difference was found in the proportion of blaCTX-M coding CTX-M group 1 and CTX-M group 9 between the isolates coming from water, biofilm and fish guts ($\chi^2 = 3.07, p = 0.2151$). Similarly, among the blaCTX-M coding CTX-M group 1, no difference was found in the proportion of blaCTX-M1 and blaCTX-M15 between the isolates from water, biofilm and fish ($\chi^2 = 2.18, p = 0.3360$).

Sequences showing 100% identity with the intI1 sequence occurred in 53% of the strains (Table 4).

**FIGURE 3** | Proportion of ESBL producing E. coli isolates harboring additional antibiotic resistances (10 antibiotics tested).

**DISCUSSION**

The objectives of the study were to monitor the occurrence of total E. coli and ESBL-Ec in a small watershed (Ouche river) targeting environmental matrices (water, biofilm) as well as fishes that live in the aquatic ecosystem. This watershed is impacted by anthropic activities, i.e., urban wastewater treatment but also by farm effluents that might also impact bacteriological water quality of the river.
Environmental Dissemination of ctxR Ec and Particularly of ESBL-Ec

First, water sampling revealed the presence of E. coli all along the Ouche river, at all sampling sites excepted from the river source. Interestingly, ctxR Ec contaminations of water and biofilm appeared downstream from the first WWTP discharge in the Ouche river (site #3 in Bligny-sur-Ouche, Figure 1). Thus, our observations confirm that WWTP are probably the main source point of water contamination by ctxR Ec (Blaak et al., 2014; Brechet et al., 2014). Among ctxR Ec isolates, at least 69% produced a CTX-M type ESBL. The other strains (not showing ESBL phenotype) may produce cephalosporinases or other beta-lactamases. The Ouche watershed ecosystem contamination by ESBL-Ec seems to be linked to WWTP discharge, which is in agreement with previously published data indicating that discharge of treated wastewater is a source point of ESBL-Ec for freshwater ecosystems (Brechet et al., 2014; Czekalski et al., 2014; Vivant et al., 2016). ESBL-Ec might thus serve as an indicator of the anthropic impact (urban or farming) in aquatic ecosystems. The ratio of ctxR Ec/total E. coli seems to be overall higher in biofilm samples than in water samples, thus highlighting the role of biofilm as a potent reservoir for ctxR Ec.

### Table 4: Genotyping of ESBL producing E. coli originated from the Ouche river.

| Strain number | Sampling site | Habitat      | Sequence Type (clonal complex when existing) | bla<sub>CTX-M</sub> gene (CTX-M group) | Occurence of intI1 sequence |
|---------------|---------------|--------------|---------------------------------------------|----------------------------------------|-----------------------------|
| MIAE02070     | 6# Mâlain     | Biofilm      | 10 (10)                                     | 15 (1)                                 | −                           |
| MIAE02071     | 8# Dijon      | Biofilm      | 212                                         | 1 (1)                                  | +                           |
| MIAE02072     | 9# Dijon      | Biofilm      | 38 (38)                                     | 27 (9)                                 | −                           |
| MIAE02073     | 9# Dijon      | Biofilm      | 367 (23)                                    | 1 (1)                                  | +                           |
| MIAE02074     | 9# Dijon      | Biofilm      | 540                                         | 1 (1)                                  | +                           |
| MIAE02077     | 9# Dijon      | Biofilm      | 410 (23)                                    | 15 (1)                                 | −                           |
| MIAE02078     | 9# Dijon      | Biofilm      | 2851                                        | 15 (1)                                 | +                           |
| MIAE02079     | 9# Dijon      | Biofilm      | 1167                                        | 1 (1)                                  | −                           |
| MIAE02080     | 9# Dijon      | Biofilm      | 410 (23)                                    | 15 (1)                                 | −                           |
| MIAE02081     | 9# Dijon      | Biofilm      | 10 (10)                                     | 1 (1)                                  | +                           |
| MIAE02082     | 10# Varanges  | Biofilm      | 181 (168)                                   | 14 (9)                                 | −                           |
| MIAE02083     | 10# Varanges  | Biofilm      | 23 (23)                                     | 14 (9)                                 | +                           |
| MIAE02084     | 3# Bligny-sur-Ouche | Water       | 10 (10)                                    | 1 (1)                                  | +                           |
| MIAE02085     | 4# Pont d’Ouche | Water       | 410 (23)                                    | 1 (1)                                  | −                           |
| MIAE02086     | 6# Mâlain     | Water        | 1431                                        | 15 (1)                                 | +                           |
| MIAE02087     | 8# Dijon      | Water        | 10 (10)                                     | 15 (1)                                 | +                           |
| MIAE02088     | 9# Dijon      | Water        | 1196                                        | 1 (1)                                  | −                           |
| MIAE02089     | 11# Tart l’Abbaye | Water       | 69 (69)                                     | 15 (1)                                 | +                           |
| MIAE02090     | 11# Tart l’Abbaye | Water       | 457                                         | 14 (9)                                 | −                           |
| MIAE02091     | 13# Saint-Jean-de-Losne | Water | 744                                         | 1 (1)                                  | −                           |
| MIAE02092     | 14# Saint-Jean-de-Losne | Water | 90 (23)                                     | 15 (1)                                 | +                           |
| MIAE02093     | 14# Saint-Jean-de-Losne | Water | 90 (23)                                     | 15 (1)                                 | +                           |
| MIAE02094     | 8# Dijon      | Fish – roach | 38 (38)                                     | 15 (1)                                 | −                           |
| MIAE02095     | 8# Dijon      | Fish – chub  | 131 (131)                                   | 1 (1)                                  | −                           |
| MIAE02096     | 8# Dijon      | Fish – chub  | 617 (10)                                    | 15 (1)                                 | +                           |
| MIAE02097     | 8# Dijon      | Fish – chub  | 617 (10)                                    | 15 (1)                                 | +                           |
| MIAE02098     | 9# Dijon      | Fish – vairone | 38 (38)                                   | 27 (9)                                 | −                           |
| MIAE02099     | 9# Dijon      | Fish – vairone | 410 (23)                               | 14 (9)                                 | −                           |
| MIAE02100     | 9# Dijon      | Fish – vairone | 362                                         | 14 (9)                                 | −                           |
| MIAE02101     | 9# Dijon      | Fish – vairone | 362                                         | 14 (9)                                 | −                           |
| MIAE02102     | 9# Dijon      | Fish – vairone | 540                                         | 1 (1)                                  | +                           |
| MIAE02103     | 9# Dijon      | Fish – minnow | 354 (354)                                   | 14 (9)                                 | +                           |
| MIAE02104     | 9# Dijon      | Fish – minnow | 357                                         | 15 (1)                                 | −                           |
| MIAE02105     | 9# Dijon      | Fish – chub  | 10 (10)                                     | 15 (1)                                 | +                           |

Strain numbers are from the MIAE collection hosted at INRA Dijon, UMR Agroecologie. Site #13# is water from the Saône river at Saint-Jean-de-Losne and site #14 is water from the Saint-Jean-de-Losne harbor. + Presence, − absence.
Prevalence of Total *E. coli* and ctxR Ec in Fish Guts

Prevalence of total *E. coli* in fish guts varied from 0 to 92% across fish species. Our study revealed that fish diet (predator or omnivore) might significantly influence the prevalence of total *E. coli* as well as ctxR Ec. To our knowledge, this is the first description of the occurrence of ESBL-Ec in freshwater fishes in France. Our findings agree with previous work showing the occurrence of ESBL-Ec in fishes from Swiss lakes (Abgottspon et al., 2014; Zurfluh et al., 2015). Several studies have already shown the presence of ESBL-Ec in fish from farmed (Jiang et al., 2012) or wild population in Asia (Hon et al., 2016) and in Africa (Moremi et al., 2016). Although *E. coli* does not seem to be a permanent inhabitant of fish guts (Austin, 2006), these bacteria are able to colonize the fish gut, especially in omnivorous fishes that, at least partially, feed from contaminated biofilms. The higher ratio of ctxR Ec/total *E. coli* in biofilm samples compared to water might thus also explain the higher contamination of omnivorous fishes by ctxR Ec and ESBL-Ec. Further studies are needed, including time lapse survival of ESBL-Ec in the gut of various fish species (omnivorous or predatory), to explore the adaptation of *E. coli* and ESBL-Ec to fish gut and potential contamination of fish muscles.

In our survey, the three species of wild predatory fish harbored very little contamination by ESBL-Ec. This could be explained by the anatomy and physiology of these species. Indeed, as for most animals, predatory fishes have significantly shorter guts than omnivorous or herbivorous fishes (Keith and Allardi, 2001). This physical characteristic could be less conducive to the survival and persistence of bacteria. Moreover, high gastric acidity of predator fishes could also play a significant role in the lack of persistence of these bacteria. For example, Western (Western, 1971) showed that in *C. gobio* the gastric contents were maintained at pH 2.0 while food remains in the stomach. However, such acidity could not be sufficient to reduce the number of *E. coli*. Indeed, Foster (Foster, 2004) showed that *E. coli* could resist in strongly acidic environments.

Finally, the difference in the number of ESBL-Ec between predator and omnivorous fishes may be explained by a combination of two parameters. First, their feeding behavior could be a first filter to contamination, with omnivorous species more susceptible to be contaminated than predatory species, combined with a second filter represented by the difference in the length of their gut, less favorable to the persistence of bacteria in predators.

Genotyping Analysis

Our study highlighted a noticeable diversity of ESBL-Ec, since 22 different STs were detected among the set of 36 environmental and fish isolates analyzed. Some STs (mainly ST10) seem to be ubiquitous and are detected in both environmental and WWTP isolates. However, some STs (namely ST410 and 13 others) occurred only in environmental ESBL-Ec and not in those from WWTP effluents. This result might indicate the presence of other sources for ESBL-Ec: i.e., farming activities, livestock effluents, manure application in fields. We demonstrated that *blaCTX-M* genes that are dominant in clinical isolates (Sauget et al., 2016), namely *blaCTX-M1*, *blaCTX-M15* and *blaCTXM14*, also occurred in environmental and fish isolates. No obvious correlation was observed between the ESBL-Ec ST and the type of *blaCTX-M* gene carried or with the presence of the *intI1* gene sequence. One fish gut isolate belonged to the well-known ST131 that groups clinical epidemic clones (Sauget et al., 2016) thus indicating that this genotype might occur and/or persist in the environment.
The genetic diversity of ESBL-Ec did not seem to corelate with a special habitat or with a sampling site. Whatever the source of our strains, all these STs were already described in a large diversity of habitats and hosts. These STs could group isolates either pathogenically or not for Humans (see text footnote2).

Although there was no obvious relationship between the occurrence of the intI1 sequence and the multi-resistance phenotype, the potential presence of class 1 integrons in half of the strains might explain the MDR phenotype of some isolates. Further complete sequencing of these integrons might shed light on their potential role in the antibiotic resistance phenotype of environmental strains. Indeed, Gillings et al. (2015) proposed to use the class 1 integron-integrase gene as a potential marker of the anthropic impact on the environment (anthropogenic pollution). Occurrence of clinical class 1 integrons in our strains obviously indicates their human origin and thus reinforces the hypothesis that the main origin of this contamination is due to effluent discharge from WWTP. Such discharges can induce serious public health problems. Indeed, these resistant strains found in freshwater environments might contribute to antibiotic resistance currently spreading in the human population, through the consumption of fish, or by water reuse for crop irrigation, or through recreational activities (bathing). Moreover, aquatic environments might act as long term or permanent reservoirs for antibiotic resistant bacteria and/or antibiotic resistance genes. Indeed, in their review, Marti et al. (2014) pointed out that bacteria found in natural aquatic ecosystems are organized in biofilms, which facilitate their survival, dispersal, but also, due to high cell density and proximity, the possible horizontal gene transfer (HGT) between bacteria. Thus, biofilms can serve as long-term reservoirs for antibiotic resistant genes and contribute to the emergence and the dissemination of multi resistant strains.

In this scheme, fishes, and especially fishes that feed on biofilm, could host strains and might be sentinels of environmental contamination through antibiotic resistant bacteria. However, further studies are needed to evaluate the duration of survival of ESBL-Ec in the environment and in fish guts. Finally, the fitness of ESBL-Ec versus wild type E. coli under environmental and fish gut conditions should be analyzed.

At the same time, this study emphasizes the need for complementary treatments of WWTP effluents (tertiary treatments). These treatments should be implemented in order to decrease the spread of such antibiotic resistant isolates. Various tertiary treatments might be implemented: i.e., constructed wetlands [combination of open water ponds and planted bed filters as described by Vivant et al. (2016)], UV treatment, effluent filtration or ozonation.

ETHICS STATEMENT

Electrofishing was performed in accordance with the recommendations of the French National Agency for Water and Aquatic Environments (ONEMA) and authorized by the prefecture from Côte d’Or (France). This study conforms to the legal requirements of France. The experiment has received the agreement of the Animal Care and Ethical Committee of the Université de Bourgogne, Dijon, France.

AUTHOR CONTRIBUTIONS

LB had substantial contributions to the conception or design of the work as well as for the drafting of the manuscript. EB had done the practical work and analyzed the results. GD had substantial contributions to the acquisition, analysis, and interpretation of data for the work and also for the drafting of the manuscript. SM contributed to the acquisition of data. CN revised the manuscript critically for important intellectual content. JM provided approval for publication of the content. AH had substantial contributions to the conception or design of the work and to the drafting of the manuscript.

FUNDING

This work was supported by ANSES through the PNR EST program CIREC grant 2013-1-254.

REFERENCES

Abgottspon, H., Nuesch-Inderbinen, M. T., Zurfluh, K., Althaus, D., Hachler, H., and Stephan, R. (2014). Enterobacteriaceae with extended-spectrum- and pAmpC-type beta-lactamase-encoding genes isolated from freshwater fish from two lakes in Switzerland. Antimicrob. Agents Chemother. 58, 2482–2484. doi: 10.1128/aac.02689-13

Alikhan, N.-F., Zhou, Z., Sergeant, M. J., and Achtman, M. (2018). A genomic overview of the population structure of Salmonella. PLoS Genet. 14:e1007261. doi: 10.1371/journal.pgen.1007261

Austin, B. (2006). The bacterial microflora of fish, revised. Sci. World J. 6, 931–945. doi: 10.1100/tsw.2006.181

Blaak, H., Lynch, G., Italiaander, R., Hamidjaja, R. A., Schets, F. M., and Husman, A. M. D. (2015). Multidrug-resistant and extended spectrum beta-lactamase-producing Escherichia coli in Dutch surface water and wastewater. PLoS One 10:e0127752. doi: 10.1371/journal.pone.0127752

Blaak, H., van Hoek, A. H. A. M., Veenman, C., van Leeuwen, A. E. D., Lynch, G., van Overbeek, W. M., et al. (2014). Extended spectrum beta-lactamase- and constitutively AmpC-producing Enterobacteriaceae on fresh produce and in the agricultural environment. Int. J. Food Microbiol. 168, 8–16. doi: 10.1016/j.ijfoodmicro.2013.10.006

Bonnedahl, J., Drobník, M., Gauthier-Clerc, M., Hernandez, J., Granholm, S., Kayser, Y., et al. (2009). Dissemination of Escherichia coli with CTX-M type ESBL between humans and yellow-legged gulls in the south of France. PLoS One 4:e5958. doi: 10.1371/journal.pone.0005958

Bonnedahl, J., Drobník, M., Johansson, A., Hernandez, J., Melhus, A., Stedt, J., et al. (2010). Characterization, and comparison, of human clinical and black-headed gull (Larus ridibundus) extended-spectrum beta-lactamase-producing bacterial isolates from Kalmar, on the southeast coast of Sweden. J. Antimicrob. Chemother. 65, 1939–1944. doi: 10.1093/jac/dkq222

Bonnet, R. (2004). Growing group of extended-spectrum beta-lactamases: the CTX-M enzymes. Antimicrob. Agents Chemother. 48, 1–14. doi: 10.1128/aac.48.1.1-14.2004

Brahmi, S., Touati, A., Dunyach-Remy, C., Sotto, A., Pantel, A., and Lavigne, J. P. (2018). High prevalence of extended-spectrum-b-lactamase-producing Enterobacteriaceae in wild fish from the Mediterranean
sea in Algeria. Microb. Drug Resist. 24, 290–298. doi: 10.1089/mdr.2017.0149

Brechet, C., Plantin, J., Sauget, M., Thouverez, M., Talon, D., Cholley, P., et al. (2014). Wastewater treatment plants release large amounts of extended-spectrum beta-lactamase-producing Escherichia coli into the environment. Clin. Infect. Dis. 58, 1658–1665. doi: 10.1093/cid/ciu190

Carter, D. L., Docherty, K. M., Gill, S. A., Baker, K., Teachout, J., and Vonhof, M. J. (2018). Antibiotic resistant bacteria are widespread in songbirds across rural and urban environments. Sci. Total Environ. 627, 1234–1241. doi: 10.1016/j.scitotenv.2018.01.343

CLSI (2012a). Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria: Approved Standard. CLSI Document M11-A8. 8th Edn. Wayne, PA: Clinical and Laboratory Standards Institute.

CLSI (2012b). Performance Standards for Antimicrobial Susceptibility Testing. Twenty-Second Informational Supplement M100-S22. Wayne, PA: Clinical and Laboratory Standards Institute.

Costa, D., Poeta, P., Saenz, Y., Vinue, L., Rojo-Bezares, B., Jouini, A., et al. (2006). Detection of Escherichia coli harbouring extended-spectrum beta-lactamases of the CTX-M, TEM and SHV classes in faecal samples of wild animals in Portugal. J. Antimicrob. Chemother. 58, 1311–1312. doi: 10.1093/jac/dkl415

Czekalski, N., Dier, E. G., and Burgmann, H. (2014). Wastewater as a point source of antibiotic-resistant genes in the sediment of a freshwater lake. ISME J. 8, 1381–1390. doi: 10.1038/ismej.2014.48

Foster, J. W. (2004). Escherichia coli acid resistance: tales of an ancient acidophile. Nat. Rev. Microbiol. 2, 898–907. doi: 10.1038/nrmicro860

Franz, E., Veeman, C., van Hoek, A. H. A. M., Husman, A. D., and Błażk, H. (2015). Pathogenic Escherichia coli producing extended-spectrum beta-lactamases isolated from surface water and wastewater. Sci. Rep. 5:14372. doi: 10.1038/srep14372

Garner, P. (1996). Microhabitat use and diet of 0+ cyprinid fishes in a lentic, regulated reach of the River Great Ouse, England. J. Fish Biol. 48, 367–382. doi: 10.1111/j.1095-8469.1996.tb01434.x

Gilling, M. R., Gaze, W. H., Pruden, A., Smalla, K., Tiedje, J. M., and Zhu, Y.-G. (2011). Use of the class 1 integron-integrase gene as a proxy for anthropogenic pollution. ISME J. 9, 1269–1279. doi: 10.1038/ismej.2014.226

Goncalves, A., Igerejas, G., Radhousami, H., Estepa, V., Pacheco, R., Monteiro, R., et al. (2012). Iberian wolf as a reservoir of extended-spectrum beta-lactamase-producing Escherichia coli of the TEM, SHV, and CTX-M groups. Microb. Drug Resis. 18, 215–219. doi: 10.1089/mdr.2011.0145

Guenther, S., Ewers, C., and Wieler, L. H. (2011). Extended-spectrum beta-lactamase producing E. coli in wildlife, yet another form of environmental pollution? Front. Microbiol. 2:246. doi: 10.3389/fmicb.2011.00246

Hartmann, A., Locatiell, A., Amouroux, L., Depret, G., Jolivet, C., Gueneau, E., et al. (2012). Occurrence of CTX-M producing Escherichia coli in soils, cattle, and farm environment in France (Burgundy Region). Front. Microbiol. 3:83. doi: 10.3389/fmicb.2012.00083

Hon, N. T. T., Hoa, T. T. T., Thinh, N. Q., Hinenoya, A., Nakayama, T., Harada, K., et al. (2015). Spread of antibiotic and antimicrobial resistance of Escherichia coli from rural areas to urban and marine environments. Sci. Rep. 5, 1658–1665. doi: 10.1038/srep14372

Hulsink, M. R., Zaza, S., Schaufler, K., Roschanski, N., Sarwar, F., Semmler, T., et al. (2017). High prevalence of CTX-M-15-Type ESBL-producing E. coli from migratory avian species in Pakistan. Front. Microbiol. 8:2476. doi: 10.3389/fmicb.2017.02476

Moremi, N., Manda, E. V., Falgenhauer, L., Ghosh, H., Imizalioglu, C., Matee, M., et al. (2016). Predominance of CTX-M-15 among ESBL producers from environment and fish gut from the shores of Lake Victoria in Mwanza, Tanzania. Front. Microbiol. 7:1862. doi: 10.3389/fmicb.2016.01862

Nicholas, K. B., and Nicholas, H. B. Jr. (1997). GeneDoc: A Tool for Editing and Annotating Multiple Sequence Alignments. Available at: http://www.psc.edu/biomed/genedoc

Pereira, A., Santos, A., Tacao, M., Alves, A., Henriquez, I., and Correia, A. (2013). Genetic diversity and antimicrobial resistance of Escherichia coli from Tagus estuary (Portugal). Sci. Total Environ. 461, 65–71. doi: 10.1016/j.scitotenv.2013.04.067

Poirel, L., Potron, A., De La Cuesta, C., Cleyrat, T., Nordmann, P., and Munoz-Piccot, L. S. (2012). Wild coastline birds as reservoirs of broad-spectrum-beta-lactamase-producing Enterobacteriaceae in Miami Beach, Florida. Antimicrob. Agents Chemother. 56, 2756–2758. doi: 10.1128/aac.05982-11

Popova, O. A., and Sytana, L. A. (1977). Food and feeding relation of Eurasian perch (Perca fluviatilis) and pikeperch (Stizostedion lucioperca) in various waters of the USSR. J. Fish. Res. Board Can. 34, 1559–1570. doi: 10.1139/f77-219

Reinthaler, F. F., Blaak, H., Schets, F. M., and Husman, A. M. D. (2010). Spread of extended-spectrum beta-lactamase-producing Enterobacteriaceae in Austrian sewage sludge. J. Appl. Microbiol. 109, 60–67. doi: 10.1111/j.1365-2672.2009.04572.x

Rieckert, P., Reiner, M., Fischer, S., and Liedtke, C. J. (2016). Trends of extended-spectrum beta-lactamase-producing Enterobacteriaceae isolated from surface water in the Rhine River catchment. Environ. Sci. Technol. 50, 92–99. doi: 10.1021/acs.est.5b01888

Sabate, M., Miro, E., Navarro, F., Verge, C., Aliaga, R., Mirelís, B., et al. (2002). Beta-lactamasas involved in resistance to broad-spectrum cephalosporins in Escherichia coli and Klebsiella spp. clinical isolates collected between 1994 and 1996, in Barcelona (Spain). J. Antimicrob. Chemother. 49, 898–997. doi: 10.1093/jac/dk575

Schoefs, B., Bocquet, M., and Routy, B. (2015). Antibiotic resistance of environmental Enterobacteriaceae in a wetland in the Paris Basin. J. Appl. Microbiol. 118, 1442–1451. doi: 10.1111/jam.12752

Stokes, H. W., and Gilling, M. R. (2011). Gene flow, mobile genetic elements and the recruitment of antibiotic resistance genes into gram-negative pathogens. FEMS Microbiol. Rev. 35, 790–819. doi: 10.1111/j.1574-6976.2011.00273.x
Tacao, M., Correia, A., and Henriques, I. (2012). Resistance to broad-spectrum antibiotics in aquatic systems: anthropogenic activities modulate the dissemination of blaCTX-M-like genes. *Appl. Environ. Microbiol.* 78, 4134–4140. doi: 10.1128/AEM.00359-12

Taylor, N. G. H., Verner-Jeffreys, D. W., and Baker-Austin, C. (2011). Aquatic systems: maintaining, mixing and mobilising antimicrobial resistance? *Trends Ecol. Evol.* 26, 278–284. doi: 10.1016/j.tree.2011.03.004

Tenover, F. C. (2006). Mechanisms of antimicrobial resistance in bacteria. *Am. J. Infect. Control* 34, S3–S10. doi: 10.1016/j.ajic.2006.05.219

Vittecoq, M., Godreuil, S., Prugnolle, F., Durand, P., Brazier, L., Renaud, N., et al. (2016). Antimicrobial resistance in wildlife. *J. Appl. Ecol.* 53, 519–529. doi: 10.1111/1365-2664.12596

Taylor, N. G. H., Verner-Jeffreys, D. W., and Baker-Austin, C. (2011). Aquatic systems: maintaining, mixing and mobilising antimicrobial resistance? *Trends Ecol. Evol.* 26, 278–284. doi: 10.1016/j.tree.2011.03.004

Tenover, F. C. (2006). Mechanisms of antimicrobial resistance in bacteria. *Am. J. Infect. Control* 34, S3–S10. doi: 10.1016/j.ajic.2006.05.219

Weatherley, N. S. (1987). The diet and growth of 0-group dace, *Leuciscus leuciscus* (L.), and roach, *Rutilus rutilus* (L.), in a lowland river. *J. Fish Biol.* 30, 237–247. doi: 10.1111/j.1095-8649.1987.tb05749.x

Western, J. R. H. (1971). Feeding and digestion in two cottid fishes, freshwater *Cottus gobio* L. and the marine *Enophrys bubalis* (Euphrasen). *J. Fish Biol.* 3, 225–246. doi: 10.1111/j.1095-8649.1971.tb03667.x

Wirth, T., Falush, D., Lan, R., Colles, F., Mensa, P., Wieler, L. H., et al. (2006). Sex and virulence in *Escherichia coli*: an evolutionary perspective. *Mol. Microbiol.* 60, 1136–1151. doi: 10.1111/j.1365-2958.2006.05172.x

Woerther, P.-L., Angebault, C., Lescat, M., Suppé, E., Skurnik, D., Mniai, A. E., et al. (2010). Emergence and dissemination of extended-spectrum beta-lactamase-producing *Escherichia coli* in the community: lessons from the study of a remote and controlled population. *J. Infect. Dis.* 202, 515–523. doi: 10.1086/654883

Zhou, Z., Alikhan, N.-F., Sergeant, M. J., Luhmann, N., Vaz, C., Francisco, A. P., et al. (2017). GrapeTree: visualization of core genomic relationships among 100,000 bacterial pathogens. *Genome Res.* 28, 1395–1404. doi:10.1101/gr.232397.117

Zurfluh, K., Gler, M., Hachler, H., and Stephan, R. (2015). Replicon typing of plasmids carrying bla(CTX-M-15) among *Enterobacteriaceae* isolated at the environment, livestock and human interface. *Sci. Total Environ.* 521, 75–78. doi: 10.1016/j.scitotenv.2015.03.079

Zurfluh, K., Hachler, H., Nuesch-inderben, M., and Stephan, R. (2013). Characteristics of extended-spectrum beta-lactamase- and carbapenemase-producing *Enterobacteriaceae* Isolates from rivers and lakes in Switzerland. *Appl. Environ. Microbiol.* 79, 3021–3026. doi: 10.1128/Aem.00054-13

**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.