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Charles Henriot

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Charles Henriot. Déterminismes environnementaux de la diffusion et de la survie des bacilles à Gram négatif commensaux et pathogènes dans les hydrosystèmes. Bactériologie. Université Bourgogne Franche-Comté, 2021. Français. NNT : 2021UBFCE016 . tel-03643429

**HAL Id: tel-03643429**

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**THESE DE DOCTORAT DE L'ETABLISSEMENT UNIVERSITE BOURGOGNE  
FRANCHE-COMTE**

**Préparée au Laboratoire Chrono-environnement – UMR 6249 CNRS**

Ecole doctorale n°554

Environnements – Santé

Doctorat de Médecine, santé publique, environnement et société

Par

**M. Henriot Charles**

**Déterminismes environnementaux de la diffusion et de la survie  
des bacilles à Gram négatif commensaux et pathogènes dans les  
hydrosystèmes**

Thèse présentée et soutenue à Besançon, le 29 septembre 2021

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des bacilles à Gram négatif commensaux et pathogènes dans les  
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Charles Henriot



## REMERCIEMENTS

Tout d'abord, je remercie Dr. Sylvie Nazaret et Pr. Philippe Usseglio-Polatera d'avoir jugé mon travail de recherche. Merci pour vos retours très positifs, vos encouragements et vos conseils constructifs. Merci à Dr. John Poté, Dr. Ed Topp et Dr. Pierre Rossi d'avoir accepté de prendre part au jury de ma soutenance. Merci à l'attention que vous avez portée à mon travail.

Merci à Pr. Didier Hocquet et Dr. Gudrun Bornette d'avoir dirigé et encadré ce travail de doctorat. Didier, merci pour ta disponibilité, tes conseils et ton soutien. C'est un plaisir et une chance de travailler avec toi. Gudrun, dans ton bureau de directrice d'UMR ou en waders dans les lûnes de l'Ain, j'ai toujours pu compter sur ta disponibilité. Merci pour nos échanges enrichissants, aussi bien scientifiques que personnels. Mais surtout, merci à vous deux pour la bienveillance que vous avez portée à mon égard durant ces années. Merci pour les échanges et les rires.

Merci aux collègues qui sont rapidement devenus des amis et des personnes sur qui j'ai pu compter. Je ne peux pas tous vous citer ici. Jonas, Anne-Lise, Corentin, Vanessa, Amandine, Marine, Émilie, Stéphane, Lisa et les autres : ça a été une joie immense de travailler et d'échanger avec vous. Une mention spéciale pour toi Daniel, qui m'a épaulé, surtout à mes débuts avec la microbiologie. Merci pour ton aide et ton soutien, et aussi (surtout) pour tous les rires, le sport, les verres. Quentin et Chloé, vous êtes des co-bureaux d'exception ! Vous êtes vrais, sincères et gentils. Ça a été un réel plaisir de vous côtoyer chaque jour. J'ai énormément de souvenirs qui me reviennent en écrivant ces lignes : les pauses goûter ou p'tit déjeuner, les tarots interminables, les nombreuses soirées, le ski et les fondues ... Merci à vous tous d'être les personnes que vous êtes. Merci d'avoir fait de ces années de dur labeur, des années d'échange, de joie et de rires. Merci de m'avoir permis de réaliser ce travail en m'amusant.

Merci aux personnes avec qui j'ai travaillé, de près ou de loin, durant ces trois années. Merci à l'ensemble du laboratoire Chrono-environnement, à vous tous qui m'avez aidé à un moment donné ou un autre, parfois sans même le savoir.

Merci à mes amis de longue date. Lucas, j'ai l'impression qu'on révisait le bac il y a quelques années seulement. Merci d'être là depuis tout ce temps. Julia, on partage la même galère de la thèse à l'autre bout du monde. Merci pour tes encouragements et ton soutien. Maxime, Carolane, Eloi, Bastien, Laloue, Lucile c'est aussi grâce à vous que j'en suis là aujourd'hui. Léo et Leïla, mes amis polytechniciens, merci pour les rires et le soutien, même à distance ! Merci à mes amis bisontins : Laurette, Caroline, Camille, Julia, Pierre, Mélodie et Kévin. Florian, merci beaucoup pour ton soutien.

Un énorme merci à ma famille qui m'a soutenu et épaulé durant tout mon cursus. Un énorme merci à ma sœur et mes frères, Emma, Louis et Jules, qui ont toujours été là pour moi, et pour qui je serai toujours là. Merci à mes parents, Blandine et Pascal, qui ont toujours mis la priorité sur le libre choix de nos orientations. Merci à ma mamie. Merci à Estelle, Jérémy, Lise, Aaron et mon filleul, Benjamin.

Thibaut, je te remercie profondément pour tes encouragements et ton soutien. Merci d'avoir pris soin de moi, et merci de m'avoir (sup)porté pendant les semaines intenses de rédaction. Merci d'être qui tu es. Je suis sincèrement heureux et fier de t'avoir à mes côtés.



## AVANT-PROPOS

Cette thèse a bénéficié d'un contrat doctoral attribué par l'école doctorale Environnement-Santé de l'Université de Bourgogne Franche-Comté. Ce contrat de thèse a été financé par le Ministère de l'Enseignement Supérieur, de la Recherche et de l'Innovation de la République Française. Le travail de thèse a été réalisé au sein de l'Unité Mixte de Recherche (UMR) 6249 Chrono-environnement à Besançon, qui est sous la double tutelle de l'Université de Franche-Comté (UFC) et du Centre National de la Recherche Scientifique (CNRS). Les travaux de thèse se sont déroulés à l'Unité de Formation et de Recherche (UFR) Sciences et Techniques, à l'UFR Sciences de la Santé de l'UFC et au Centre Hospitalier Universitaire (CHU) de Besançon. Ce contrat a également été le support de plusieurs avenants doctoraux d'enseignement, dont la majorité des 80 h équivalent TD a été enseignée dans le département Sciences de la Vie de l'UFC.

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## LISTE DES PUBLICATIONS ET COMMUNICATIONS

### Articles dans des revues scientifiques internationales

- **Henriot, C. P.**, Martak, D., Genet, S., Bertrand, X., Hocquet, D. & Bornette, G. (2021). Origin, fluxes, and reservoirs of human-associated bacteria in floodplain ecosystems. (*Manuscript en révision à Water Research*)
- Martak, D., Guther, J., Verschuuren, T., Valot, B., Conzelmann, N., Bunk, N., Riccio, M. E., Salamanca, E., Meunier, A., **Henriot C. P.**, Brossier, C., Bertrand, X., Cooper, B. S., Harbarth, S., Tacconelli, E., Fluit, A. C., Rodriguez-Baño, J., Kluytmans, J. A. J. W., Peter, S., Hocquet, D., on behalf of the MODERN WP3 study group (2021). Populations of extended-spectrum  $\beta$ -lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* are different in human-polluted environment and food in five European cities. *Clinical Microbiology and Infection*. DOI : 10.1016/j.cmi.2021.07.022
- Chiapponi, E., **Henriot, C. P.**, Bertrand, X., Hocquet, D., & Bornette, G. (2020). Using GFP-tagged *Escherichia coli* to investigate the persistence of fecal bacteria in vegetated wetlands: an experimental approach. *Antibiotics*, 9(6), 335. DOI : 10.3390/antibiotics9060335
- Martak, D., **Henriot, C. P.**, Broussier, M., Couchoud, C., Valot, B., Richard, M., Couchot J., Bornette G., Hocquet D. & Bertrand, X. (2020). High prevalence of human-associated *Escherichia coli* in wetlands located in eastern France. *Frontiers in Microbiology*, 11, 2200. DOI : 10.3389/fmicb.2020.552566
- **Henriot, C. P.**, Martak, D., Cuenot, Q., Loup, C., Masclaux, H., Gillet, F., Bertrand X., Hocquet D. & Bornette, G. (2019). Occurrence and ecological determinants of the contamination of floodplain wetlands with *Klebsiella pneumoniae* and pathogenic or antibiotic-resistant *Escherichia coli*. *FEMS microbiology ecology*, 95(8), fiz097. DOI : 10.1093/femsec/fiz097

### Chapitre d'ouvrage

- **Henriot C. P.**, Martak D., Dagot C., Petit F., Topp E., Bertrand X., Bornette G., & Hocquet D. (2020). The fate of antibiotic-resistant bacteria, in *Emerging Contaminants Vol. 1 - Occurrence and Impact, Environmental Chemistry for a Sustainable World*, volume number 65. ISBN : 978-3-030-69078-6. DOI : 10.1007/978-3-030-69079-3\_4.

## Communications dans des congrès internationaux

- **Henriot C.P.**, Martak D., Valot B., Bertrand X., Bornette G. & Hocquet D., Presence and functional determinants of *Escherichia coli* in floodplain wetlands, “World One Health Congress”, 02/11/2020, videoconference, Edimbourg. Communication orale.
- **Henriot C.P.**, Martak D., Hocquet D., Bertrand X. & Bornette G., Déterminisme de la présence des bactéries pathogènes et antibiorésistantes associées à l'Homme dans les zones humides alluviales, Colloque “I.S. Rivers”, 05/06/2018, Université Lyon II, Lyon. Communication orale.

## Autres communications

- **Henriot C.P.**, Martak D., Valot B., Bertrand X., Bornette G. & Hocquet D., Présence et déterminants écologiques et fonctionnels d'*Escherichia coli* dans les zones humides alluviales, Journée Des Doctorants, 17/01/2020, UMR6249 Chrono-environnement, Besançon. Communication orale.
- **Henriot C.P.**, Martak D., Cuenot Q., Loup C., Masclaux H., Gillet F., Bertrand X., Hocquet D. & Bornette G., Occurrence et déterminants écologiques de la contamination des zones humides alluviales par *Klebsiella pneumoniae* et *Escherichia coli* pathogène ou résistant aux antibiotique, Forum des Jeunes Chercheurs, 13/06/2019, Université de Bourgogne, Dijon. Communication orale.
- **Henriot C.P.**, Martak D., Hocquet D., Bertrand X. & Bornette G., Déterminisme de la présence des bactéries pathogènes et antibiorésistantes associées à l'Homme dans les zones humides alluviales, Journée Des Doctorants, 17/11/2018, UMR6249 Chrono-environnement, Besançon. Poster.
- **Henriot C.P.**, Martak D., Hocquet D., Bertrand X. & Bornette G., Déterminisme de la présence des bactéries pathogènes et antibiorésistantes associées à l'Homme dans les zones humides alluviales, Concours de la Société de Biologie de Besançon, 13/12/2018, Université de Franche-Comté, Besançon. Communication orale. Prix de la meilleure communication.
- **Henriot C.P.**, Martak D., Hocquet D., Bertrand X. & Bornette G., Déterminisme de la présence des bactéries pathogènes et antibiorésistantes d'origine humaine dans les zones humides alluviales : étude de *Escherichia coli*, *Klebsiella pneumoniae* et *Pseudomonas aeruginosa*, Colloque “Eau, Santé, Environnement”, 22-23/11/2018, Université Evry val d'Essonne et université Paris-Saclay, Evry. Poster. Prix du meilleur poster.
- **Henriot C.P.**, Martak D., Hocquet D., Bertrand X. & Bornette G., Déterminisme de la présence des bactéries pathogènes et antibiorésistantes associées à l'Homme dans les zones humides alluviales, Workshop “PICS éco-épidémiologie des maladies infectieuses”, 13/11/2018, UMR6249 Chrono-environnement, Besançon. Communication orale.

## LISTE DES ABRÉVIATIONS, SIGLES ET ACRONYMES

3GC : *third generation cephalosporin*  
3GC-R : *resistant to third generation cephalosporin*  
ADN : Acide désoxyribonucléique  
ARB : *antibiotic-resistant bacteria*  
ARG : *antibiotic resistance gene*  
ARN : acide ribonucléique  
BGN : bacilles à Gram négatif  
BGN-CP : bacilles à Gram négatif commensaux et pathogènes  
BLSE :  $\beta$ -lactamase à spectre étendu  
BRA : bactérie résistante aux antibiotiques  
C3G : céphalosporines de troisième génération  
C3G-R : résistant aux céphalosporines de troisième génération  
CFU : *colony forming unit*  
CTCF : *corrected total cell fluorescence*  
CTX-M : céfotaximase-Munich  
DOC : *dissolved organic carbon*  
DPMT : *degenerate primer MOB typing*  
EPA : *Environmental Protection Agency*  
ESBL : *extended spectrum  $\beta$ -lactamase*  
ExPEC : *extraintestinal E. coli*  
GMWL : *global meteoric water line*  
GFP : *green fluorescent protein*  
GRA : gène de résistance aux antibiotiques  
HGT : *horizontal gene transfer*  
Inc : incompatibilité / *incompatibility*  
LOD : *limit of detection*  
MALDI-TOF : *Matrix Assisted Laser Desorption Ionization – Time Of Flight*  
McF : McFarland  
MLST : *Multi-Locus Sequence Typing*  
MPN : *most probable number*  
pAmpC : céphalosporinase à support plasmidique  
PBRT : *PCR-based replicon typing*  
PCR : *polymerase chain reaction*  
PRE : pays à revenus élevés  
PRFI : pays à revenus faibles et intermédiaires  
STEU : station d'épuration des eaux usées  
ST : *sequence type*  
THG : transfert horizontal de gènes  
TOC : *total organic carbon*  
UFC : unité formant colonie  
VBNC : *viable but non-cultivable*  
WHO : *World Health Organization*  
WMMWL : *western Mediterranean meteoric water line*  
WWTP : *wastewater treatment plant*





## RÉSUMÉ

Le concept *One Health* suppose que les santés humaine, animale et environnementale sont interconnectées. Il promeut une approche holistique et transdisciplinaire pour appréhender les problèmes de santé publique. Les risques infectieux et la résistance aux antibiotiques sont des préoccupations majeures du concept *One Health*. L'environnement est souvent relégué au second plan lors de la mise en place d'approches *One Health*. Ce travail de recherche vise à combler le manque de connaissances quant à la diffusion des bacilles à Gram négatif commensaux et pathogènes (BGN-CP) dans l'environnement. *Escherichia coli*, *Klebsiella pneumoniae* et *Pseudomonas aeruginosa* sont trois espèces bactériennes associées à l'Homme qui acquièrent facilement des déterminants de résistance aux antibiotiques. Ces BGN-CP contaminent l'environnement, principalement *via* les eaux usées et les activités agricoles. Dans ce travail, nous avons étudié le devenir des BGN-CP dans les écosystèmes aquatiques des plaines alluviales. *E. coli*, *K. pneumoniae* et *P. aeruginosa* sont largement dispersées dans les hydrosystèmes. Leur survie, influencée par les facteurs abiotiques et biotiques, est généralement faible dans l'environnement. Toutefois, certaines conditions favorisent leur survie, voire leur prolifération dans des écosystèmes spécifiques. Certaines zones humides peuvent constituer des réservoirs environnementaux de BGN-CP. L'environnement joue un rôle mineur dans la contamination par les BGN-CP dans les pays à revenus élevés, où la transmission interhumaine prédomine. En revanche, la situation peut être beaucoup plus complexe dans les pays à revenus faibles et intermédiaires où les compartiments humain et environnement sont plus connectés. Bien que l'environnement est supposé jouer un rôle limité dans la rétro contamination de l'Homme par les BGN-CP dans les pays à revenus élevés, sa prise en compte dans l'approche *One Health* est indispensable car les changements induits par l'Anthropocène pourraient accroître le risque sanitaire pour les populations humaines.

**Mots clés :** *One Health*, bacilles à Gram négatif, antibiorésistance, *Escherichia coli*, écosystèmes alluviaux, zones humides

## ABSTRACT

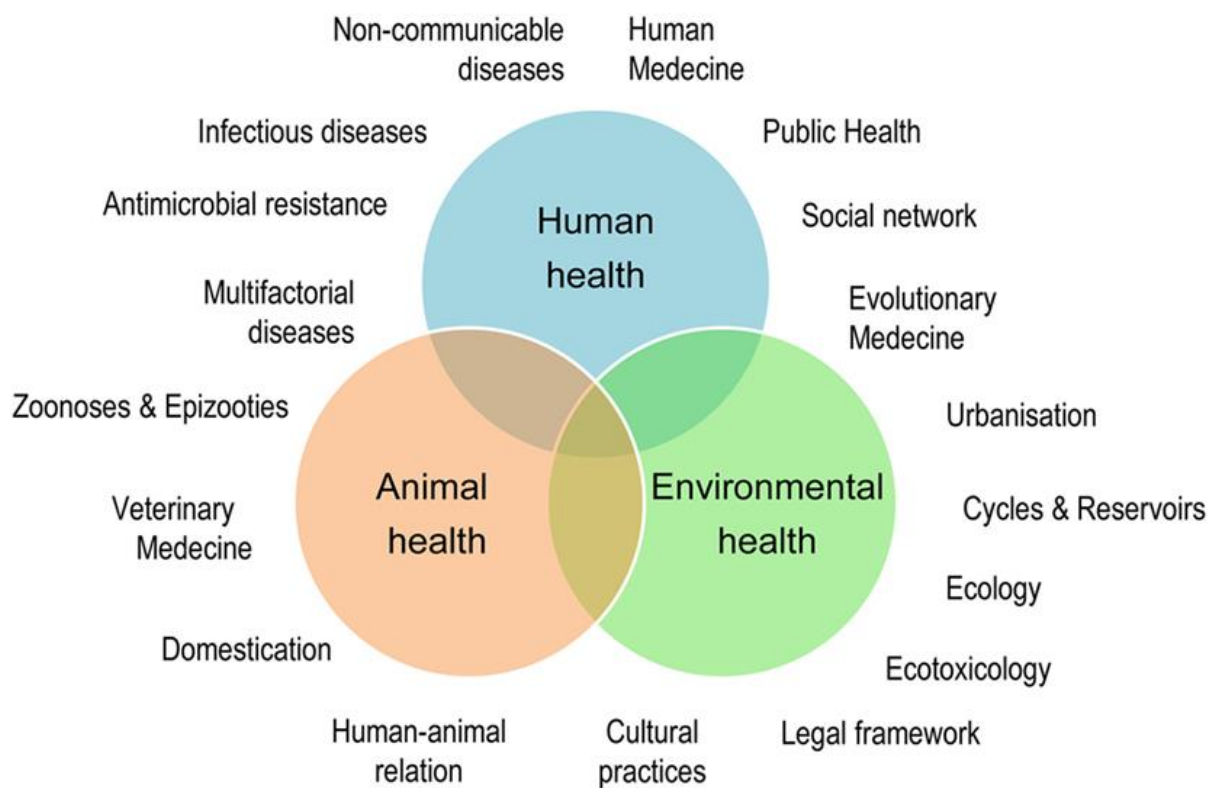
The One Health concept assumes that human, animal and environmental health are interconnected. It promotes a holistic and transdisciplinary approach to public health issues. Infectious risks and antibiotic resistance are key issues of the One Health concept. The environment is often neglected when implementing One Health approaches. This research work aims to fill the lack of knowledge about the diffusion of commensal and pathogenic Gram-negative bacteria (CP-GNB) in the environment. *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* are three bacterial species associated with humans that readily acquire antibiotic resistance determinants. These CP-GNB contaminate the environment, mainly *via* wastewater and agricultural activities. In this work, we studied the fate of CP-GNB in aquatic ecosystems of floodplains. *E. coli*, *K. pneumoniae* and *P. aeruginosa* are widely dispersed in hydrosystems. Their survival, influenced by abiotic and biotic parameters, is generally low in the environment. However, specific factors favour their survival, and even they thrive in specific ecosystems. Some wetlands can be environmental reservoirs of CP-GNB. The environment plays a minor role in the human contamination with CP-GNB in high-income countries, where human-to-human transmission predominates. In contrast, the situation could be much more complex in low- and middle-income countries, where the human and environment reservoirs are more connected. Although the environment is thought to play a limited role in the retro contamination of human by CP-GNB in high-income countries, its consideration is essential in the One Health approach since changes induced by the Anthropocene could increase the health risk for human populations.

**Key words:** One Health, Gram-negative bacteria, antibiotic-resistance, *Escherichia coli*, floodplain ecosystems, wetlands



## 1. INTRODUCTION GÉNÉRALE

"Le concept *One Health* ou Une Seule Santé en français, est mis en avant depuis le début des années 2000, avec la prise de conscience des liens étroits entre la santé humaine, celle des animaux et l'état écologique global"<sup>1</sup>. En d'autres termes, le concept *One Health* promeut une approche holistique et transdisciplinaire intégrant des compétences multidisciplinaires pour traiter de la santé humaine, animale et environnementale (Figure 1). En considérant la santé et non plus seulement la maladie, le concept *One Health* a permis de passer d'une approche "santé médicale" à une approche "santé publique".



**Figure 1. Le concept *One Health*.** Ce concept promeut une approche holistique et transdisciplinaire intégrant des compétences multidisciplinaires pour traiter de la santé humaine, animale et environnementale. Destoumieux-Garzón *et al.*<sup>2</sup>.

L'approche *One Health* implique que les santés humaine et animale sont interdépendantes et liées à la qualité des écosystèmes dans lesquels les êtres vivants coexistent, supposant que l'altération des écosystèmes induit un risque pour les santés humaine et animale. Cela se vérifie par exemple aisément avec la prolifération des cyanobactéries dans les écosystèmes fortement pollués (eutrophisation, dystrophisation), laissant peser un risque sur les santés humaine et animale<sup>3,4</sup>. Or, l'application du concept *One Health* n'est pas toujours évidente. C'est le cas pour

les maladies chroniques non-infectieuses (*e.g.* obésité, diabète, cancers) ou les maladies mentales. L'implication des compartiments "animal" et "environnement" est moins évidente que pour les maladies infectieuses, même si elle est de plus en plus étudiée et reconnue<sup>2</sup>.

Au niveau mondial, c'est dans les pays à revenus faibles et intermédiaires (PRFI) que l'émergence de zoonoses est la plus élevée. Cela peut être imputé à plusieurs phénomènes, dont (i) la forte diversité des écosystèmes plus à même d'abriter un agent infectieux transmissible à l'Homme et (ii) le contact important entre le compartiment "humain" et le compartiment "environnement" (*e.g.* altération et perte d'habitat, changement d'occupation des sols, consommation de produits de la faune sauvage, *etc.*)<sup>5,6</sup>. Au-delà de la bonne santé intrinsèque des écosystèmes, la perméabilité entre les différents compartiments *One Health* joue un rôle primordial dans la transmission des pathogènes.

L'approche *One Health* est particulièrement importante lorsque la perméabilité entre les trois compartiments "humain", "animal" et "environnement" est élevée. C'est le cas dans la plupart des PRFI, où les infrastructures d'hygiène individuelles et collectives efficaces manquent. Il faut ajouter à cela la fragilité des systèmes de santé des PRFI qui exacerbe la transmission interhumaine des agents infectieux, mais aussi entre les trois compartiments "humain", "animal" et "environnement"<sup>7</sup>. En revanche, l'approche *One Health* est souvent moins évidente dans les pays à revenus élevés (PRE) où la perméabilité entre humains-animaux-écosystèmes est plus faible, notamment grâce à des infrastructures d'hygiène développées et efficaces, des populations davantage sensibilisées et éduquées aux pratiques d'hygiène, la sécurité et l'hygiène alimentaire, des systèmes de surveillance opérationnels ou encore des réglementations claires, cohérentes et strictes<sup>8,9,10</sup>.

Le concept *One Health* est facilement applicable aux zoonoses et à la transmission de pathogènes animaux à l'Homme. En revanche, le rôle des compartiments "animal" et "environnement" est moins évident lorsque l'on parle des bacilles à Gram négatif commensaux et pathogènes (BGN-CP). D'une part, certaines études décrivent la transmission d'*Escherichia coli* entéropathogène d'animaux d'élevages à l'Homme, majoritairement *via* un contact étroit avec les animaux d'élevage<sup>11,12</sup>. D'autres études rapportent une similarité génétique entre les isolats d'*E. coli* et de *Klebsiella pneumoniae* humains, animaux et environnementaux<sup>13,14,15</sup>, supposant une perméabilité entre les trois compartiments. D'autre part, plusieurs études montrent que les isolats d'*E. coli* et de *K. pneumoniae* sont génétiquement distincts suivant le compartiment d'origine<sup>16,17,18</sup>, supposant une perméabilité limitée entre les trois compartiments "humain", "animal" et "environnement", diminuant ainsi la pertinence de l'approche *One*

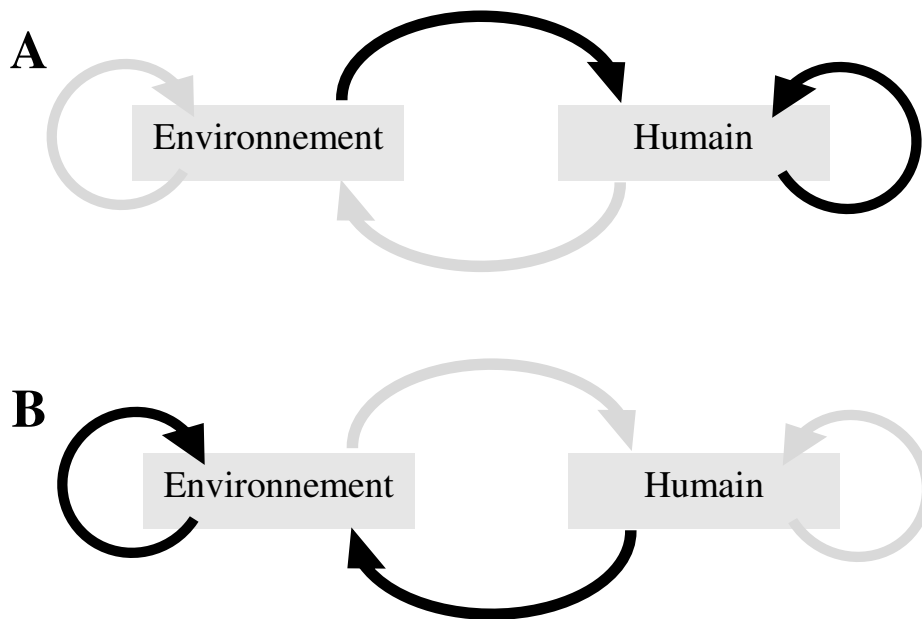
*Health* lorsqu'appliquée aux BGN-CP. Nous savons que les BGN-CP sont majoritairement rejetés dans l'environnement *via* les effluents des stations d'épuration des eaux usées (STEU) et l'agriculture<sup>19,20</sup>. En revanche, le devenir et le comportement de ces bactéries associées à l'Homme dans les écosystèmes est beaucoup moins étudié que dans les compartiments "humain" et "animal".

La lutte contre la résistance aux antibiotiques est un point central de l'approche *One Health*<sup>21</sup>. La résistance aux antibiotiques sélectionnée *in vitro* représente un coût biologique susceptible d'affecter la *fitness* des BGN-CP en l'absence de sélection par les antibiotiques<sup>22,23,24,25</sup>. Comparées à leurs clones sensibles, les BGN-CP résistants aux antibiotiques semblent donc défavorisées en l'absence de sélection par les antibiotiques. D'autres études, minoritaires, considèrent au contraire que le coût biologique lié à l'antibiorésistance est faible<sup>26,27</sup>. Ces différentes études aux résultats contradictoires, souvent réalisées *in vitro*, soulignent la méconnaissance globale du devenir des bactéries résistantes aux antibiotiques (BRA) dans l'environnement.

En résumé, la diffusion et les échanges des BGN-CP restent mal connus au sein du tryptique humains-animaux-écosystèmes. Bien que les trois compartiments *One Health* ne soient pas imperméables à la diffusion des BGN-CP, il semblerait néanmoins que la diffusion des BGN-CP soit limitée depuis les compartiments "animal" et "environnement" au compartiment "humain", au moins dans les PRE, mieux étudiés. La plupart des études existantes s'est intéressée à la perméabilité entre les compartiments "animal" et "humain", laissant de côté le compartiment "environnement", comme c'est fréquemment le cas lors de la mise en place d'approches *One Health*<sup>2</sup>. L'approche *One Health* a souvent pour finalité la santé humaine, et est couramment appliquée dans le sens "animal" → "humain" ou "environnement" → "humain". Hors, cette approche n'a de sens que si l'on considère les interactions (*de facto* réciproques) entre les différents compartiments : l'environnement est contaminé par l'Homme et les animaux, et peut également être une voie d'infection pour l'Homme. Afin de comprendre comment l'Homme peut être contaminé par un BGN-CP *via* son environnement, il est d'abord opportun de comprendre comment ces espèces bactériennes se comportent dans les écosystèmes et dans quelles mesures les écosystèmes sont résistants face à la contamination par les BGN-CP. Un écosystème à l'équilibre (*i.e.* "en bonne santé"), ou un écosystème dans lequel la biodiversité est élevée, est-il plus résistant qu'un écosystème en situation de déséquilibre (*i.e.* altéré), ou à faible biodiversité, face aux BGN-CP relargués dans l'environnement ? Les écosystèmes

peuvent-ils constituer des réservoirs de BGN-CP, conduisant ainsi à une probabilité de contamination "environnement" → "humain" plus élevée ?

Le présent travail a donc pour but général de combler le manque de connaissances et de compréhension qu'il existe quant à la contamination environnementale par les BGN-CP, et surtout leur devenir dans les écosystèmes aquatiques (Figure 2). Ce travail a plusieurs objectifs particuliers. Premièrement, au-delà des sources déjà connues (STEU, agriculture), il vise à reconstruire les voies de dispersion des BGN-CP dans et entre les écosystèmes aquatiques. Deuxièmement, il veut identifier les facteurs favorisant la survie et la prolifération des BGN-CP dans les écosystèmes aquatiques. Ce travail a donc un objectif descriptif, avec la description des patrons d'occurrence, d'abondance et des structures de population des BGN-CP dans l'environnement, et surtout un objectif explicatif, avec l'étude de l'origine et du devenir des BGN-CP dans l'environnement, et l'analyse du contrôle biotique des BGN-CP dans les écosystèmes aquatiques.



**Figure 2. Objectif général du présent travail de doctorat.** **A.** Approche traditionnelle, centrée sur la santé humaine : transmission des BGN-CP de l'environnement à l'Homme, et transmission interhumaine. **B.** Approche du présent travail de recherche, centré sur l'environnement : contamination de l'environnement par les BGN-CP, et comportement dans les écosystèmes.

Ce document est articulé autour de quatre chapitres. Le premier détaille l'état actuel de la connaissance sur le devenir des BGN-CP dans l'environnement, en portant un intérêt particulier à la diffusion de la résistance aux antibiotiques, grâce à une synthèse bibliographique. Ensuite, l'origine, la dispersion et la survie des BGN-CP sont étudiées dans les écosystèmes alluviaux. Afin de saisir l'importance de l'échelle considérée, les deuxième et troisième chapitres utilisent une approche inter- et intra-hydrosystèmes, respectivement. Le quatrième chapitre s'intéresse aux interactions biotiques pouvant exister entre les BGN-CP et les écosystèmes : plus précisément, l'interaction *E. coli*-végétation est étudiée. Finalement, les résultats exposés dans ces quatre chapitres sont discutés dans un contexte plus global.





## 2. MÉTHODOLOGIES GÉNÉRALES

### 2.1 Espèces bactériennes d'intérêt

#### 2.1.1. *Escherichia coli*, *Klebsiella pneumoniae* et *Pseudomonas aeruginosa*

Les trois espèces bactériennes d'intérêt étudiées dans ce travail sont *E. coli*, *K. pneumoniae* et *P. aeruginosa* (Table 1). Ces trois bactéries sont des bacilles à Gram négatif (BGN). *E. coli* est un commensal du microbiote intestinal des vertébrés. Cette espèce bactérienne est également retrouvée dans une multitude d'habitats secondaires, de l'environnement hospitalier, aux écosystèmes naturels. Certaines souches d'*E. coli* sont responsables d'infections intra- et extra-intestinales (Table 1). L'espèce *E. coli* est divisée en huit phylogroupes différents : A, B1, B2, C, D, E, F et G<sup>28,29</sup>. Les phylogroupes B2, F et G sont les plus virulents<sup>29,30</sup>. Les clones pathogènes majeurs sont ST10, ST12, ST38, ST69, ST73, ST95, ST131<sup>31</sup>. L'espèce bactérienne *K. pneumoniae* est retrouvée dans plusieurs réservoirs et fait partie du microbiome humain. Entre 6 et 23 % des humains sont porteurs de *K. pneumoniae* dans les PRE<sup>32,33</sup>. L'espèce bactérienne *P. aeruginosa* est ubiquitaire, mais considérée comme particulièrement abondante dans les milieux humides contaminés par les antibiotiques, notamment hospitaliers<sup>34</sup>. *K. pneumoniae* et *P. aeruginosa* sont des pathogènes opportunistes.

Les souches d'*E. coli* sont fréquemment rencontrées dans les infections dans la communauté, dans laquelle cette espèce diffuse facilement<sup>35</sup>. A l'inverse, les souches de *K. pneumoniae* et *P. aeruginosa* diffusent davantage dans les établissements de santé et sont principalement rencontrées dans les infections liées aux soins<sup>36,37</sup>. *E. coli*, *K. pneumoniae* et *P. aeruginosa* sont responsables de 23,6 %, 5,6 % et 6,3 % des infections liées aux soins en France, respectivement<sup>37</sup>. Ces BGN-PC contaminent l'environnement *via* les selles (effluents de STEU, agriculture). *E. coli*, et dans de moindres mesures, *K. pneumoniae* et *P. aeruginosa*, sont ainsi fréquemment utilisées comme marqueurs de la contamination environnementale par les BGN-CP.

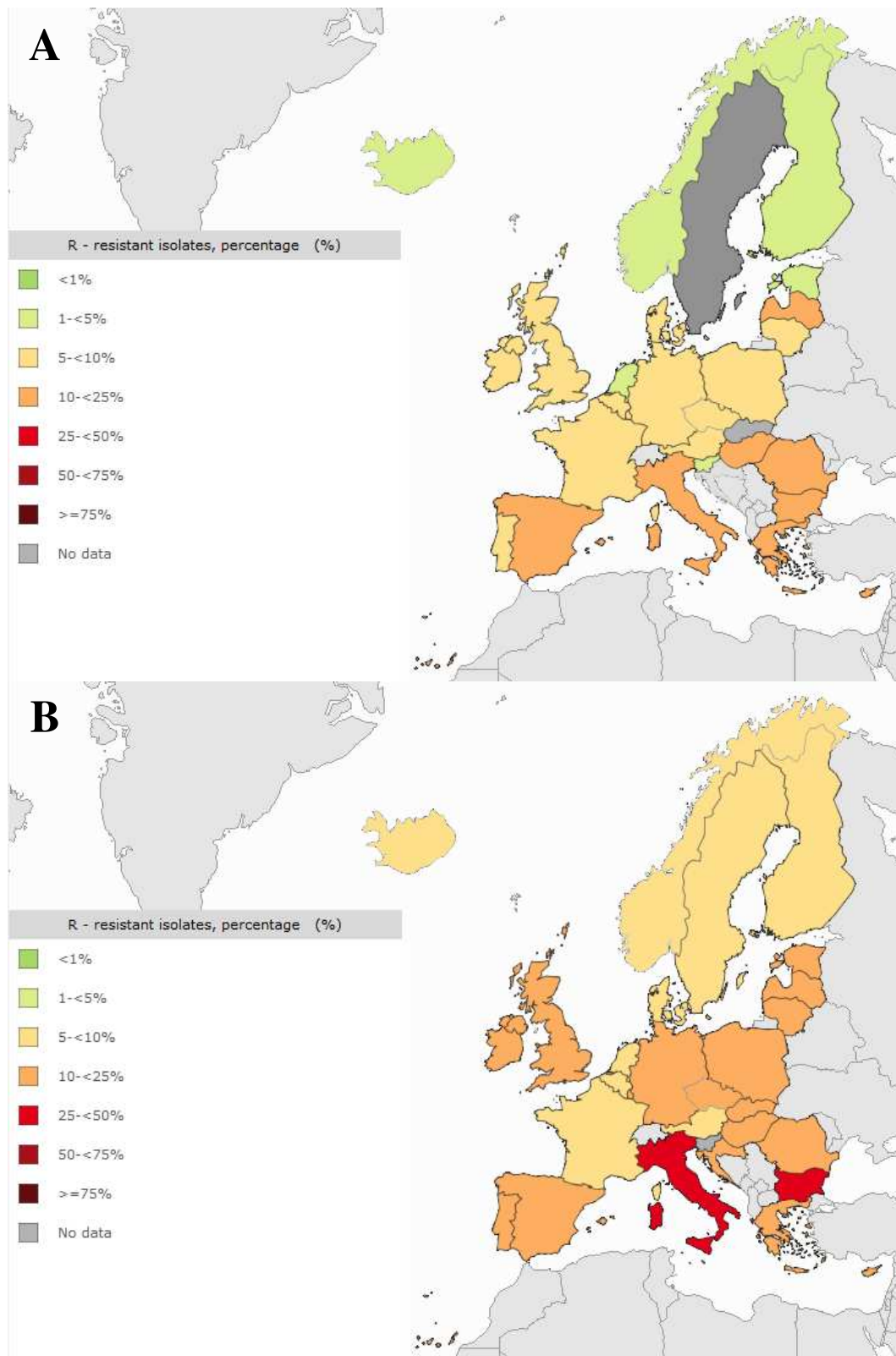
#### 2.1.2. Résistance aux antibiotiques

En rendant les traitements des infections bactériennes moins efficaces, la résistance aux antibiotiques – en particulier aux céphalosporines de troisième génération (C3G) – est devenue

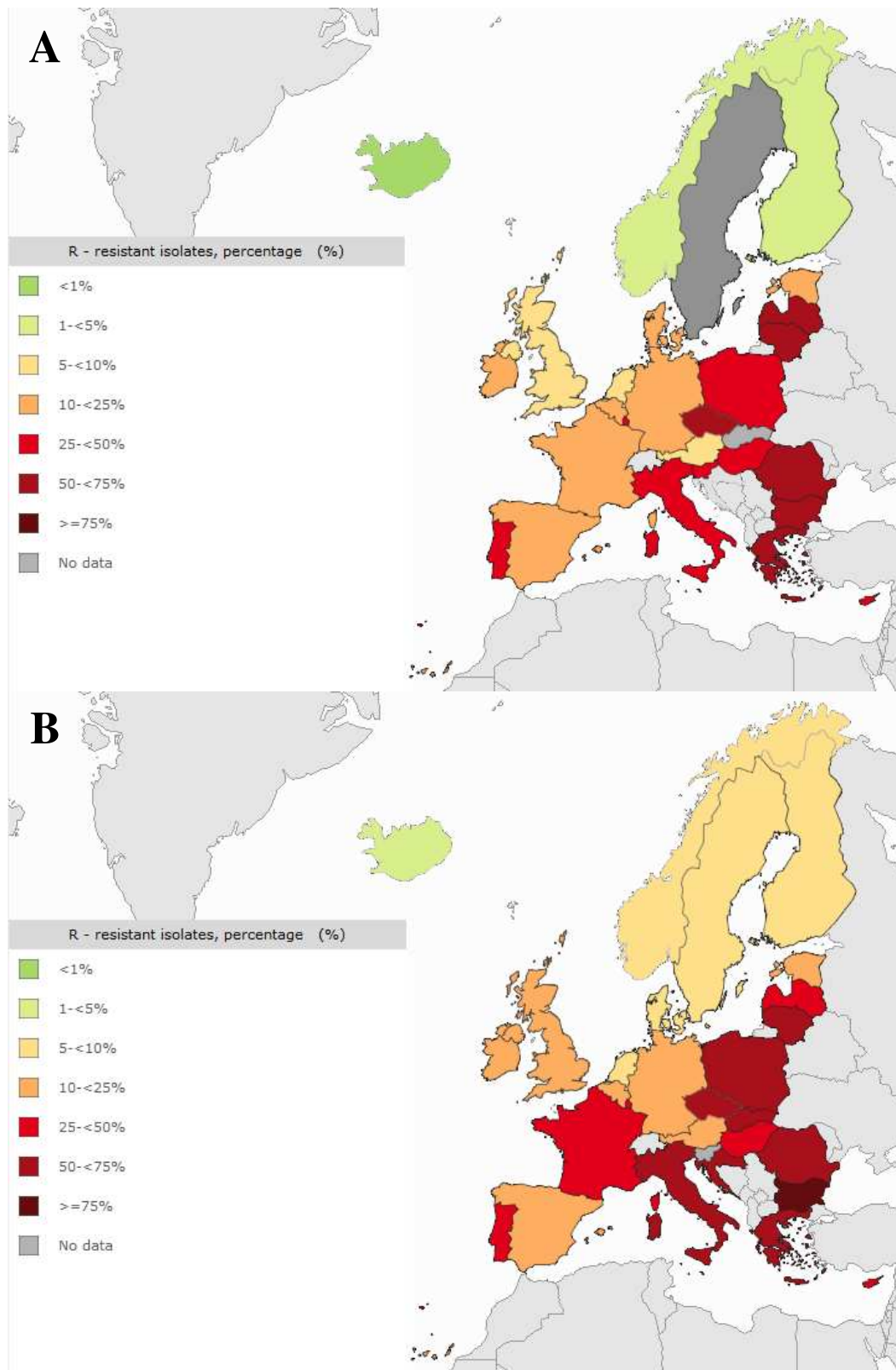
une menace croissante pour la santé mondiale<sup>38,39</sup> (Figure 3 et 4). Les trois espèces bactériennes d'intérêt de ce travail de recherche sont capables d'acquérir de nombreux déterminants de résistance aux antibiotiques<sup>40</sup>. La production de  $\beta$ -lactamases à spectre étendu (BLSE) est la principale cause de la résistance aux C3G chez les *Enterobacteriaceae*<sup>40</sup>. La quantification des isolats d'*E. coli* producteurs de BLSE est fréquemment utilisée comme marqueur de la contamination environnementale par les BRA. Dans ce travail, les isolats d'*E. coli* producteurs de BLSE et les *K. pneumoniae* producteurs de BLSE sont utilisés comme marqueurs de la contamination environnementale par les BGN résistants aux antibiotiques.

**Table 1. Généralités sur *E. coli*, *K. pneumoniae* et *P. aeruginosa*.**

	<i>E. coli</i>	<i>K. pneumoniae</i>	<i>P. aeruginosa</i>
<b>Type</b>	Bacille à Gram négatif	Bacille à Gram négatif	Bacille à Gram négatif
<b>Classification</b>	Règne : <i>Bacteria</i> Phylum : <i>Proteobacteria</i> Classe : <i>Gamma Proteobacteria</i> Ordre : <i>Enterobacteriales</i> Famille : <i>Enterobacteriaceae</i> Genre : <i>Escherichia</i>	Règne : <i>Bacteria</i> Phylum : <i>Proteobacteria</i> Classe : <i>Gamma Proteobacteria</i> Ordre : <i>Enterobacteriales</i> Famille : <i>Enterobacteriaceae</i> Genre : <i>Klebsiella</i>	Règne : <i>Bacteria</i> Phylum : <i>Proteobacteria</i> Classe : <i>Gamma proteobacteria</i> Ordre : <i>Pseudomonadales</i> Famille : <i>Pseudomonadaceae</i> Genre : <i>Pseudomonas</i>
<b>Pathologies associées</b>	Diarrhées, gastro-entérites, infections urinaires, méningites, septicémies, <i>etc.</i>  Huit pathovars connus <sup>35</sup> : 6 intra-intestinaux ( <i>E. coli</i> entéropathogène, <i>E. coli</i> entérohémorragique, <i>E. coli</i> entérotoxigène, <i>E. coli</i> entéro-invasif, <i>E. coli</i> entéro-agrégatif, <i>E. coli</i> à adhérence diffuse) et 2 extra-intestinaux ( <i>E. coli</i> uropathogène, <i>E. coli</i> à méningite néonatale).	Infections pulmonaires et urinaires, septicémies, <i>etc.</i>	Infections pulmonaires, urinaires, oculaires, cutanées, <i>etc.</i>
<b>Résistance aux C3G</b>	Taux d'isolats invasifs C3G-R en 2019 : 6-37 % en Europe, 9 % en France <sup>41</sup>	Taux d'isolats invasifs C3G-R en 2019 : 4-76 % en Europe, 30 % en France <sup>41</sup>	Espèce naturellement résistante aux C3G



**Figure 3. Evolution de la résistance aux antibiotiques chez *E. coli* en Europe entre 2009 et 2019.** Taux d'isolats invasifs d'*E. coli* résistants aux C3G, dont la majorité produisent une BLSE, entre 2009 (A) et 2019 (B) en Europe. Atlas de la surveillance des maladies infectieuses, Centre européen de prévention et de contrôle des maladies<sup>41</sup>.



**Figure 4. Evolution de la résistance aux antibiotiques chez *K. pneumoniae* en Europe entre 2009 et 2019.** Taux d'isolats invasifs de *K. pneumoniae* résistants aux C3G, dont la majorité produisent une BLSE, entre 2009 (A) et 2019 (B) en Europe. Atlas de la surveillance des maladies infectieuses, Centre européen de prévention et de contrôle des maladies<sup>41</sup>.

## 2.2 Approche inter-hydrosystème

Les BGN-CP – dont *E. coli*, *K. pneumoniae* et *P. aeruginosa* – atteignent l'environnement principalement *via* les effluents de STEU<sup>42,43,44,20</sup>, les pratiques agricoles<sup>45,46,47,48</sup> et aquacoles<sup>49,50,51</sup>. Les écosystèmes aquatiques apparaissent donc particulièrement exposés à la contamination par les BGN-CP, car (i) ils constituent un récepteur direct des contaminations humaines, et (ii) ils reçoivent des eaux de ruissellement (superficielles) et de lessivage (souterraines) potentiellement contaminées. Ce travail se concentre donc sur le devenir et le comportement des BGN-CP dans les environnements aquatiques, et plus particulièrement dans les écosystèmes alluviaux. Les plaines d'inondation sont composées d'écosystèmes aquatiques ou sub-aquatiques diversifiés, dont la rivière principale, les affluents, les nappes phréatiques et une multitude de zones humides (*e.g.* annexes aquatiques, prairies, forêts inondables).

La définition d'une zone humide est vaste et variable. La Convention de Ramsar définit les zones humides comme "des étendues de marais, de fagnes, de tourbières ou d'eaux naturelles ou artificielles, permanentes ou temporaires, où l'eau est stagnante ou courante, douce, saumâtre, ou salée, y compris des étendues d'eau marines dont la profondeur à marée basse n'excède pas six mètres"<sup>52</sup>. Plus de 250 millions et plus de 28 millions d'hectares de zones humides sont protégées par la Convention de Ramsar en 2021 à l'échelle mondiale et européenne, respectivement<sup>53</sup>. En France, 3,7 millions d'hectares sont inscrits à la Convention de Ramsar en 2021, dont 1 million d'hectares en France métropolitaine<sup>53</sup>. Législativement en France, une zone humide est définie comme "un terrain, exploité ou non, habituellement inondé ou gorgé d'eau douce, salée ou saumâtre de façon permanente ou temporaire, ou dont la végétation, quand elle existe, y est dominée par des plantes hygrophiles pendant au moins une partie de l'année" (Article L211-1 du Code de l'environnement, France, 2020). Les zones humides sont des réservoirs d'eau douce et de biodiversité. Les zones humides alluviales sont majoritairement des bras morts de la rivière principale, qui sont alimentées par un mélange d'eaux de surface et d'eaux souterraines. Les zones humides alluviales sont des écosystèmes hétérogènes en termes d'alimentation et donc de caractéristiques physico-chimiques de l'eau, mais également en termes d'hydrologie, de géomorphologie, d'écologie, de végétation, *etc*<sup>54</sup>. Cette diversité fonctionnelle naturelle permet d'étudier la capacité d'écosystèmes fonctionnellement contrastés à recevoir et abriter sur le long terme les BGN-CP étudiés.

Afin de répondre aux objectifs généraux du devenir des BGN-CP dans l'environnement, le deuxième chapitre du présent travail explore l'origine, la dispersion et la survie d'*E. coli*, d'*E. coli* producteur de BLSE, de *K. pneumoniae*, de *K. pneumoniae* producteur de BLSE et de

*P. aeruginosa* dans plusieurs hydrosystèmes. Trois hydrosystèmes de l'est de la France ont été étudiés : les plaines alluviales de l'Ain, du Doubs et de la Loue. Un intérêt particulier a été porté aux zones humides de ces trois plaines alluviales, très hétérogènes en termes de fonctionnement et d'eutrophisation, constituant ainsi une large gamme d'écosystèmes aquatiques. Deux articles scientifiques traitant de cet aspect inter-hydrosystèmes, publiés dans *FEMS Microbiology Ecology* et *Frontiers in Microbiology*, sont présentés dans le deuxième chapitre du présent document.

### 2.3 Approche intra-hydrosystème

Après l'étude globale du devenir des BGN-CP dans les écosystèmes aquatiques alluviaux de plusieurs hydrosystèmes, le troisième chapitre s'est attardé sur une unique plaine alluviale afin de tracer avec précision la ou les sources des BGN-CP, leur circulation dans les cours d'eau et dans les différents écosystèmes de la plaine alluviale, et leur capacité à survivre au cours de leur dispersion et dans les écosystèmes récepteurs. La plaine alluviale de l'Ain a été préférée pour plusieurs raisons. Premièrement, les écosystèmes aquatiques alluviaux sont fonctionnellement variés dans cette plaine d'inondation, notamment en termes géomorphologique (et par conséquent, hydrologique, hydrogéologique et écologique) et en termes d'anthropisation. Deuxièmement, le fonctionnement et les écosystèmes de la plaine alluviale de l'Ain sont bien décrits. Troisièmement, comme nous le verrons plus tard, le deuxième chapitre suppose des contaminations aussi bien humaines qu'agricoles de la plaine alluviale de l'Ain par les BGN-CP, ce qui en fait un cas d'étude de choix. Enfin, le lit majeur de la rivière d'Ain est majoritairement composé d'écosystèmes méso-oligotrophes, permettant (i) de faciliter la compréhension des interactions entre BGN-CP en caractéristiques physico-chimiques des écosystèmes, et (ii) d'étudier la résistance des écosystèmes peu dégradés face à la contamination par les BGN-CP dans un contexte *One Health*. Une grande diversité d'écosystèmes aquatiques de la plaine alluviale de l'Ain a été densément échantillonnée : la rivière principale, les affluents, les zones humides alluviales et les eaux souterraines. Un manuscrit soumis à *Water Research* traitant de cet aspect intra-hydrosystème est présenté dans le troisième chapitre du présent document.



## 2.4 Interaction bacilles à Gram négatif – végétation

Afin de mettre en évidence les relations entre les abondances des BGN-CP retrouvés dans les écosystèmes alluviaux et les caractéristiques biotiques des écosystèmes, nous avons étudié l'interaction entre les BGN-CP et la végétation. Les paramètres biotiques – la végétation dans le cas présent – jouent-ils un rôle sur la survie des BGN-CP dans les écosystèmes alluviaux ? Si oui, quels processus écologiques entrent en jeu ? Plusieurs études ont montré que les zones humides artificielles végétalisées réduisent la charge bactérienne, soulignant les propriétés de biorémédiation de certaines espèces végétales<sup>55,56,57</sup>. D'autres rapportent plus précisément que la production de métabolites secondaires par les macrophytes réduit les abondances de cyanobactéries et de phytoplancton<sup>58,59</sup>. Afin d'aborder cette question, nous avons mis en place une expérimentation *in vitro* visant à étudier le contrôle biotique des BGN-CP dans les écosystèmes aquatiques. Pour cela, nous avons suivi l'abondance d'*E. coli* en présence de quatre espèces de macrophytes présentant, d'après la littérature, des capacités de production de métabolites secondaires, dont certains reconnus comme affectant la croissance bactérienne. Dans un souci de cohérence, les macrophytes utilisés dans cette expérimentation sont typiques d'écosystèmes aquatiques calmes et tempérés, et sont largement retrouvés dans les plaines alluviales de l'Ain, du Doubs et de la Loue. Les individus utilisés proviennent directement de zones humides de la plaine alluviale de l'Ain étudiées dans le chapitre précédent. Un article scientifique traitant de l'interaction *E. coli*-végétation, publié dans *Antibiotics*, est présenté dans le quatrième chapitre du présent document.

*CHAPITRE 1*

LE DEVENIR DES BACTÉRIES RÉSISTANTES  
AUX ANTIBIOTIQUES DANS  
L'ENVIRONNEMENT



### 3. CHAPITRE 1 : LE DEVENIR DES BACTÉRIES RÉSISTANTES AUX ANTIBIOTIQUES DANS L'ENVIRONNEMENT

**Titre de l'ouvrage :** Contaminants émergents Vol. 1 (*Emerging Contaminants Vol. 1*)

**Titre du chapitre :** Le devenir des bactéries résistantes aux antibiotiques dans l'environnement

**Maison d'édition :** Springer International Publishing

**État :** publié, 2021

**Éléments essentiels :**

- Les rejets des STEU (eaux usées traitées et biosolides) sont les principales sources de déterminants de la résistance aux antibiotiques dans l'environnement dans les PRE, en particulier si les STEU sont reliées à un réseau d'égouts unitaires ;
- Les bactéries résistantes aux antibiotiques (BRA) sont retrouvées dans de nombreux écosystèmes, principalement aquatiques ;
- Malgré la dispersion de la résistance aux antibiotiques dans notre environnement, le risque associé semble limité dans les PRE ;
- La résistance aux antibiotiques qui se propage dans l'environnement et tout au long du continuum *One Health* est extrêmement problématique dans les PRFI.

## Résumé

La découverte des antibiotiques au XX<sup>e</sup> siècle a largement amélioré la santé humaine. Cependant, l'utilisation croissante et disproportionnée de ces médicaments a favorisé l'émergence et la diffusion de bactéries résistantes aux antibiotiques (BRA). Ces BRA se propagent dans la communauté humaine. Notre environnement est pollué par ces contaminants émergents, principalement par les effluents des stations d'épuration des eaux usées et par l'amendement des sols avec des déchets organiques résiduels. L'agriculture, les pâturages intensifs et les aquacultures sont également des sources de BRA dans l'environnement. Une fois dans l'environnement, les BRA se propagent principalement dans les écosystèmes aquatiques où elles peuvent persister à l'état cultivable ou non cultivable. Les antimicrobiens (*i.e.* antibiotiques, métaux, biocides) ont les mêmes sources que les BRA et peuvent exercer une pression sélective sur les communautés bactériennes. Dans quelle mesure les environnements contaminés par des BRA constituent-ils un nouveau risque pour les populations humaines ? Les Hommes des pays à revenus faibles et intermédiaires sont plus fréquemment exposés que ceux des pays à revenus élevés, qui bénéficient de traitements efficaces des eaux usées. En effet, diverses mesures de gestion peuvent réduire la probabilité d'exposition humaine à la résistance aux antibiotiques dans l'environnement ; ces mesures comprennent des traitements efficaces des eaux usées, la mise en œuvre de politiques locales et l'échange de connaissances. En outre, la propagation de la résistance aux antibiotiques étant un problème de santé publique et environnementale, ce problème ne pourra être résolu que par une approche multidisciplinaire impliquant toutes les parties prenantes.

# Chapter 4

## The Fate of Antibiotic-Resistant Bacteria in the Environment



Charles P. Henriot, Daniel Martak, Christophe Dagot, Fabienne Petit, Edward Topp, Xavier Bertrand, Gudrun Bornette, and Didier Hocquet

**Abstract** The discovery of antibiotics in the twentieth century largely improved health conditions. However, the increasing and disproportionate use of these drugs has led to the emergence of antibiotic-resistant bacteria. Currently, these antibiotic-resistant bacteria are spreading within the human community. Our environment is polluted by these emerging contaminants, mostly through the effluents of wastewater treatment plants and soil amendments with residual organic waste. Farming, intensive pasturing, and aquacultures are also sources of antibiotic-resistant bacteria in the environment. Once released into the environment, antibiotic-resistant bacteria spread mostly in aquatic ecosystems where they can persist in culturable or non-culturable states. Together with antibiotic-resistant bacteria, antimicrobials (i.e.

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antibiotics, metals and biocides) are released most often from the same sources and can exert selective pressure on bacterial communities. To what extent are environments contaminated with antibiotic-resistant bacteria a new risk for human populations? Humans in low and middle income countries are more frequently exposed than humans in high income countries, benefiting from the efficient collection and treatment of wastewater. Indeed, various management measures can be implemented to reduce the likelihood of human exposure to environmental antibiotic resistance and the subsequent risk to human health; such measures include efficient wastewater treatments, the implementation of local policies and knowledge exchange. Moreover, as the spread of antibiotic resistance is both a public and an environmental health concern, this issue can only be understood through a multidisciplinary approach that involves all stakeholders.

**Keywords** Antibiotic resistance · Antibiotic-resistant bacteria · Wastewater treatment plant · Environmental exposure · Water environment

### Abbreviations

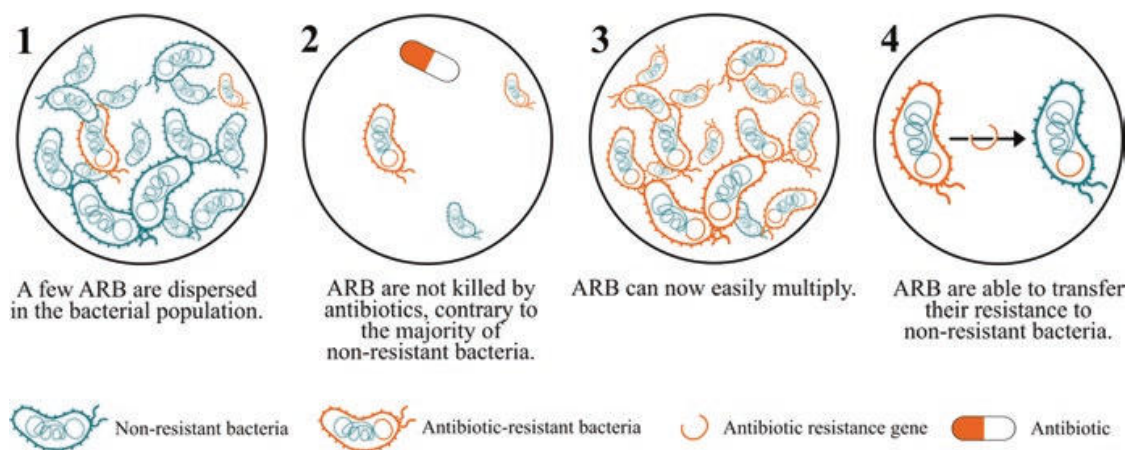
ARB	antibiotic-resistant bacteria
ARG	antibiotic resistance gene
CFU	colony-forming unit
ESBL	extended-spectrum $\beta$ -lactamase
VBNC	viable but non-culturable
WWTP	wastewater treatment plant

## 4.1 Introduction

The discovery of antibiotics in the twentieth century largely improved health conditions. Antibiotics are natural, semi-synthetic or synthetic molecules capable of destroying bacteria (bactericidal antibiotics) or inhibiting their growth (bacteriostatic antibiotics). A microorganism is said to be resistant when it multiplies in the presence of a given antibiotic. Antibiotic-resistant bacteria are thus bacteria able to grow in the presence of a given antibiotic. This ability to resist can be intrinsic and specific to all the isolates of a given bacterial species or genus (natural resistance). Metagenomic approaches have shown that the soil microbiota, the source of many antibiotic-producing microorganisms, is a reservoir of antibiotic-resistant genes, with nucleotidic sequences close or identical to those found in clinically important pathogenic bacteria (Forsberg et al. 2012; Nesme and Simonet 2015; Jiang et al. 2017). In contrast, acquired resistance differs from natural resistance since it is only observed in a subpopulation of a species that is susceptible to the considered antibiotic. Acquired resistance relies either on the mutation of chromosomal genes

(modification of the genetic capital) or the acquisition of genes by horizontal gene transfer. Among human pathogens, some bacterial species are intrinsically resistant to certain antibiotics (e.g. *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Enterobacter cloacae*) and can acquire resistance to other antibiotics. However, the majority of human pathogenic bacteria are not intrinsically resistant but can acquire resistance to different antibiotics (e.g. *Escherichia coli* and *Staphylococcus aureus*).

The misuse and increasing use of antibiotics has led to the emergence of antibiotic-resistant bacteria (ARB), mainly within bacterial strains of clinical relevance. ARB are therefore emerging contaminants of concern. Antibiotics give ARB a selective advantage over susceptible bacteria (Fig. 4.1). Antibiotics have become less effective at treating bacterial infections, and the proportion of bacteria resistant to a specific antibiotic has increased (Cecchini et al. 2015). The pace at which we are discovering new antibiotics has slowed, and bacteria have invariably become resistant to new antibiotics (Review on Antimicrobial Resistance 2016). Cecchini et al. (2015) highlighted three major threats related to antibiotic-resistant microorganisms: (i) antibiotic-resistant microorganisms prolong morbidity and increase the time spent ill with the infection, (ii) antibiotic-resistant microorganisms increase the likelihood of developing other comorbidities or complications, and (iii) by requiring more intensive treatments, patients are more likely to experience adverse reactions or secondary effects. The authors estimated that patients infected with antibiotic-resistant microorganisms are up to three times more likely to die than are other patients (Cecchini et al. 2015). More than 25,000 European citizens are estimated to die annually due to ARB infections (European Centre for Disease Prevention and Control, European Medicines Agency 2009). Globally, antimicrobial-resistant infections are estimated to cause over 0.7 million deaths per year (Cecchini et al. 2015), although reliable estimates of the true number are scarce. Between 2007 and 2015 in the European Union, cases attributed to ARB infections increased from ~240,000 to 603,000 cases, and the associated deaths increased from ~11,000 to



**Fig. 4.1** A schematic representation illustrating the selective advantage of antibiotic-resistant bacteria and explaining their spread in the presence of antibiotics compared with that of susceptible bacteria of the same species (ARB antibiotic-resistant bacteria; adapted from the World Health Organization)



27,000 (Cassini et al. 2018). A continued increase in resistance by 2050 could result in the death of ten million people worldwide each year (Review on Antimicrobial Resistance 2016). Africa and Asia are expected to be particularly impacted by antimicrobial resistance. Hence, by 2050, antimicrobial-resistant infections could cause 22,000 deaths yearly in Oceania, 317,000 deaths in North America, 390,000 deaths in Europe, 392,000 deaths in Latin America, 4,150,000 deaths in Africa, and 4,730,000 deaths in Asia (Review on Antimicrobial Resistance 2016).

Among bacteria, the class of  $\gamma$ -proteobacteria, especially the order *Enterobacteriaceae*, contains a particularly large number of human pathogens that may acquire antibiotic resistance and therefore pose a major threat to human populations (Cassini et al. 2018). The authors showed that four ARB populations were particularly harmful to humans in Europe: third-generation cephalosporin-resistant *E. coli*, methicillin-resistant *S. aureus*, carbapenem-resistant *P. aeruginosa* and third-generation cephalosporin-resistant *K. pneumoniae*. In the same geographical area, the authors estimated that these four species annually cause approximately 9100, 7000, 4200, and 3700 deaths, respectively (Cassini et al. 2018). Bacterial resistance to antibiotics has rapidly evolved over time. Hence, the most prevalent extended-spectrum  $\beta$ -lactamase (ESBL)-producing *Enterobacteriaceae*, which are able to hydrolyse third-generation cephalosporins, were *Enterobacter aerogenes* (34%) and *E. coli* (25%) in healthcare facilities in 2003, whereas *E. coli* (60%) and *K. pneumoniae* (18%) were dominant only seven years later in 2010 (Carbonne et al. 2012). In the G7 countries (Canada, France, Germany, Italy, Japan, the United Kingdom and the USA), Cecchini et al. (2015) estimated that in clinical settings, 12.1% of *E. coli* were resistant to third-generation cephalosporin and 27.7% were resistant to fluoroquinolones; 17.3% of *K. pneumoniae* were resistant to third-generation cephalosporin and 5.5% were resistant to carbapenems; and 30.5% of *S. aureus* were resistant to methicillin. In low and middle income countries the data are scarce (World Health Organization 2014). Some global ARB prevalence data (World Health Organization 2014) are reported in Table 4.1. Currently, these ARB are spreading within the human community. In 2006, 0.6% of healthy subjects living in the Paris area were carrying ESBL *E. coli* in their gut, while in 2011, this rate was 6%, reflecting a ten-fold increase in 5 years (Nicolas-Chanoine et al. 2012).

The intestinal microbiome is the primary reservoir of human-associated bacteria engaged in mutual symbiotic or commensal symbiotic relationships with their host. Thus, the intestinal microbiota of antibiotic-treated humans or livestock become ecological niches where ARB are selected (Casals-Pascual et al. 2018). For these reasons, hospitals (Hocquet et al. 2016) and intensive farms (McKinney et al. 2018; Topp et al. 2018) are the two main reservoirs with the highest prevalence of ARB. Bacteria from the intestinal microbiota, among which ARB, are dispersed into the environment *via* faeces. Therefore, the main sources of ARB emissions into the environment are human and animal excretions. Human excretions mostly end up in sewage systems in high income countries. Animal manures may in some instances be treated prior to land application. Antibiotics and other selective agents (e.g. biocides and trace metals) are also released in wastewater and may exert co-selective pressure on ARB. Bacterial contaminants then reach wastewater treatment plants

**Table 4.1** Summary table of the antibiotic-resistant bacteria prevalence in the six regions of the world

	Third-generation cephalosporin-resistant <i>E. coli</i> (%)	Fluoroquinolone-resistant <i>E. coli</i> (%)	Third-generation cephalosporin-resistant <i>K. pneumoniae</i> (%)	Carbapenem-resistant <i>K. pneumoniae</i> (%)	$\beta$ -lactam-resistant <i>S. aureus</i> (%)
African Region National data	2–70 (n = 13)	14–71 (n = 14)	8–77 (n = 13)	0–4 (n = 4)	12–80 (n = 9)
Region of the Americas National data or report to ReLAVRA	0–48 (n = 14)	8–58 (n = 16)	4–71 (n = 17)	0–11 (n = 17)	21–90 (n = 15)
Eastern Mediterranean Region National data	22–63 (n = 4)	21–62 (n = 4)	22–50 (n = 4)	0–54 (n = 4)	10–53 (n = 4)
European Region National data or report to EARS-Net	3–82 (n = 35)	8–48 (n = 35)	2–82 (n = 33)	0–68 (n = 33)	0.3–60 (n = 36)
South-East Asia Region National reports	16–68 (n = 5)	32–64 (n = 5)	34–81 (n = 4)	0–8 (n = 4)	10–26 (n = 3)
Western Pacific Region National data	0–77 (n = 13)	3–96 (n = 16)	1–72 (n = 14)	0–8 (n = 9)	4–84 (n = 16)

Source: World Health Organization (2014)

ReLAVRA Latin American Antimicrobial Resistance Surveillance Network, EARS-Net European Antimicrobial Resistance Surveillance Network

% range, n = number of countries considered

(WWTPs), which historically were not designed to mitigate contamination by ARB. A non-negligible proportion of the bacteria entering the WWTP are ultimately released into the environment (rivers, coastal waters, lakes) (Marti et al. 2014a). In high income countries, treated wastewater is thus the main source of ARB contamination in aquatic environments (Marti et al. 2014a; Guo et al. 2017). In other regions of the world, where basic hygiene infrastructures may be poorly developed and/or accessible, the situation is quite different. Indeed, approximately 2.5 billion people lack improved sanitation (World Health Organization and United Nations Children’s Fund 2013). A sizeable part of the population practices open defecation (e.g. Niger: 68%, Chad: 67%, South Sudan: 63% and India: 26%; World Health Organization 2019), thus releasing ARB directly in the environment. The issue of ARB dispersal and the associated environmental contamination is a contemporary issue that has been of growing interest for only a few decades. The

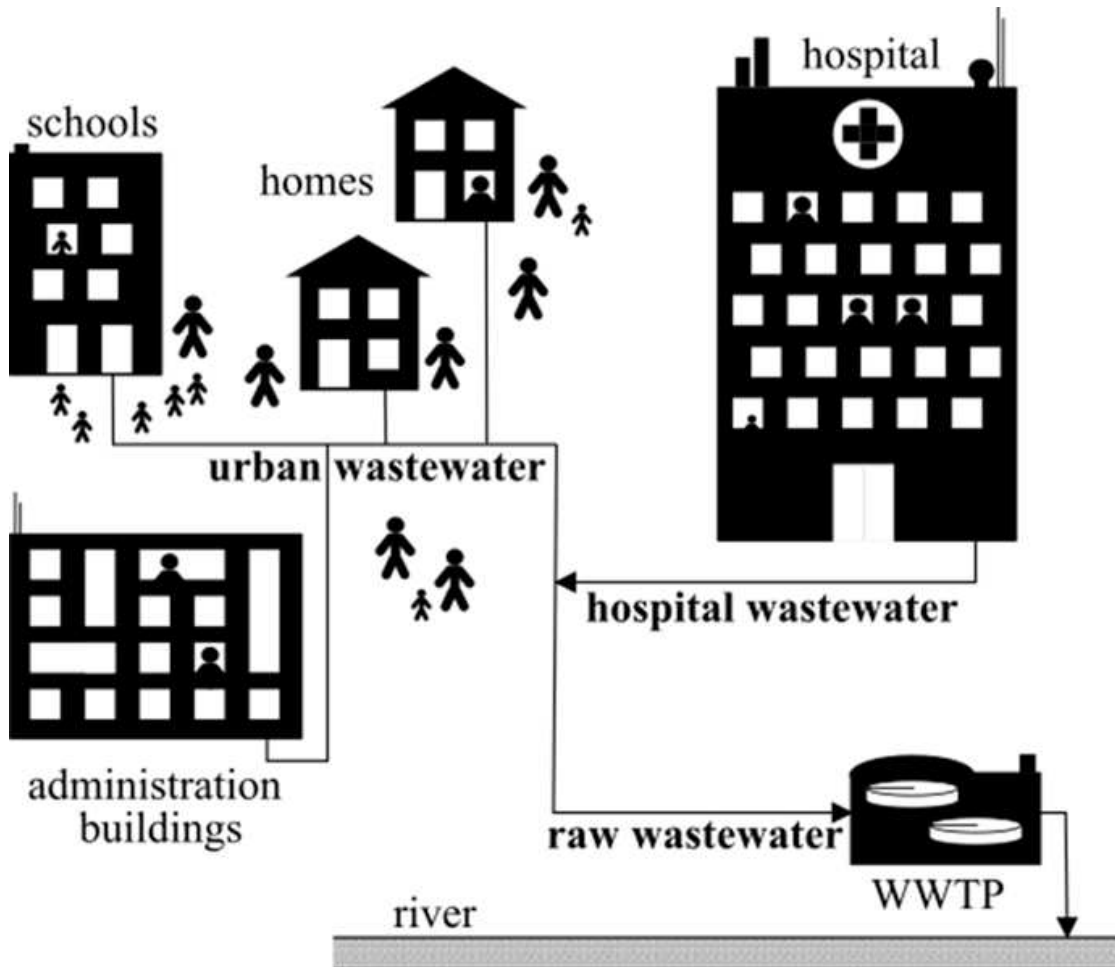
processes of dispersion, survival and selection in sewage systems – from the sewer entrance to the WWTP effluent – and in the environment still raise considerable public health concerns.

This chapter focuses on the release and dispersal of antibiotic-resistant bacteria from wastewater into the environment and the corresponding human-associated likelihood of exposure to environmental antibiotic resistance. Here, we focus on the four antibiotic-resistant bacteria that account for the major part of the resistance burden: *E. coli*, *S. aureus*, *P. aeruginosa* and *K. pneumoniae* (Cassini et al. 2018). To illustrate the release and spread of antibiotic resistance in the environment, we will also describe the spread of other bacterial species and antibiotic resistance genes. This chapter will first focus on antibiotic-resistant bacteria in hospital wastewater and urban wastewater before considering their fate in wastewater treatment plants and their release in the environment. Sources of environmental contamination other than wastewater treatment plants will also be briefly discussed. The dispersion and survival of antibiotic-resistant bacteria in several aquatic and terrestrial environments will then be studied. The resulting environmental risk associated with these antibiotic-resistant bacteria will be investigated. Finally, we will briefly discuss the mitigation processes that could reduce the environmental exposure to antibiotic-resistant bacteria.

## 4.2 Contamination of Sewage Systems by Antibiotic-Resistant Bacteria

Human faeces are a major source of antibiotic-resistant bacteria. Faeces end up in the sewage system before being treated in WWTPs. Two types of wastewaters will be of concern in this section: hospital wastewater and urban wastewater (Fig. 4.2). Together, hospital and urban wastewater combine to form the raw wastewater flow that enters WWTPs, as described in the following section. Urban wastewater contains antibiotics and antibiotic-resistant bacteria excreted by the human community. Antibiotics are widely used in hospitals, where the antibiotic-resistant bacteria prevalence is *de facto* higher than that in the community. Hospital wastewater is therefore expected to be more contaminated by antibiotic-resistant bacteria than is urban wastewater. Hospital wastewater is generally not treated before reaching a WWTP and is mixed with urban wastewater in the sewage system.

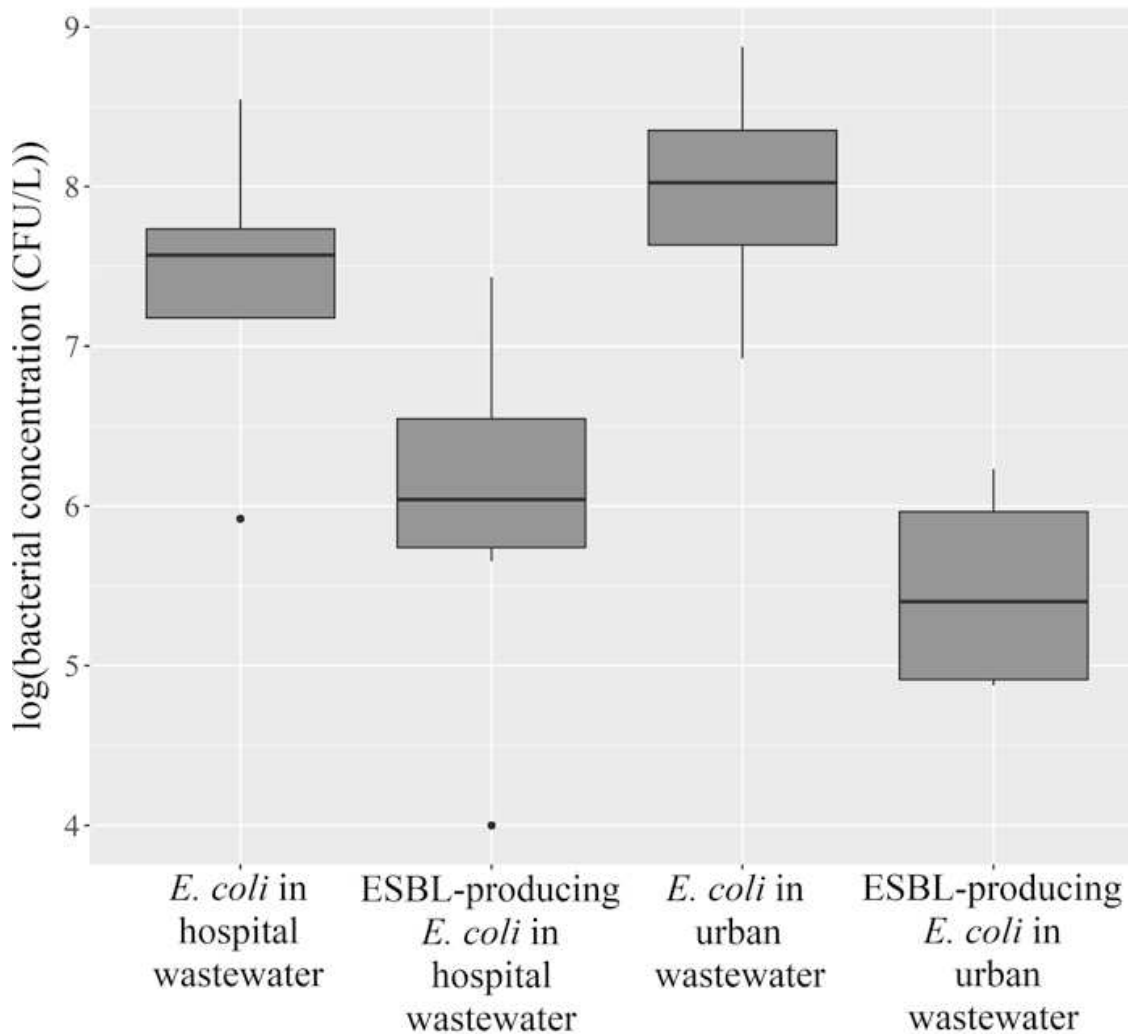
In high income countries, most houses are connected to a sewage system (e.g. ~85% in France (Ministère de la transition écologique et solidaire et Ministère des solidarités et de la santé 2019)). Other houses use non-collective sewage systems to treat their wastewater (mostly septic tanks). Their environmental impact in terms of ARB dispersion is very heterogeneous in view of the different contexts and treatment systems and such variations have been poorly studied. For these reasons, this chapter will not address this issue.



**Fig. 4.2** Simplified scheme of a sewage system. Hospital wastewater reaches urban wastewater to form the raw wastewater. The raw wastewater is treated at a wastewater treatment plant, where it releases the treated outflow into the environment (WWTP wastewater treatment plant)

### 4.2.1 Hospital Wastewater

Servais and Passerat (2009) found a higher prevalence of *E. coli* strains resistant to at least one antibiotic of clinical relevance in hospital wastewater (71%) than in urban wastewater effluent (44%). Some authors found no difference in total *E. coli* concentrations in hospital and urban wastewater but found that the proportion of ESBL-producing *E. coli* was higher in hospital wastewater than in urban wastewater (7.55% vs. 0.10% of the total *E. coli* strains, respectively, in Bréchet et al. (2014), or 3.8–39% vs. 0.1–19% of the total *E. coli* strains ( $10^5$ – $10^6$  in both urban and hospital wastewaters), respectively, in Hocquet et al. (2016)). *P. aeruginosa* concentrations have been found to be higher in hospital wastewater than in urban wastewater, and *P. aeruginosa* found in hospital wastewater was more frequently resistant to one or more antibiotics (Slekovec et al. 2012). ARB are therefore more concentrated in hospital wastewater than in urban wastewater (Fig. 4.3). In German clinical/urban wastewater, 28.2% of all isolates harboured a carbapenemase-encoding gene, whereas this proportion was 1.4% in rural systems, identifying clinical/urban



**Fig. 4.3** Comparison of *E. coli* and ESBL-producing *E. coli* in hospital and urban wastewater (CFU colony forming units, ESBL extended-spectrum  $\beta$ -lactamase; data collected from Servais and Passerat 2009; Galvin et al. 2010; Oberlé et al. 2012; Bréchet et al. 2014; Kwak et al. 2015; Drieux et al. 2016)

wastewater as a source of dissemination of bacteria resistant to last-resort antibiotics in the environment (Müller et al. 2018). Similarly, Cahill et al. (2019) reported that 59% and 14% of *Enterobacterales* were resistant to ertapenem and/or meropenem in hospital wastewater and prehospital wastewater, respectively. Furthermore, Paulus et al. (2019) detected the presence of some antibiotics (namely, fluconazole, sulfaclozine and trimethoprim) only in hospital wastewater. These examples show high and specific contamination levels in hospital wastewater by antibiotics and ARB compared to those in urban wastewater.

The minimal selective concentration is the lowest antibiotic concentration at which the bacteria resistant to an antibiotic are favoured compared to the susceptible subpopulation. In other words, the minimal selective concentration is the minimum antibiotic concentration at which the biological cost of resistance is compensated. In addition, Gullberg et al. (2011) reported that subinhibitory

concentrations of antibiotics can also select ARB. Hence, antibiotic concentrations found in hospital wastewater could be sufficient for selecting new ARB and to favour their growth (Rodriguez-Mozaz et al. 2015; Buelow et al. 2018). For example, Paulus et al. (2019) reported ciprofloxacin concentrations of 2.7–3.7 µg/L in two hospital wastewaters in the Netherlands. These concentrations are much higher than the minimum inhibitory concentration of ciprofloxacin for wild-type *E. coli* reported by Gullberg et al. (2011), which was 0.023 µg/L.

#### 4.2.2 *Urban Wastewater*

Urban wastewater is relatively more contaminated by faecal bacteria but is less contaminated by ARB than is hospital wastewater, as described in the previous section (Servais and Passerat 2009; Slekovec et al. 2012; Bréchet et al. 2014; Hocquet et al. 2016; Fig. 4.3). However, urban wastewater has been described as more diverse than hospital wastewater in terms of antibiotic resistance (Stalder et al. 2013). Furthermore, urban wastewater includes much larger volumes of wastewater than hospital wastewater. For example, hospital wastewater was reported to represent only 2.2% of the volume of raw wastewater in France (Kümmerer 2004; Buelow et al. 2018), reflecting a much higher absolute number of ARB in urban wastewater. Therefore, the majority of ARB that reach WWTPs come from urban wastewater.

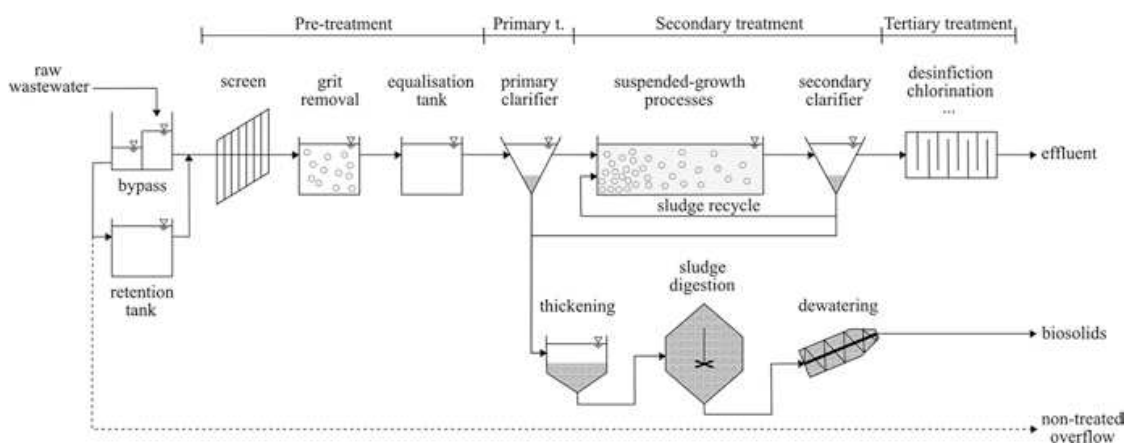
#### 4.2.3 *Would It Be Preferable to Treat Hospital Wastewater Separately from Urban Wastewater?*

In the influents of three Austrian WWTPs, the highest *E. coli* resistance rates were found at the only WWTP treating both hospital and urban wastewater (Reinthaler et al. 2003). However, Buelow et al. (2018) showed that the relative abundance of human-associated ARB at the influent of WWTPs was comparable with or without hospital wastewater. The authors reported that even if they release high loads of ARB, hospitals contributed little to the quantity and diversity of ARB in the investigated sewage systems. Buelow et al. (2020) monitored the microbiota and resistome dynamics of untreated and treated hospital and urban wastewaters over three years. Each wastewater presented a robust, distinct and stable antibiotic resistance signature over time. The different signatures mirrored the differences in antibiotic exposure for each wastewater type. The authors also studied the resistome of mixed hospital and urban wastewaters for one year: the resistome of the mixed wastewater closely resembled that of urban wastewater. Although Buelow et al. (2020) confirmed high rates of antibiotic resistance in hospital wastewater, they also highlighted that separate treatments of hospital and urban wastewater would not reduce the release of ARB in the environment. Since the volume ratio between each type of

wastewater used in this study is much higher than that reported in most sewage systems, the contribution of hospital-specific WWTPs would be limited. Overall, the following conclusions can be summarized as follows: (i) hospital wastewater contains specific antibiotic-resistant bacteria due to the use of hospital-specific antibiotics; (ii) the load of ARB is higher in hospital wastewater than in urban wastewater; and (iii) the high volume of urban wastewater (compared to that of hospital wastewater) makes it the major contributor to antibiotic resistance in the raw wastewater entering WWTPs.

### 4.3 Wastewater Treatment Plants Effluents as the Main Source of Antibiotic-Resistant Bacteria in the Environment

The raw wastewater is treated by wastewater treatment plants (Fig. 4.2) before the discharge of the treated effluents into the receiving environment. This process consists of a multitude of different treatment systems depending on several parameters (e.g. the specific contamination of the raw wastewater, the localization and the financial cost). In general, a WWTP implements the following five treatment steps (Crini and Lichtfouse 2019) (Fig. 4.4): (1) pre-treatment, which consists of grit removal, flow equalization, and fat and grease removal; (2) primary treatment, which includes a primary clarifier: simple decantation (sedimentation of a part of the solids present in the raw wastewater) with the collection of sludge; (3) secondary treatment, which usually consists of attached or suspended growth systems (including activated sludge) in which microorganisms (mainly bacteria and protozoa) consume biodegradable soluble organic contaminants; a secondary clarifier



**Fig. 4.4** General scheme of a conventional wastewater treatment plant (the untreated raw wastewater enters the WWTP; in combined sewage systems, a part of the entering raw wastewater can directly be rejected in the environment without any treatment, see the end of Sect. 4.3.2; once in a WWTP, the influent wastewater undergoes several treatment steps and is separated between the aqueous and solid phases, thus producing two types of output: aqueous effluents and biosolids)

usually follows and collects the sludge formed during the previous biological process; (4) in some cases, a third and even a fourth treatment is used to further improve the effluent quality before the effluent is discharged into the receiving environment; these treatments can consist of a variety of physical, chemical and biological advanced wastewater processes; and (5) sludge treatment, which includes thickening, dewatering, and eventual drying before agricultural (spreading) or thermal (incineration) recovery. In this chapter, sewage sludge refers to the solids separated during treatment at WWTPs, while biosolids refer to treated sewage sludge.

The main role of WWTPs is to remove most of the organic matter and nutrients present in raw wastewater prior to discharge into the receiving environment, usually a stream or river. The performance of a WWTP is generally monitored using global parameters such as dissolved oxygen, the oxidation-reduction potential, the pH and nutrients (mostly ammonia and nitrogen) (Martín de la Vega and Jaramillo-Morán 2018). The levels of human-associated bacteria are usually not monitored, simply because WWTPs were not designed to treat such bacteria and because clearance and monitoring would add a significant additional cost.

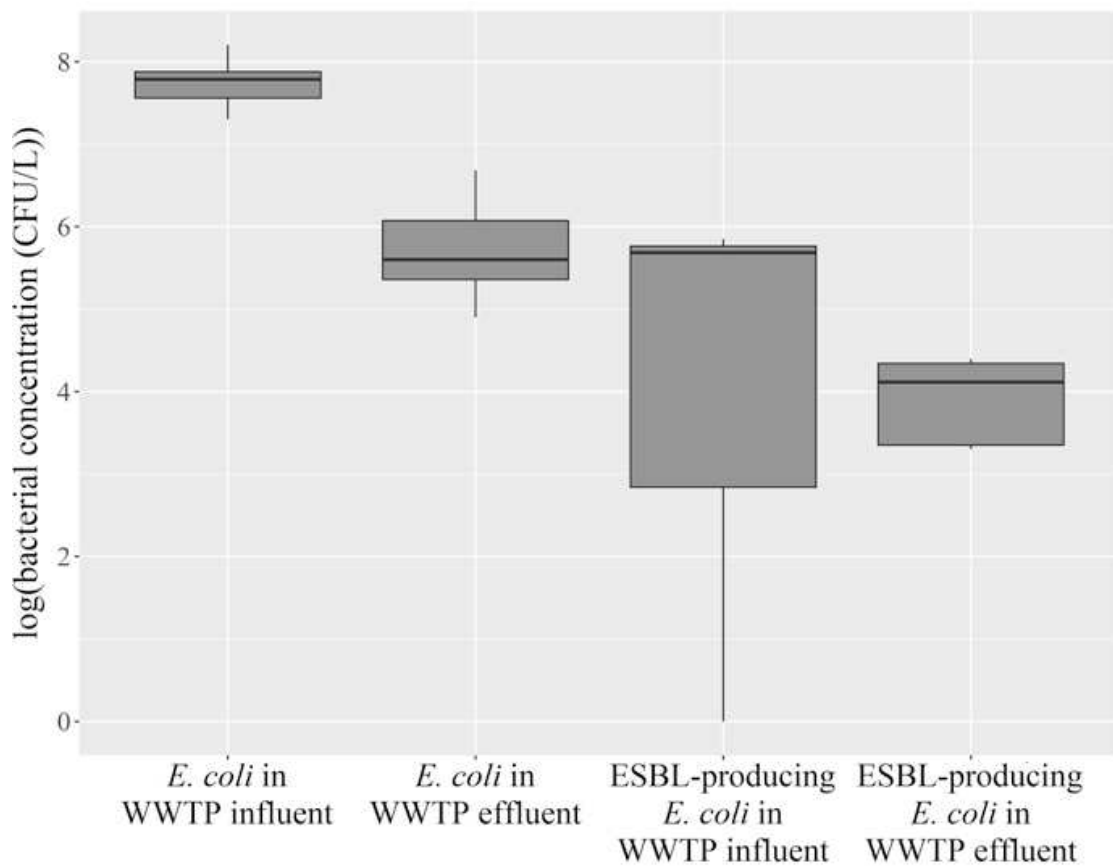
Not all bacteria survive in raw wastewater before reaching WWTPs. A Swedish study showed that the proportion of human-associated bacteria was reduced by at least 40% in the influent of WWTPs compared to the level in human faeces (Bengtsson-Palme et al. 2016). This decrease in the faecal bacteria abundance is due to a shift from anaerobic to aerobic conditions in raw wastewater, which leads to a shift from obligate anaerobic bacteria to facultative anaerobes (Bengtsson-Palme et al. 2016).

### ***4.3.1 Are Antibiotic-Resistant Bacteria Enriched in Wastewater Treatment Plants?***

This section first focuses on the aqueous phase (wastewater), from the influent to the effluent of the WWTP, before examining the solid phase (sludge) during the sludge treatment.

**In Wastewater** Most studies on the removal of ARB by WWTPs have shown good absolute clearance rates. Reinthaler et al. (2003) reported a 2.3-log reduction in the total *E. coli* concentration in the effluents of three studied WWTPs and a decrease in the resistance rates of *E. coli*. In a WWTP in Tokyo (Japan), Iwane et al. (2001) assessed coliform and *E. coli* resistance to a panel of antibiotics and found a decrease in the ARB loads during the treatment process. The percentage of *E. coli* isolates resistant to one or more antibiotics was less than two-thirds in the effluent compared with that in the influent. Schwartz et al. (2003) found that the WWTP reduced the release of vancomycin-resistant enterococci and  $\beta$ -lactam-hydrolysing *Enterobacteriaceae* into the aquatic environment. Overall, ARB loads are reduced by WWTPs (Fig. 4.5).





**Fig. 4.5** Comparison of *E. coli* and ESBL-producing *E. coli* in wastewater treatment plant influent and effluent (ESBL extended-spectrum  $\beta$ -lactamase, WWTP wastewater treatment plant; data collected from Reinthaler et al. 2003; Servais and Passerat 2009; Galvin et al. 2010; Oberlé et al. 2012; Blaak et al. 2014a; Bréchet et al. 2014; Ojer-Usoz et al. 2014; Kwak et al. 2015)

Many studies have shown a good absolute clearance rate for ARB in WWTP processes, but other studies have shown a relative enrichment in ARB throughout WWTP processes. Silva et al. (2006) investigated the clearance of antibiotic-resistant coliforms at an activated sludge WWTP in Antofagasta (Chile) and noted a significant increase in the proportion of antibiotic-resistant coliforms in the effluent. Wastewater treatment plants can therefore reduce the total number of coliforms but may increase the relative abundance of antibiotic-resistant coliforms in effluent. The same conclusions were drawn by Bréchet et al. (2014), who showed that absolute total and ESBL-producing *E. coli* concentrations both decreased within a WWTP, whereas the proportion of ESBL-producing *E. coli* increased during the WWTP treatment process, changing from 0.3% of total *E. coli* in the raw wastewater entering the WWTP to 0.6% of total *E. coli* in the effluent. Börjesson et al. (2010) confirmed that although WWTPs reduced the diversity and number of meticillin-resistant *S. aureus* strains, they simultaneously selected antibiotic-resistant strains. For other bacteria, Zhang et al. (2009b) reported that the prevalence of resistance in *Acinetobacter* isolates to amoxicillin/clavulanic acid, chloramphenicol, rifampin and  $\geq 3$  antibiotics increased from 8.7%, 25.2%, 63.1%

and 33.0%, respectively, in raw wastewater influents, to 37.9%, 69.0%, 84.5% and 72.4% respectively, in WWTP effluents. These conflicting results indicate that while WWTPs may sometimes favor ARB, these most often undergo the same clearance as their susceptible parents.

Some studies have observed a correlation between antibiotic-resistant bacteria (Gao et al. 2012) or antibiotic resistance genes (Gao et al. 2012; Mao et al. 2015; Rodriguez-Mozaz et al. 2015) and the associated antibiotics throughout the WWTP. These correlations suggest the possible selection of antibiotic resistance determinants (i.e. ARB and ARGs) by antibiotics throughout WWTP processes. Even if some studies reported high reductions in antibiotic concentrations in WWTPs (e.g. 50–98% for sulfonamides, 58–6% for tetracyclines, 56–86% for quinolones, and 58–87% for macrolides; Mao et al. 2015), the suggested selection of ARB by antibiotics in WWTPs is reinforced by the fact that Gullberg et al. (2011) revealed that ARB can be selected by subinhibitory concentrations of antibiotics. Nevertheless, we have to be careful: correlation does not imply causation. The ARB and antibiotic concentrations could be correlated because they have the same source (WWTP outflow). This enrichment is not consensually agreed upon in the scientific community. For example, Bengtsson-Palme et al. (2016) observed that despite the relatively high concentrations of tetracycline and ciprofloxacin in raw wastewater influent (above the predicted concentrations for resistance selection), there was no consistent enrichment in antibiotic resistance in the studied WWTPs. Antibiotics may poorly influence antibiotic resistance throughout the treatment process compared to the effect of other selective factors, such as oxygen availability (Bengtsson-Palme et al. 2016). Similarly, Karkman et al. (2019) reported that ARB were correlated with faecal pollution rather than with antibiotics. The previous correlation-based speculations therefore require further studies. General conclusions remain difficult to obtain because of the discrepancies in the type of bacteria and resistance investigated, type of influent wastewater, WWTP processes reported and abundance metrics used (i.e. relative/proportion or absolute value).

In addition to the question of the selection of antibiotic-resistant bacteria by antibiotics in WWTPs, the question of co-selection, in particular by metals and biocides, also arises. Trace metals can induce a similar bacterial resistance mechanism as that of antibiotics (e.g. efflux pumps), which can explain the co-selection of some ARB and ARGs by both antibiotics and metals (Mao et al. 2015; Di Cesare et al. 2016a). However, this question, which involves the selective pressure of antibiotics, is not a subject of consensus among the scientific community. For example, using metagenomics, Bengtsson-Palme et al. (2016) found limited support for co-selection between antibiotic resistance and both biocides and metals in WWTPs.

Some authors have reported that WWTPs are hotspots of horizontal gene transfer and may facilitate the dissemination of antibiotic resistance. Hultman et al. (2018) confirmed the decrease in antibiotic resistance genes during the WWTP treatment process, but found clues related to horizontal gene transfer for antibiotic resistance genes by some bacterial groups. Müller et al. (2018) also suggested that WWTPs act as “melting pots” for the horizontal gene transfer of ARGs. The proportion of ARGs associated with mobile genetic elements has been observed to increase within

WWTPs (Petrovich et al. 2018). Additionally, some studies have indicated that in WWTPs, bacteria harbour various plasmids associated with antibiotic resistance genes that can be transferred to other bacteria, favouring genetic exchange and the dissemination of antibiotic resistance among bacterial communities (Szczepanowski et al. 2009; Ju et al. 2019). Other studies revealed that the environmental conditions in WWTPs may favour horizontal gene transfer (Silva et al. 2006; Rizzo et al. 2013). All these studies have suggested that WWTPs may provide a favourable environment for the horizontal gene transfer of antibiotic resistance genes. However, studies supporting active horizontal gene transfer in WWTPs are rather scarce, and some studies assumed a limited role for horizontal gene transfer in WWTPs (Karkman et al. 2019).

**In Sludge** As briefly described before, WWTP treatment processes usually separate aqueous and solid phases twice. Primary sludge consists of the settled solids removed during the primary treatment (i.e. in the primary clarifier). Secondary sludge that is collected in the secondary clarifier includes sludge from the secondary treatment bioreactors. The sludge follows a treatment circuit parallel to that of the wastewater. The sludge is usually first thickened before being dewatered (through centrifugation, filtration and/or evaporation). The dewatered sludge is then digested (through anaerobic digestion, aerobic digestion, and/or composting) and sometimes stabilized (e.g. lime and heat stabilization). Finally, the resulting treated sludge is either incinerated (in a minority of cases, due to the cost of the process) or spread as fertilizer in fields.

Gao et al. (2012) reported an enrichment of antibiotic-resistant bacteria in WWTP sludge, as they observed that the tetracycline- and sulfamethoxazole-resistant bacteria concentrations were higher in dewatered sludge than in primary sludge (2-log and 1-log, respectively). When normalized by the total count of bacteria, the concentrations of tetracycline-resistant bacteria were also higher in dewatered sludge than in surplus sludge (from the activated sludge tank) and in primary sludge, suggesting the potential selection of antibiotic-resistant bacteria during the WWTP sludge treatment process. Anaerobic digestion and lime or heat stabilization can reduce the level of antibiotic resistance of sewage sludge compared to that of conventional dewatering and gravity-thickening methods (Munir et al. 2011; Murray et al. 2019). Previous studies suggested that the persistence of antibiotic-resistant bacteria depends on the bacterial species considered and the type of treatment applied to the WWTP sewage sludge. Before being spread on agricultural fields, biosolids are often composted. Composting is commonly reported to reduce the abundance of antibiotic-resistant bacteria (Sadikalay 2018). However, some studies have shown that the antibiotic resistance reduction is not systematic during composting (Chen et al. 2019), or that the abundance and diversity of some ARGs are enriched (Gao et al. 2012; Bengtsson-Palme et al. 2016).

Antibiotic-resistant bacteria (Brooks et al. 2007; Munir et al. 2011; Gao et al. 2012) and antibiotic resistance genes (Auerbach et al. 2007; Zhang et al. 2009a; Munir et al. 2011; Gao et al. 2012; Bengtsson-Palme et al. 2016) have been reported to be more concentrated in biosolids than in WWTP aqueous effluent (Auerbach

et al. 2007; Munir et al. 2011), supporting the sorption of antibiotic resistance determinants by sludge. Yang et al. (2014) reported a lower clearance of ARGs in sludge (20.70%, between activated sludge and anaerobic digestion sludge) than in wastewater (99.82%, between influent and effluent). Mao et al. (2015) drew the same conclusion by studying 30 ARGs (conferring resistance to tetracyclines, sulfonamides, quinolones and macrolides) in two WWTPs (North China), with higher ARG concentrations in dewatered than in aqueous WWTP effluent. Munir et al. (2011) reported that tetracycline- and sulfonamide-resistant bacteria had comparable concentrations in biosolids and in raw wastewater, reflecting the low efficiency of WWTP sludge processes in reducing ARB levels. Biosolids may therefore contribute to the spread of antibiotic resistance in the environment.

### 4.3.2 *Environmental Contamination by Wastewater Treatment Plant Effluents*

Wastewater treatment plant effluents are highly concentrated with antibiotic-resistant bacteria (e.g. in the range of  $5 \times 10^2$ – $6 \times 10^5$  CFU/100 mL; Munir et al. 2011). In their review, Hocquet et al. (2016) estimated that WWTPs, depending on their size, release between  $10^{10}$  and  $10^{12}$  ESBL-producing *E. coli* each day in receiving environments, depending on the released discharge. Similarly, Bréchet et al. (2014) estimated that  $6 \times 10^{11}$  ESBL-producing *E. coli* are released daily in the Doubs River (France), and Blaak et al. (2014a) reported that approximately  $10^{10}$ – $10^{11}$  ESBL-producing *E. coli* are discharged daily in four surface freshwater bodies in the Netherlands from four corresponding WWTPs. LaPara et al. (2011) demonstrated that even with a tertiary treatment, WWTP effluents still constitute a source of dissemination of ARB and ARGs into the environment. As expected and demonstrated previously, WWTP effluents highly contribute to the dissemination of ARB in the environment (Table 4.2).

The proportion of antibiotic-resistant bacteria has been reported to be considerably higher in WWTP effluents compared to that in the receiving river upstream from the WWTP effluent (Iwane et al. 2001; Galvin et al. 2010). Blaak et al. (2014a) reported that the concentrations of ESBL-producing *E. coli* were similar in the investigated WWTP effluents and receiving rivers just downstream of WWTP discharges and 1- to 2-log levels higher than concentrations upstream of the WWTPs. Iwane et al. (2001) showed that the proportion of ARB increased in the receiving river downstream of an effluent discharge; notably, 5.6% vs. 29.4% of coliforms were ampicillin-resistant upstream and downstream of the studied WWTP, respectively. In the Doubs River (France), Bréchet et al. (2014) reported 20 vs. 1300 ESBL-producing *E. coli* per litre upstream and downstream of a WWTP effluent, respectively; additionally, 0.7% and 0.9% of total *E. coli* produced ESBL upstream and downstream of the WWTP, respectively. In the same case study, Slekovec et al. (2012) showed that the studied WWTP did not decrease the proportion of

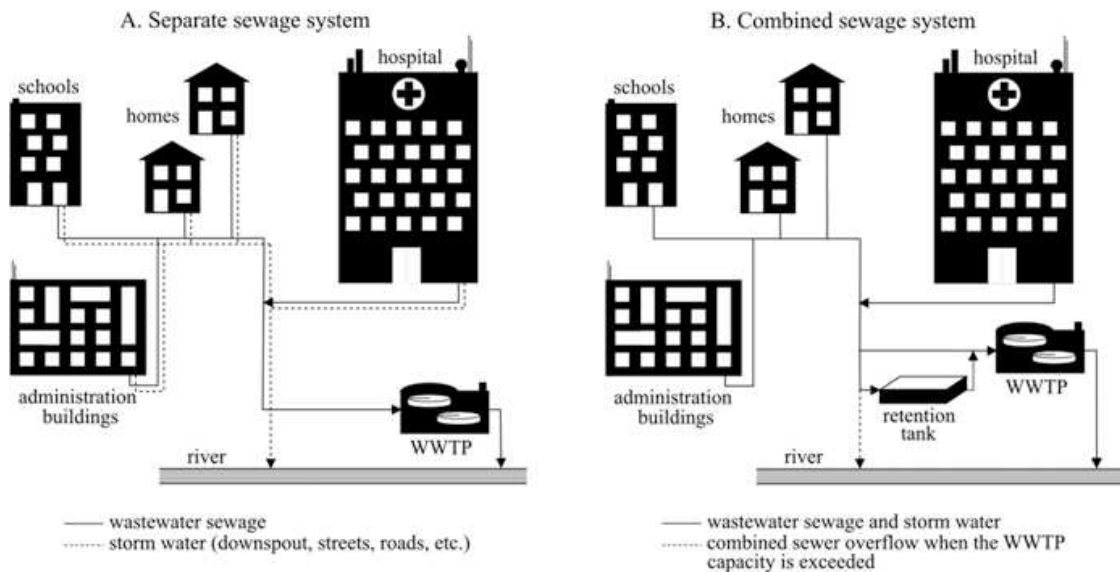
**Table 4.2** Summary table of environmental contamination with antibiotic-resistant bacteria according to the different sources mentioned in this chapter

	WWTPs	Agriculture	Aquaculture
ARB- or ARG-contaminated media	Effluents and biosolids	Manure, animals, and biosolids from WWTPs	Ponds and fishes
ARB- or ARG-contaminated environments	Mostly aquatic (e.g. rivers, lakes, and coastal waters)	Agricultural lands and the surrounding aquatic environments.	Mostly aquatic (e.g. rivers, lakes, and coastal waters)
Selection of ARB and ARG	Generally low (excepted downstream effluents of pharmaceutical industries)	Low	High
Dispersion of ARB and ARGs	Water fluxes	Spreading, runoff and leaching	Water fluxes
Survival of ARB and ARGs	Generally low, but higher in biofilms	Low for ARB and higher for ARGs	High
Associated health hazard	Water reuse, downstream water consumption, and downstream recreational activities	Food consumption, especially poultry, and workers	Fish consumption, downstream water consumption, downstream recreational activities, and workers

ARB antibiotic-resistant bacteria, ARG antibiotic resistance gene, WWTP wastewater treatment plant

antibiotic-resistant strains of *P. aeruginosa* and that the concentration of *P. aeruginosa* was higher in the river downstream of the WWTP effluent, demonstrating environmental contamination by hospital-associated antibiotic-resistant strains *via* WWTPs. The greater the contribution of the WWTP effluent to the river discharge is, the greater the concentration of antibiotic resistance determinants in the receiving stream (Proia et al. 2016). In addition to receiving waters, the sediment near WWTP discharge points is also directly contaminated and harbours higher levels of antibiotic resistance determinants compared with the upstream sediment (Marti et al. 2014a; Chu et al. 2018). Marti et al. (2014a) also highlighted significant differences in the community structure and composition of biofilms between upstream and downstream sediment samples, suggesting that WWTP effluents not only disseminate antibiotic resistance determinants in the environment, but may also impact the bacterial communities of the receiving bodies.

**Separate *Versus* Combined Sewage Systems** WWTPs can become an even more important source of antibiotic resistance during high-precipitation periods. Indeed, there are two types of sewage systems used to collect wastewater before reaching a WWTP: a separate sewage system collects wastewater separately from runoff water (storm water), whereas a combined sewage system collects wastewater and runoff water *via* the same network (Fig. 4.6). Combined sewage systems are more widespread than separate systems because they are older, cheaper and easier to implement.



**Fig. 4.6** Schematic representation of (a) separate and (b) combined sewage systems (a separate sewage system collects wastewater separately from runoff water – storm water; a combined sewage system collects wastewater and runoff water *via* the same network; combined sewer overflows, i.e. the release of untreated wastewater into the receiving environment, are frequent in combined sewage systems during rainy periods; WWTP wastewater treatment plant)

In a combined sewage system, runoff water, together with raw wastewater, enters a WWTP to be treated. This system therefore encounters two main problems during rainy periods or storm events. First, the dilution of wastewater reduces the WWTP efficiency and the subsequent clearance of pollutants, including antibiotic-resistant bacteria (Capodaglio 2004). Second, an excessive influent flux can saturate the WWTPs, which are designed and optimized to treat a given inflow generally estimated during non-rainy periods. This scenario results in combined sewer overflows (i.e. untreated raw wastewater is deviated from the WWTP by a bypass to the receiving environment; Capodaglio 2004; Jalliffier-Verne et al. 2015; Honda et al. 2020). The discharge of ARB from combined sewer overflows occurs occasionally, depending on precipitations. Honda et al. (2020) studied the combined sewage system in Kanazawa, Japan (29,900 inhabitants, continental climate) over a one-year period. The authors found that 23% of the volume of combined sewage was discharged untreated *via* combined sewer overflows, estimating that the yearly total discharge of antibiotic-resistant *E. coli* from combined sewer overflows in the environment was 3.7-log larger than that from WWTP effluent. Combined sewer overflows therefore appeared to be the primary source of faecal and antibiotic-resistance contamination in the receiving river (Jalliffier-Verne et al. 2015; Honda et al. 2020). The impact of combined sewer overflows obviously depends on climate, and Honda et al. (2020) reported that even if combined sewer overflows are intense in tropical regions, combined sewer overflows in temperate regions could release more pollutant loads, including ARB, due to limited dilution and long-duration rainfall events. To avoid this catastrophic situation, bypasses are generally built upstream of WWTPs (Fig. 4.4). These bypasses hold the raw wastewater in retention tanks that

are later discharged in the WWTP. In France and the United States, retention tanks effectively reduce pollutant loads and faecal coliform levels in combined sewer overflows (Jacopin et al. 1999; U.S. Environmental Protection Agency 1999). If a WWTP is not equipped with retention tanks or if the retention tanks are full, the raw wastewater is discharged untreated directly into the receiving environment (Figs. 4.4 and 4.6), as described by Honda et al. (2020; WWTP without a retention tank). The receiving environment is therefore directly exposed to antibiotic-resistant bacteria contamination. The spread of antibiotic resistance can therefore be considerably higher during high-precipitation periods due to combined sewer overflows.

### 4.3.3 *Environmental Contamination by the Spreading of Wastewater Treatment Plant Sewage Sludge and Biosolids*

In most countries, the majority of biosolids produced by WWTPs are spread on agricultural lands, raising the question of the dispersal of antibiotic resistance.

Studies have reported the persistence of antibiotic-resistant bacteria in the biosolids produced by WWTPs. Briefly, Brooks et al. (2007) reported that the ARB concentration varied from  $7.8 \times 10^5$  to  $4.5 \times 10^8$  CFU/g in the studied biosolids, and Munir et al. (2011) found ARB concentrations ranking between  $3.2 \times 10^4$  and  $1.9 \times 10^9$  CFU/g. Compared to the ARB levels found in WWTP effluents ( $5.0 \times 10^0$  –  $6.1 \times 10^3$  CFU/mL; Munir et al. 2011), biosolids are more concentrated in ARB. Specifically, biosolids can contain  $2.6 \times 10^5$  ESBL-producing *E. coli*/g (Bréchet et al. 2014) and  $5 \times 10^2$  antibiotic-resistant *P. aeruginosa*/g (Slekovec et al. 2012). Thus, the spread of biosolids on agricultural lands could play a role in the dispersion of antibiotic resistance in the environment (Table 4.2).

Brooks et al. (2007) determined the prevalence of culturable ARB in soil after the application of biosolids and reported that the application of biosolids did not change the proportion of ARB in agricultural soils. In contrast, Yang et al. (2018) reported an increase in ARG concentrations in soils after the application of biosolids. Similarly, Murray et al. (2019) measured the increase in several ARG abundances in the week following biosolid application. Chen et al. (2016) reported that soils that have received biosolids for multiple years were enriched in ARGs. The application of sewage sludge or biosolids therefore implies an increase in antibiotic resistance in agricultural soils by increasing the load of ARGs rather than viable ARB (Table 4.2).

The risk of using biosolids as a fertilizer is widely debated. Their application on agricultural land is limited or even prohibited in favour of incineration and energy production in some countries, such as Switzerland and Sweden.

#### 4.3.4 *Other Sources of Antibiotic Resistance in the Environment*

Wastewater treatment plants are hotspot sources for antibiotic-resistance in the environment. However, the contamination of water bodies away from WWTPs indicates that other sources of antibiotic-resistant bacteria exist (Laroche et al. 2010; Blaak et al. 2014a; Henriot et al. 2019).

**Aquaculture Farms** Between 1986 and 2006, the number of industrial aquaculture farms increased by a factor of 20 (Cabello 2006), especially in Asia and South America (Cabello et al. 2013). The high densities of fish increase the risk of infection, and antibiotics are widely used prophylactically (Cabello 2006). In the early 2000s, high income countries generally restricted the use of antibiotics at aquaculture farms, but this was not necessarily the case globally (Cabello 2006; Bravo et al. 2005). For example, in Chile, 10–12 t of quinolones were used for humans, compared to 100–110 t for animals annually, mainly for aquaculture (Cabello 2006; Bravo et al. 2005). Antibiotics are involved in the selection of ARB in the water column and in sediment (Baquero et al. 2008). Food containing antibiotics, faeces contaminated by antibiotics and antibiotic-resistant bacteria are released in the water column. Ng et al. (2018) retrieved multidrug-resistant *E. coli* from fish farms in Singapore, among which 81.8% were ESBL producers. Fish farms are generally connected with the local aquatic environment, or fish are directly farmed in natural environments (e.g. coastal water), thus facilitating the dissemination of ARB by water fluxes (Table 4.2). Gordon et al. (2007) observed an increase in bacteria resistant to oxytetracycline, quinolones, trimethoprim-sulfamethoxazole, florfenicol and amoxicillin in the vicinity of a fish farm. Cabello (2006) warned about the potential alteration of the composition of sediment bacterial communities (Tamminen et al. 2011a). Cabello (2006) also warned about the potential transfer of antibiotic resistance to animal (both wild and domestic) and human pathogenic bacteria (Rhodes et al. 2000). Tamminen et al. (2011b) demonstrated the long-term impact of aquaculture farms on the surrounding environment by showing the persistence of ARGs after the ban of ARG-specific antibiotics.

**Agriculture** In 2017, in 31 European countries, 6703 t of antibiotics were consumed in agriculture (European Medicines Agency 2019). Antibiotic residues and ARB are excreted in animal faeces that are directly released on pasture or are collected in manure/slurry. Manure application generally increases the abundance of antibiotic resistance in agricultural soils (McKinney et al. 2018). Once in agricultural soils, ARB can spread in the environment through water fluxes during rainy periods *via* runoff (surface drainage) and leaching (underground drainage). For example, Laroche et al. (2010) investigated a rural watershed with only agriculture and livestock breeding activities and away from any WWTP, and they found that the transport of resistant *E. coli* in the study area was dominated by runoff and leaching phenomena. During a wet period without grazing or during a dry period with



grazing, less than 7% of the isolated *E. coli* were antibiotic-resistant. In contrast, during rainfall events with grazing, 30–55% of the *E. coli* were antibiotic-resistant (Laroche et al. 2010). ARB of agricultural origin can further spread in natural aquatic environments (Table 4.2). Beattie et al. (2018) observed that riverbed sediment can temporally accumulate antibiotic resistance determinants during manure fertilization periods.

In addition to the spread of manure and slurry, agricultural areas are also sources of antibiotic resistance because of (i) the application of sewage sludge and biosolids from WWTPs and (ii) the reuse of treated water (WWTP effluents) to irrigate agricultural lands in remote regions (Silva et al. 2006) (Table 4.2).

**Slaughterhouses** Very few studies have been conducted on antibiotic resistance at slaughterhouses. Slaughterhouse wastewater is also contaminated by antibiotic-resistant bacteria (Um et al. 2016), as the majority of slaughtered farm animals have been treated with antibiotics and can therefore carry ARB. The wastewater at slaughterhouses is either collected in an urban wastewater network or treated in an independent WWTP for larger slaughterhouses. Slaughterhouse WWTPs are estimated to release approximately  $10^5$ – $10^6$  CFU/L of *E. coli* into the environment (i.e. on the same order as the concentration for urban WWTPs). With respect to ARB, Um et al. (2016) showed that treatment of slaughterhouse wastewater does not alter the proportion of resistant *E. coli* strains. Sludge for land application produced by slaughterhouse WWTPs contains up to  $10^5$  CFU/g of *E. coli*, less than that produced by urban WWTPs ( $10^7$ – $10^8$  CFU/g; Bréchet et al. 2014). Slaughterhouses are therefore not important sources of environmental contamination by ARB compared to WWTPs.

**Pharmaceutical Industries** Pharmaceutical industries can release large amounts of antibiotics into the environment and therefore favour the persistence and dispersion of antibiotic-resistant bacteria. The contribution of pharmaceutical industries to environmental antibiotic resistance will be further discussed in Sect. 4.4.2.

Overall, the following conclusions can be summarized: (i) although WWTPs reduce the absolute ARB load in raw wastewater, aqueous effluents and biosolids release ARB into the environment; (ii) in terms of relative abundance, it is difficult to draw clear conclusions; ARB are neither advantaged nor disadvantaged over their susceptible counterparts during treatment at WWTPs; (iii) WWTP products, including aqueous effluents and biosolids, are the main sources of contamination by antibiotic resistance in the environment; (iv) fish farms and agriculture also contribute to the dissemination of antibiotic-resistance in the environment.

## 4.4 Dispersion and Persistence of Antibiotic Resistance in the Environment

### 4.4.1 *Environmental Contamination Driven by the Level of Anthropization*

In addition to suggesting that WWTPs have a strong impact on the receiving environments in terms of antibiotic-resistant bacteria contamination, the literature shows that this impact depends on the degree of anthropization of the watershed considered. Servais and Passerat (2009) investigated the source of faecal ARB in the Seine watershed (France). They observed that the abundance of antibiotic-resistant *E. coli* increases from forest non-point sources (2% of total *E. coli*) to agricultural non-point sources (16% of total *E. coli*), municipal wastewater (44% of total *E. coli*) and hospital wastewater (71% of total *E. coli*). Along a river continuum characterized by two opposite density gradients for animals (cattle and wild animals) and human populations, Petit et al. (2017) showed that in the upstream part, the low abundance of resistant *E. coli* (5% of total *E. coli*) could be explained by the low number of cattle receiving antibiotic therapy; in contrast, the higher frequency of resistant *E. coli* (27% of total *E. coli*) reflected the human contamination of water at downstream urban sites. Along a medical centre-retirement home-wastewater treatment plant-river continuum in France, the relative proportion of *Enterococcus faecium* that was resistant to antibiotics decreased, and subpopulations of *E. faecium* less resistant to antibiotics progressively increased (Leclercq et al. 2013). Guyomard-Rabenirina et al. (2017) investigated the antimicrobial resistance of *Enterobacteriaceae* to fluoroquinolones and third-generation cephalosporins in 70 surface water samples obtained primarily from two rivers in Guadeloupe (France). The authors confirmed that resistant *Enterobacteriaceae* were absent or rare near the river sources but frequent near the surrounding human activities dominated by housing and farming. Similarly, this increase in antibiotic resistance in anthropized environments has also been demonstrated in lake ecosystems, with a decrease in antibiotic resistance as the distance from WWTP discharge points increased (LaPara et al. 2011; Czekalski et al. 2014; Czekalski et al. 2015). These increased antibiotic resistance rates in the vicinity of WWTP discharges and the associated anthropization-dependent levels of ARB in natural environments are intuitive but also suggest that the dissemination of ARB seems limited, as their survival is rather poor in nonfavourable environments such as freshwaters or coastal waters.

#### 4.4.2 *Exogenous Parameters that Influence the Dispersion and Survival of Antibiotic-Resistant Bacteria in Natural Environments*

In the absence of antibiotic pressure, Berthe et al. (2013) demonstrated in a microcosm study that antibiotic-resistant bacteria survived less (< 4 days) than susceptible bacteria in water (> 14 days), suggesting a cost of resistance to bacterial fitness. However, antibiotic-resistant bacteria are released into the environment along with other pollutants, some of which potentially promote the survival of ARB.

**Antibiotics** These substances are released by several sources into the environment (e.g. WWTPs, agriculture, fish farms and pharmaceutical industry effluents). Multiple studies/monitoring networks have measured the concentrations of antibiotics in surface water. Three of them will be detailed by way of example. Valcárcel et al. (2011) determined the concentration of several antibiotics in five Spanish rivers and reported ciprofloxacin, clarithromycin, erythromycin, metronidazole, norfloxacin, ofloxacin, sulfamethoxazole, tetracycline and trimethoprim median concentrations of 0.003, 0.235, 0.320, 1.195, 0.010, 0.179, 0.326, 0.023 and 0.424 µg/L, respectively. In China, norfloxacin, ofloxacin, ciprofloxacin and oxytetracycline have been frequently detected at concentrations up to 5.770, 1.290, 0.653 and 0.652 µg/g in sediments from the Pearl River, Yellow River, Hai River and Liao River (Zhou et al. 2011). Finally, Johnson et al. (2015) reported expected means of 0–0.04, 0–0.04, 0–0.06 and 0–0.06 µg/L for ciprofloxacin, sulfamethoxazole, trimethoprim and erythromycin, respectively, in European rivers. The concentrations of antibiotics generally increase from upstream to downstream, with a maximum observed downstream of the main WWTP discharge outfalls (Dinh et al. 2017).

Some of the previous antibiotic concentrations measured in rivers are higher than the “environmental predicted no-effect concentrations” reported by Tell et al. (2019), suggesting a possible selection of ARB in natural environments. Moreover, Gullberg et al. (2011) reported that the minimal selective concentrations of streptomycin, ciprofloxacin and tetracycline are approximately 1/4, 1/10 and 1/100 of their minimal inhibitory concentrations respectively. Subinhibitory concentrations of antibiotics can therefore favour ARB. For example, Gullberg et al. (2011) determined that the minimal inhibitory concentration of ciprofloxacin for wild type *E. coli* was 0.023 µg/ml, and the corresponding minimal selective concentration could be estimated at 0.0023 µg/ml. The concentrations of ciprofloxacin previously reported in rivers can exceed this minimal selective concentration and therefore exert a selective pressure on resistant *E. coli*. On the other hand, some studies did not find any relation between antibiotic concentrations and ARB abundances in rivers (Oberlé et al. 2012). Karkman et al. (2019) observed that antibiotic resistance was correlated with faecal contamination downstream of WWTP effluents rather than with antibiotic concentrations. However, there is no simple relationship between the presence of ARB and the concentrations of antibiotics in water because ARB have been previously selected in humans and animals treated with numerous

antibiotics, whereas only persistent compounds can be detected in water (e.g. sulfonamides, fluoroquinolones, macrolides and diaminopyrimidine).

Antibiotic-resistant bacteria have been reported to be selected in rivers downstream of pharmaceutical industries. Milaković et al. (2019) showed that the discharge of an azithromycin manufacturing factory shifted the bacterial community composition in the downstream sediment of the Sava River (Croatia), and this community displayed higher antibiotic resistance than the bacterial communities located upstream from the WWTP. The five studied ARGs persisted in sediment until 11 km downstream from the effluent discharge point. China and India produce the majority of the world's antibiotics (Changing Markets, Ecostorm 2016). Two studies that investigated the role of pharmaceutical industries producing penicillin G and oxytetracycline in China reported higher multiresistance frequencies in bacteria isolated in the rivers downstream of industrial discharges than in other areas (Li et al. 2009, 2010). Li et al. (2010) found that almost all bacteria (97%) from the wastewater and the river water downstream of the effluent discharge points were multiresistant to antibiotics, while they were less frequent (28%) in upstream river samples. In Patancheru (India), the antibiotic concentration was extremely high in the effluent of the WWTP that served approximately 90 bulk drug manufacturers (Larsson et al. 2007). For example, the concentration of ciprofloxacin, which reached 31,000 µg/L, exceeded the level of toxicity to some bacteria by over 1000-fold. Kristiansson et al. (2011) investigated the impact of these effluents on the sediment of the receiving river. Antibiotics can persist for up to 30–40 years in the sediment (Tamtam et al. 2011). Kristiansson et al. (2011) confirmed the selection of antibiotic resistance determinants downstream of pharmaceutical industries in the sediment and also suggested the mobilization of antimicrobial resistance within bacterial communities, potentially from environmental bacteria to human-associated bacteria, including human pathogens.

In the natural environment, antibiotic pollution could favour antibiotic-resistant bacteria. However, environmental matrices are complex, polyspecific and contaminated by multiple pollutants, unlike most experimental matrices. Bacteria survive in communities in the liquid phase or biofilm and are not systematically culturable. Moreover, the bioavailability of antibiotics is poorly known. The resulting selection of antibiotic resistance by antibiotics occurs under specific conditions in natural environments (e.g. downstream pharmaceutical industry effluents, WWTP effluents particularly concentrated in antibiotics and areas near fish farms). Low and middle income countries appear to be more exposed than high income countries to the selection of antibiotic resistance in natural environments. This difference is mostly due to the lower removal of ARB and antibiotics in wastewaters, as well as the many pharmaceutical industries in these countries and their poorly treated effluents (Changing Markets, Ecostorm 2016).

**Metals and Biocides** Contamination with metals could favour the maintenance of ARB in the environment (Baker-Austin et al. 2006; Wales and Davies 2015). These associated resistances can be explained either by the presence of genes resistant to metals and antibiotics on the same genetic carrier (co-resistance) or by the existence

of common mechanisms conferring co-resistance (cross-resistance; e.g. changes in membrane permeability) (Pal et al. 2017). In the environment, many studies have shown a correlation between metal contamination and bacterial populations co-resistant to metals and antibiotics (Dickinson et al. 2019; Milaković et al. 2019). Wales and Davies (2015) and Poole (2017) suggested that some metals, particularly copper and zinc, are capable of complexing with antibiotics, thereby enhancing their antimicrobial activity depending on the molecule and promoting the emergence of co-resistant strains. However, this was not the case reported by Vos et al. (2020). Recently, the contribution of metagenomics has made it possible to gain a comprehensive understanding of this issue. An in-depth genomic analysis of a collection of 5436 genomes from 39 phyla from differing habitats (human, water, soil) showed that the co-occurrence of genes encoding resistance to antibiotics or to metals was very high in the genomes of bacteria isolated from humans and moderate in bacterial genomes from water or soil (Li et al. 2017). Maximum values were found in pathogenic bacteria belonging to *Enterobacteriaceae*.

The relationship between bacterial resistance to antimicrobial biocides and the use of antibiotics has long been known (Chaplin 1952). Like metals, biocides can promote the spread of antimicrobial resistance through co- and cross-resistance mechanisms (Davin-Regli and Pagès 2012; Wales and Davies 2015). Metal and biocide resistance genes were found in 86% of bacterial genomes and coexisted with ARGs in 17% of cases (Pal et al. 2015). Environmental contamination by biocides and metals could therefore favour the maintenance of ARB in natural environments. Agricultural environments are particularly exposed to this risk, especially because of the combined use of antibiotics, biocides and metals (mainly copper and zinc).

However, the data currently available in the literature do not allow to clearly understand the effect of the presence of metals or biocides on the dissemination of antimicrobial resistance in the environment. Thus, in the conclusion of their review, Pal et al. (2017) proposed further studies to assess the relationship between the level of exposure to metals, including their bioavailability and the selection/persistence of antibiotic-resistant bacteria in differing environments.

**Microplastics** These new emerging contaminants are other exogenous products released by WWTPs into natural environments, which can favour the dissemination of antibiotic resistance determinants. Microplastics have been reported to constitute a reservoir/niche for the bacterial community and more specifically for ARB and ARGs (Arias-Andres et al. 2018; Yang et al. 2019), thus increasing the horizontal gene transfer of ARGs between human-associated or environmental bacteria and the dispersion of ARB and ARGs over long distances (Arias-Andres et al. 2018). Using metagenomics, Yang et al. (2019) studied the ARGs present in microplastics in the Northern Pacific. They showed that both the diversity and richness indices of ARGs were higher in microbiota on plastics compared to those for seawater microbiota. Multidrug and multi-metal resistance genes were the main classes of ARGs detected in the studied microbiota on plastics, suggesting the high horizontal gene transfer of antibiotic resistance determinants (Yang et al. 2019). Recent studies reported high concentrations of microplastics in natural – mainly aquatic – environments (Ivleva

et al. 2017), highlighting the risk of antibiotic resistance dissemination by microplastics. Further investigations should focus on the following questions: are microplastics more favourable to the spread of antibiotic resistance than other suspended matter in aquatic environments? In any case, microplastics offer an additional dispersion route for antibiotic resistance.

### 4.4.3 *Dispersal and Survival of Antibiotic-Resistant Bacteria in Receiving Environments*

#### 4.4.3.1 Rivers

These aquatic water bodies, i.e. rivers, are the main receivers of wastewater treatment plant effluents and are also the most studied and exposed in terms of antibiotic-resistant bacteria contamination. Antibiotic-resistant bacteria have been studied in the water column and in sediments.

**Case of the Water Column** In the upstream part of the Seine watershed, Servais and Passerat (2009) found that 83% of enterococci and 42% of *E. coli* were resistant to at least one antibiotic of clinical interest. Forty-nine percent of enterococci and 35% of *E. coli* were multiresistant to antibiotics. In another study, 30–57% of *E. coli* isolated from estuarine water in the Seine watershed were resistant to at least one antibiotic, and of these, 60.5–80% were resistant to  $\geq 2$  antibiotics (Laroche et al. 2009). The authors determined that  $3.5 \times 10^9$  antibiotic-resistant *E. coli* circulated in the Seine estuary each second. The estuary of the Seine River contained a proportion of ARB similar to that at a WWTP situated ~50 km upstream (48.3%), suggesting the dispersion of ARB over long distances. Estuary environments, since they are the final destination for ARB, might serve as reservoirs for antibiotic resistance (Laroche et al. 2009). Temporarily, high loads of ARB are often measured in rivers during high precipitation periods (Laroche et al. 2009; Jalliffier-Verne et al. 2015; Henriot et al. 2019), mostly due to combined sewer overflows but also to the runoff and leaching of contaminated water from the surrounding agricultural lands. However, high ARB concentrations are also reported in summer due to the low flow of receiving streams and thus the decreased dilution of pollutants (Zhang et al. 2009b).

Garcia-Armisen and Servais (2004) studied the viable but non-culturable (VBNC) bacteria in microcosms and natural environments by comparing the concentrations of viable *E. coli* assessed by a culture-based method with that of the VBNC *E. coli* with a culture-independent method. On the one hand, 200 hours after inoculation, only 1% of the bacteria were still culturable, whereas 21% were VBNC. On the other hand, along the Seine River downstream of a large WWTP, the ratio of the *E. coli* concentrations VBNC/viable varied from 11 (directly downstream of the WWTP discharge) to 71 (120 km downstream of the WWTP). A metagenomic approach confirmed the high proportion of VBNC bacteria in the

effluents of WWTP release in surface water (Jäger et al. 2018a). This suggests that harsh conditions for bacteria (in WWTP or in natural environments) favour the VBNC state. Faecal bacteria, including ARB, are therefore capable of surviving over long distances downstream of WWTP discharges in the environment. The role of VBNC in ARB as a vehicle for the spread of antibiotic resistance in the environment still needs to be explored.

Once in natural environments, antibiotic resistance determinants can be spread by water fluxes and disrupt environmental bacterial communities, as shown above. The ARB can also be disseminated through fauna (Bollache et al. 2018). ESBL-producing *E. coli* can be transferred from WWTPs to receiving rivers and then to biofilms grazed by omnivorous fishes that they are able to colonize (Bollache et al. 2018). Most compartments of the aquatic food networks can thus become vectors in the environmental dispersion of antibiotic resistance. However, the survival of such human-associated bacteria in the gut of fish and other aquatic animals is poorly understood.

**Case of the Sediment** Few studies have investigated ARB in riverbed sediment, but several have done so for total faecal bacteria. Some studies have reported the rapid decay of bacteria in sediment, mostly due to the absence of water column mixing and thus the lack of dissolved oxygen (Anderson et al. 2005). In contrast, many authors considered sediment to be a favourable habitat for bacteria, as it provides nutrients (Knox et al. 2008) and protection against predators, parasites, desiccation, UV, toxins and antibiotics (Brennan et al. 2014). Other studies have shown that the concentration of bacteria in the river water column is influenced by the concentration in the sediment because of sediment remobilization by water movements (Smith et al. 2008). However, Berthe et al. (2008) observed that in the Seine estuary mudflats, abundance of faecal bacteria decreased with the sediment depth. This suggests that the sediment does not favour the survival of a culturable form of ARB. The authors described that bacteria associated with easily settleable particles were deposited on mudflats, which can temporally constitute a storage site, and lost their culturability. They also suggested that bacteria may be favoured at low temperatures on mudflats. Although the sediment can contain higher bacterial loads than the water column, resuspension moderately impacts the ARB concentration in the water column (Heß et al. 2018).

#### 4.4.3.2 Riverine Wetlands

River annexes, mainly wetlands, are supplied by different types of water, mostly river water, groundwater and runoff water (Amoros and Bornette 2002), that can potentially contaminate wetlands with ARB. The physico-chemical characteristics of these ecosystems greatly vary according to the geomorphological, anthropic and hydrogeological contexts (Amoros and Bornette 2002; Bornette et al. 2008).

Very few studies have been conducted on the antibiotic resistance contamination of riverine wetlands. Henriot et al. (2019) studied 16 isolated wetlands in three

floodplains in eastern France. ESBL-producing *E. coli* was able to reach the riverine wetlands, but only during high-flow periods, meaning that the main stream of the river was the leading source of antibiotic resistance in these low-anthropized ecosystems (Henriot et al. 2019). This seasonal presence of ESBL-producing *E. coli* in wetlands suggests that although ARB are transferred by rivers over long distances, their culturability quickly decreases in natural environments. Moreover, some physico-chemical parameters (i.e. phosphate, pH and nitrate levels) were positively related to the abundance of total *E. coli* in wetlands (Henriot et al. 2019). Nevertheless, ESBL-producing *E. coli* loads were independent of the studied physico-chemical parameters and only depended on the connectivity of wetlands with a river. This result demonstrates that although ESBL-producing *E. coli* are able to be dispersed from rivers to wetlands, they struggle to survive there. The higher abundance of ESBL-producing *E. coli* during high-flow periods than in other periods suggests that the retrieved isolates are coming from untreated discharges associated with combined sewer overflows and/or that both runoff and leaching from the surrounding areas contribute to the contamination of wetlands by ARB.

Due to lower flow and favourable physico-chemical conditions, riverine wetlands contain more abundant and diversified vegetation than the main stream of most rivers. Aquatic vegetation possibly affects ARB. Several studies have explored the sheltering role of bacteria by aquatic plants, thus promoting the bacterial fixation, the formation of biofilm on the surface of submerged parts and the presence in vegetal tissues (Knox et al. 2008; Kleinheinz et al. 2009). Furthermore, secondary compounds produced by aquatic plant species can reduce the survival of bacteria (Özbay and Alim 2009; Chiapponi et al. 2020). Finally, due to its high nutrient content (Berg et al. 2005), the rhizosphere could favour bacterial concentration and interactions, including horizontal gene transfer. However, these different processes have been poorly studied, especially for ARB. Further investigations are needed to better understand the role of vegetation in the spread of antibiotic resistance in aquatic environments.

#### 4.4.3.3 Lakes

To the best of our knowledge, no studies have been conducted on the survival of the four major bacterial species considered in this chapter in lakes. However, several studies have investigated ARGs in lakes. Chu et al. (2018) showed the direct influence of WWTP discharges in the sediment of Lake Michigan (USA). Their results suggest that WWTP effluents modify indigenous microbial communities in the sediment through the simple deposition of ARGs and horizontal gene transfer. Low concentrations of ARGs were found in the centre of Lake Michigan (100–200 km from a WWTP effluent), reflecting the dilution and poor dispersion of antibiotic resistance in lakes. In Lake Geneva (Switzerland), Czekalski et al. (2014) showed that ARG abundances near a WWTP effluent were up to 200-fold higher than those measured at the centre of the lake (~7 km from the effluent source). However, some ARGs were transported over long distances in the lake. Czekalski et al. (2015)



measured the ARG concentrations in 21 Swiss lakes and found that some ARGs were directly related to the anthropization level of the catchment area of the lakes. In contrast, ARG concentrations have been correlated with several ecological parameters in lakes (i.e. positively with dissolved oxygen and negatively with chlorophyll-a (Di Cesare et al. 2015), as well as positively with water residence times and the eutrophication status (Czekalski et al. 2015)). This finding suggests that freshwater lakes may constitute a niche for antibiotic resistance determinants. However, this role of reservoirs is very limited, as antibiotic resistance determinants are generally found near WWTP effluents (Czekalski et al. 2014; Chu et al. 2018).

#### 4.4.3.4 Coastal Water

These environments can be contaminated with antibiotic resistance determinants transported by river estuaries, WWTP effluents, surface runoff and contaminated groundwater (Laroche et al. 2009). WWTP outfalls in the open sea and cruise ship discharges cannot be neglected. Entering seawater, faecal bacteria face several abiotic (light radiation and the associated oxidative stress, salinity and nutrient deprivation) and biotic stresses (predation protozoa, competition and bacteriophage) (Rozen and Belkin 2001). However, antibiotic-resistant *E. coli* have been detected in several coastal waters (Blaak et al. 2014a; Leonard et al. 2018a, b). Leonard et al. (2018a) estimated that each *E. coli* retrieved in 13 studied coastal waters (England) harboured an average of 1.24 ARGs. Fifteen percent of 97 bathing water samples obtained off the coast of England and Wales, contained third-generation cephalosporin-resistant *E. coli*, with 0.12% of all collected *E. coli* being third-generation cephalosporin-resistant *E. coli* (Leonard et al. 2018a). Contrary to the findings of Blaak et al. (2014a), Leonard et al. (2018a, b) studied sites that were not under the direct influence of a WWTP, and the results suggested the possible dispersion of antibiotic resistance in coastal water. Coastal water is generally less polluted than rivers due to the dilution from the mouth of a river to the sea (Guyomard-Rabenirina et al. 2017).

In addition to direct contamination sources, fauna can be a vector for the spread of antibiotic resistance to coastal waters. Vittecoq et al. (2017) investigated the presence of carbapenem-resistant strains of *E. coli* in two species of gulls in southern France: one living in close association with humans (*Larus michahellis*) and the other feeding at sea (*Chroicocephalus genei*). The authors found that 19% and 0% of ‘anthropized’ and ‘non-anthropized’ gulls carried the gene *bla<sub>VIM-1</sub>* (encoding a carbapenemase) in their gut, respectively. This result confirmed that fauna can be contaminated with ARB directly by the ingestion of water or of particulate matter as plastic previously contaminated by ARB and thus could spatially disseminate the antibiotic resistance determinants.

#### 4.4.3.5 Agricultural soils

Agricultural soils can be contaminated by antibiotic-resistant determinants through the application of biosolids from WWTPs and manure from agricultural farms.

**Case of Sewage Sludge or Biosolid Application** As previously described, Brooks et al. (2007) reported that the application of biosolids did not change the proportion of culturable ARB in soils. In contrast, Chen et al. (2016) demonstrated that the long-term application of sewage sludge significantly modified the composition and structure of the bacterial soil community by increasing ARG abundance and diversity in the studied soils. In addition, the authors found that mobile genetic elements were positively correlated with total ARGs in soils. This result suggests that the dissemination of ARGs in agricultural soils is partly related to mobile genetic elements and the associated horizontal gene transfer, which in turn could favour the accumulation and persistence of ARGs in amended soils. Similarly, Yang et al. (2018) reported an increase in ARG abundances in soils after the application of biosolids. They also reported that the application of biosolids could drive a shift in ARGs through horizontal gene transfer.

**Case of Manure and Slurry Application** Five percent of cattle faeces sampled in eastern France carried ESBL-producing *E. coli* (Hartmann et al. 2012). Crops harvested one year after manure application carried the same clones detected in manure, suggesting their persistence for at least one year in the field environment. In a Canadian study performed in an area with a humid continental climate, viable *E. coli* and *Enterococcus* spp. populations quantified by plate counts fell below the detection limit within six months of a swine or dairy manure application (Marti et al. 2014b). In contrast, ARG concentrations determined by qPCR remained higher in manured plots than in non-manured controls throughout two growing seasons, a period of approximately a year and a half. The long-term application of chicken manure and short-term application (one week) of composted and liquid swine manures increased the abundance and diversity of ARGs in soil, with the abundance returning to background levels six months after application (Chen et al. 2016, 2019). In contrast, analyses of archived soils from field plots treating with composted swine manure indicated that some gene targets persisted for at least five years post application (Scott et al. 2018). Overall, ARG targets persist in soils far longer than typical viable indicator bacteria. However, it is unknown whether the gene targets quantified using culture-independent methods are carried in viable bacteria.

The persistence of antibiotic-resistant bacteria is variable in amended soils and is condition dependent (type of fertilizer applied, type of soil). Combined, these results demonstrate that the ARG concentration increases after manure application and suggest that ARGs can persist in soils (several weeks to more than one year). Soils are not very favourable habitats for enteric bacteria, and a non-negligible part of them can become VBNC.

#### 4.4.3.6 Groundwater

This environment can be contaminated with antibiotic-resistant bacteria by the leaching of contaminated soils depending on the land use in the watershed and the hydrological conditions. In general, groundwater is minimally contaminated due to the poor survival conditions of human-associated bacteria within aquifers (John and Rose 2005). This section will not describe riverine aquifers that can be contaminated by water fluxes coming from contaminated rivers and streams. We will rather focus on karstic aquifers that are comparatively easily contaminated due to their high porosity and the resulting rapid infiltration. As for rivers in rural areas, the presence of ARB in aquifers can be explained by soil leaching during rainy events (Laroche et al. 2010; Ribeiro et al. 2012). Laroche et al. (2010) investigated a rural karstic hydrosystem not influenced by any WWTP but only by livestock breeding activities. They found that despite the permanent circulation of *E. coli* in the studied karstic hydrosystem, antibiotic-resistant *E. coli* infiltrated the groundwater during rainy events, indicating leaching from agricultural surfaces. Petit et al. (2018) reported the same presence of antibiotic-resistant *E. coli* in a karstic hydrosystem during high-rainfall events. Laroche et al. (2010) and Petit et al. (2018) observed a decrease in culturable bacteria in a spring (downstream of a karstic hydrosystem) compared to the bacteria level in a creek (upstream of the karstic hydrosystem) of 1–2 log. This decrease may be due to the dilution of ARB within the aquifer, the trapping/decay in the karst and/or the loss of cultivability.

Groundwater has been described as a permanent reservoir of VBNC *E. coli* (Petit et al. 2018). Further studies are needed to better investigate the ARGs in groundwater and improve our knowledge of antibiotic resistance dispersion in these particular systems. Aquifers – even karstic ones – seem less exposed to antibiotic resistance than surface waters.

Overall, the following conclusions can be summarized: (i) the high dispersion of antibiotic resistance is reported in aquatic environments; the level of anthropization of an ecosystem is a determinant, and ARB are quickly diluted with the distance from a source; (ii) the dissemination of antibiotic resistance depends on precipitation and land use in a watershed; (iii) in a natural environment, the selective pressure is generally low, with the exception of environments that receive effluents from pharmaceutical industries; (iv) the survival of ARB is difficult to quantify due to VBNC forms; natural environments do not allow the long-term culturability/survival of ARB, but it could be interesting to assess the persistence of genetic support in such environments; (v) ARB survival can depend on the specific conditions of the receiving environment; (vi) the environment could therefore be a secondary reservoir of antibiotic resistance, but the ARB present in natural environments are rather the result of spatial dissemination than of proliferation; (vii) fauna and flora could favour ARB spatial dispersion and possibly survival in the environment, but their role needs further investigation; and (viii) the persistence of VBNC ARB and the corresponding genetic information have been poorly investigated and require special attention.

## 4.5 What Are the Risks for Humans ?

The presence of antibiotic-resistant bacteria in natural environments raises the question of the risk for human population health. The concept of risk involves aspects of both hazard and exposure. The risk is the exposure to a hazard, here antibiotic resistance, and more specifically, human contamination by ARB. In human health, risk assessment is generally used to estimate the probability of illness or death for individuals in a given population exposed to a given hazard (Bürgmann et al. 2018). However, the risk is comparatively complex to assess considering antibiotic resistance. Indeed, several parameters must be taken into account, such as the pathogenicity of the resistant bacterial strains, the importance of the antibiotic to which resistance is confirmed, the ease of establishment, the dissemination of resistant strains in individuals and the possibility of transfer of resistance within the microbiota of the population studied (Bürgmann et al. 2018). Because of the difficulty of assessing all of the above parameters, very few studies have assessed the risk for human population conferred by antibiotic resistance in the environment. The studies referred to in this section estimated the likelihood of exposure to antibiotic-resistant bacteria, rather than the actual risk associated with antibiotic resistance.

### 4.5.1 Exposure Through Water Consumption

Groundwater is one of the main sources of water consumption (41% of the population of the USA depends on groundwater for its drinking water supply, National Groundwater Association 2020). Laroche et al. (2010) retrieved antibiotic-resistant *E. coli* in boreholes, a water resource intended for human consumption, demonstrating that drinking water resources withdrawn from karst groundwater are vulnerable to contamination by ARB (Laroche et al. 2010; Ribeiro et al. 2012). Admittedly, such contamination is uneven and temporary, but after pumping and distribution systems are contaminated, antibiotic-resistant bacteria could be trapped and grow in biofilms; therefore, if so, the entire system could be vulnerable, and not only during rainy periods.

Surface freshwater is also a direct or indirect source of water consumption for humans and animals. Antibiotic pollution and the spread of ARB discussed above may pose serious risks for human and animal health. In Lake Geneva (Switzerland), Czekalski et al. (2014) retrieved ARGs near the location of a drinking water pumping station. Schwartz et al. (2003) investigated antibiotic resistance in biofilms in a water distribution system in Mainz (Germany). This water distribution system is supplied with bank-filtered water (from the Rhine River) followed by UV irradiation at waterworks. Antibiotic-resistant bacteria (i.e. enterococci and *Enterobacteriaceae*) were absent from the drinking water distribution system, but ARGs (i.e. *vanA* and *ampC*) were retrieved from the same samples. This discrepancy was either due to the presence of dead bacteria that released their DNA or

contamination with VBNC forms of ARB. For example, Jeanvoine et al. (2019) found VBNC cells of the high-risk clone *P. aeruginosa* ST308 in the plumbing system of a healthcare facility. The health hazard associated with the presence of VBNC bacteria has generally been poorly evaluated (Schwartz et al. 2003), but such bacteria may pose a health risk.

In New Delhi (India), Tanner et al. (2015) investigated 19 drinking water samples from the distribution system. The drinking water in New Delhi mostly comes from rivers (Safe Water Network 2016). The authors found isolates resistant to meropenem at five sampling sites. The presence of carbapenem-resistant bacteria retrieved in this drinking water is particularly alarming since it directly exposes 18 million people to bacteria resistant to an antibiotic of last resort (Safe Water Network 2016).

Environmental water contaminated with antibiotic-resistant bacteria or antibiotic resistance genes can therefore constitute a risk for the human population through drinking water. There is an urgent need to find a low-cost and easy-implemented method to control the presence of antibiotic resistance determinants in drinking water networks, especially those conferring resistance to antibiotics of last resort and for highly exposed populations in low and middle income countries.

#### 4.5.2 Exposure Through Food Consumption

**Vegetable Contamination** Blaak et al. (2014b) found that third-generation cephalosporin-resistant *Enterobacteriaceae* were present on raw consumed fresh produce (isolated from 2.7%, 1.3% and 1.1% of vegetables from supermarkets, iceberg lettuce from farms and agricultural soil, respectively). By demonstrating the link between pig manure spreading (1–2 weeks before planting) and the contamination of vegetables, the authors also showed that the ARB contamination of fresh vegetables mirrored that of the agricultural soil in which they were grown. However, the percentages of contaminated vegetables were rather low, suggesting a low hazard for contamination through vegetable consumption for human populations. Moreover, the authors found that the ESBL-producing *Enterobacteriaceae* isolated from vegetables and the environment were all environmental species, therefore limiting the hazard for human populations (Blaak et al. 2014b). Randall et al. (2017) reported that none of the fruits and vegetables sampled from five retail stores in the UK were positive for ESBL-producing *E. coli*. The likelihood of exposure to ARB through the consumption of vegetables is limited and dependent on agricultural conditions (type of soil, type of amendment, type of vegetable).

**Meat and Fish Meat Contamination** Cabello (2006) reported the presence of antibiotic residues and ARB in fish grown in fish farms. In the same way, Abraham (2011) reported that more than 50% of the strains of human-associated bacteria retrieved in farm-raised freshwater fishes (India) exhibited resistance to multiple antibiotics, again suggesting the potential hazard associated with the consumption

of farm-raised fishes. Finally, Obaidat et al. (2015) observed that half of the sampled traded fish (from Egypt, India, and Yemen) were contaminated with *S. aureus*, and most were resistant to at least one antibiotic. The authors also found that the presence of an enterotoxin-encoding gene (a toxin causing gastrointestinal symptoms) was positively correlated with antibiotic resistance, suggesting a pathogenic hazard – in addition to the antibiotic resistance hazard – linked to the consumption of fish meats.

Concerning meat, Schwaiger et al. (2012) showed that *E. coli* strains retrieved in chicken and pork meat sampled in a slaughterhouse and a retail setting (Germany) were often resistant to penicillins, streptomycin, spectinomycin, doxycycline and sulfamethoxazole/trimethoprim. Among several coliforms of interest, *E. coli* presented the highest resistance rates. The prevalence of ARB was higher in meat from a retail store than in meat from a slaughterhouse, and resistant and multiresistant isolates were more abundant in chicken than in pork meat (Schwaiger et al. 2012). Randall et al. (2017) reported that approximately 2% of beef and pork samples and 65% of chicken samples were positive for ESBL-producing *E. coli*. These two studies suggest that poultry meat provides a higher level of resistance than pork and beef meats. Tansawai et al. (2018) showed that the prevalence of ESBL-producing *E. coli* reached 70% in poultry meat from supermarkets in Thailand. Moreover, the authors found that pathogenic *E. coli* ST131 isolates harboured ARGs, thus raising serious concerns for food safety. Monte et al. (2017) retrieved colistin-resistant *E. coli*-carrying plasmids previously identified in animals and human isolates in commercial chicken meat (Brazil), suggesting the potential dispersion of ARB between farm animals and humans. Similarly, Berg et al. (2017) found that clones of cephalosporin-resistant *E. coli* were shared by poultry and patients, thus supporting their transfer from chicken meat to humans. All these studies revealed that human contamination with ARB through meat consumption may lead to difficult-to-treat infections. However, the likelihood of exposure appears to be low except for poultry meat.

Several studies have been conducted on the contamination of vegetables and meats by antibiotic-resistant bacteria and assumed an existing but limited ARB dispersion from food to humans. Poultry meat is relatively more contaminated by ARB than are other meats, but the associated risk is very limited if the meat is well cooked. Some studies have suggested that ARB present in the food chain could constitute a reservoir of transferable ARGs among pathogens (Koo and Woo 2012). Losasso et al. (2018) measured a lower total load of ARGs in vegan diets compared to those in omnivorous and vegetarian diets, suggesting a possible role of animal-derived food consumption in the risk of contamination by antibiotic resistance determinants. However, considering each studied ARG separately, the authors found no differences among the three diet groups, thereby reflecting the results of Meijs et al. (2020) who conclude that eating meat is not an important risk factor for ESBL *E. coli* and *K. pneumoniae* carriage. Moreover, Königer et al. (2014) and Milanović et al. (2017) found that the diet did not impact ARG diversity in the gut and the load of ESBL-producing *Enterobacteriaceae* in faeces, respectively. More generally, the

likelihood of exposure is also linked to the cooking conditions (cooking, kitchen environment, hygiene measures). Based on these findings, food is associated with a moderate risk of transmission of antibiotic resistance in high income countries.

### 4.5.3 Exposure through Recreational Activities

Coastal water and freshwater, potentially contaminated by antibiotic-resistant bacteria of faecal origin, are the sites of multiple recreational activities, thus raising the question of the hazard to which human populations are exposed. The environmental health hazard associated with antibiotic resistance through recreational activities is poorly known. ESBL-producing *E. coli* have been found in coastal waters popular for swimming and other recreational activities (Blaak et al. 2014a). However, the associated risk for human populations has not been assessed. Nevertheless, Leonard et al. (2018a) investigated antibiotic-resistant *E. coli* in 13 coastal bathing waters in England. As already reported, each *E. coli* isolate retrieved from these sites harboured an average of 1.24 ARGs. Nearly 2.5 million water sports sessions occurred in England in 2016, exposing people to the ingestion of at least 100 ARGs (Leonard et al. 2018a). This estimation only took into account one species (*E. coli*) and did not report the additional resistance hazard associated with other species of bacteria. Moreover, this study was carried out on viable *E. coli*, which are able to directly colonize the human gut. This result demonstrates that humans can be exposed to ARGs borne by bacterial species able to colonize the human intestinal microbiota during recreational aquatic activities. In a second work, Leonard et al. (2018b) found that 15% of the water samples from 97 coastal bathing waters (England and Wales) contained third-generation cephalosporin-resistant *E. coli* and that 0.12% of all retrieved *E. coli* were resistant to third-generation cephalosporin (and 0.07% harboured *bla*<sub>CTX-M</sub> genes). The authors noted a low hazard for such a high level of exposure but concluded that the risk of environmental contamination does exist. Furthermore, Leonard et al. (2018b) estimated the risk of contamination for surfers by third-generation cephalosporin-resistant *E. coli*. To do so, 143 surfers were recruited together with 130 non-surfer controls. The authors found that 9.1% of the surfers were contaminated by third-generation cephalosporin-resistant *E. coli* vs. 3.1% of the non-surfers. Additionally, 6.3% of the surfers carried a *bla*<sub>CTX-M</sub> gene vs. 3.1% for the controls. The authors determined that surfers have an increased risk (3–4 times) of being exposed to antibiotic resistance through the environment than non-surfers.

Fishing is a recreational activity that includes a lower environmental contamination exposure for human populations. Bollache et al. (2018) demonstrated that fish could be contaminated by ARB (cefotaxime-resistant *E. coli* and ESBL-producing *E. coli*), therefore supporting the health hazard associated with the consumption of fish from recreational fishing. However, fishing represents a very low health risk if the fish are cooked before eating.

#### 4.5.4 *One Health or Not One Health?*

The One Health approach (initiated in the early 2000s) promotes an integrated, systemic and unified approach to public, animal and environmental health; this implies that human and animal health are interdependent and linked to the health of the ecosystems in which they coexist. We have previously seen that animals and the environment can be contaminated with antibiotic-resistant bacteria, becoming a potential source of contamination for humans.

Some studies have found a clonal relatedness between clinical and environmental antibiotic-resistant bacteria. Hence, Seni et al. (2018) showed that part of the human, animal and environmental chains in Tanzania shared some sequence types of ESBL-producing *Enterobacteriaceae*, including *E. coli* ST131. Ojer-Usoz et al. (2017) studied ESBL-producing *E. coli* retrieved in humans, food, WWTPs, water and rivers and reported that there was no sequence-type segregation according to the origin of the isolates, and Martak et al. (2020) found similar results. However, the resolution of the multilocus sequence-typing methods is too low to identify transmissions among compartments. Runcharoen et al. (2017) sequenced the genomes of clinical and environmental isolates of ESBL-producing *K. pneumoniae* collected in Bangkok (Thailand) and found no divergence between the two groups, suggesting the exchange of antibiotic-resistant bacteria between humans and the environment.

However, the majority of genomic studies generally sorted antibiotic-resistant bacteria from clinical and environmental sources into distinct clusters, thereby suggesting they are not exchanged. For example, Ludden et al. (2019) compared the genomes of ESBL-producing and ESBL-non-producing *E. coli* isolated from livestock farms and retail meat with those of *E. coli* isolates responsible for blood-stream infection in the UK. They found that livestock and patient isolates were genetically distinct, and some bacteria isolated from different farms were similar. This finding suggests that *E. coli* originating from livestock do not directly transfer to humans but can spread among animals. In a second study, the same group compared the genomes of *K. pneumoniae* retrieved in a hospital with those isolated from livestock farms, meat products, hospital sewers and WWTPs in England (Ludden et al. 2020). The authors observed a weak genetic link between hospital and extra-hospital isolates. This segregation was also observed at the gene level. Hence, although ARGs with resistance to cephalosporin were widespread, they were borne on different plasmids by hospital and extra-hospital isolates. As for *E. coli*, *K. pneumoniae* originating from livestock or meat are rarely observed in human infections. Previous results reported limited evidence of antibiotic-resistant bacteria transfer between livestock or food and humans, thus contradicting the One Health approach in the context of high income countries.

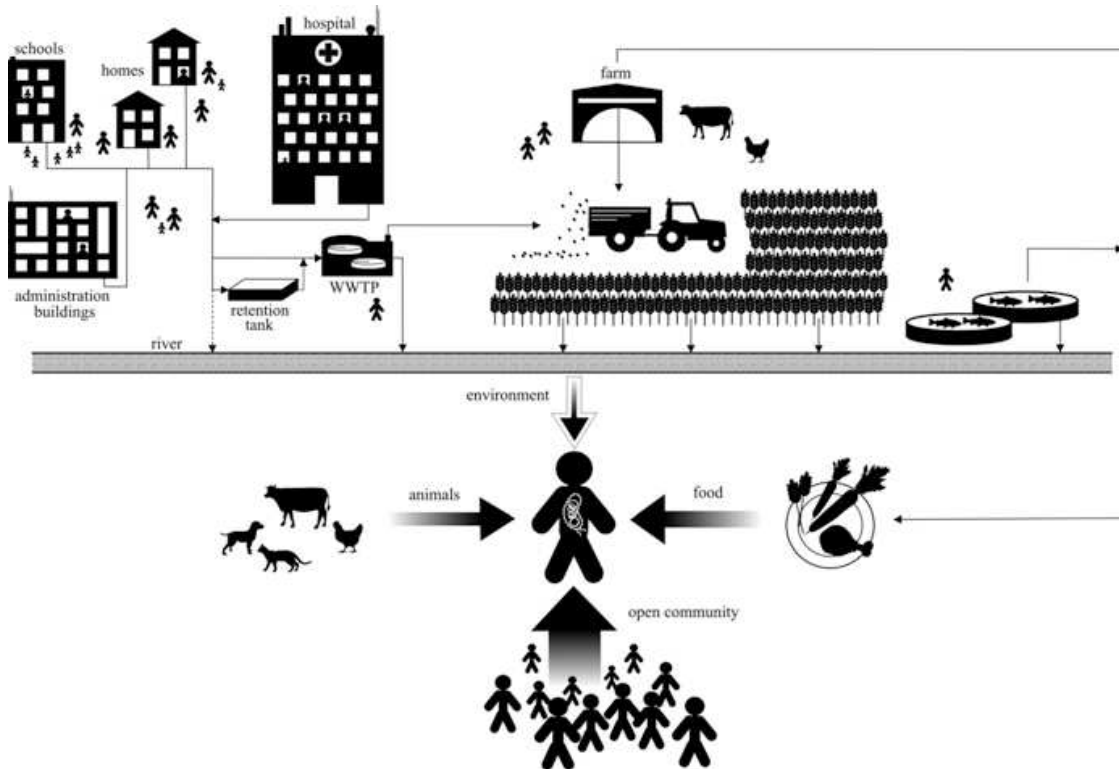
Mughini-Gras et al. (2019) conducted a population-based modelling study of ESBL-encoding and plasmid-mediated cephalosporinase genes collected from human sources (clinical patients, asymptomatic carriers in the open community, poultry and pig farmers, and people who travelled recently) and non-human sources (companion animals, farm animals, wild birds, food products and surface



freshwater). Their model demonstrated that the majority (67.0%) of the ESBL-producing and plasmid-mediated cephalosporinase *E. coli* was acquired by interhuman transmission. In descending order, food accounted for 18.9%, companion animals accounted for 7.9% and farm animals accounted for 3.6% of carriage. The environment (i.e. freshwater and wild birds) only accounted for 2.6% of the community-acquired ESBL-producing and plasmid-mediated cephalosporinase *E. coli* carriage. However, frequent swimming in freshwater increased the environmental-associated carriage of the ESBL-producing and plasmid-mediated cephalosporinase *E. coli* in summer (from 2.3% to 5.7%) (Mughini-Gras et al. 2019). This study therefore suggests that the likelihood of human contamination with antibiotic resistance determinants from the environment may be low in high income countries. The likelihood of exposure to waterborne antibiotic resistance is much higher in low and middle income countries with poor water sanitation and hygiene infrastructure than it is in high income countries with more robust water sanitation and hygiene infrastructure.

#### 4.5.5 *Climate Change and Antibiotic Resistance*

Some studies have noted that climate change could increase environmental contamination with antibiotic resistance. For example, MacFadden et al. (2018) demonstrated that a 10 °C increase in the minimum temperature could be associated with increases in antibiotic resistance of 4.2%, 2.2% and 2.7% for *E. coli*, *K pneumoniae* and *S. aureus*, respectively. Similarly, Kaba et al. (2020) confirmed the potential impact of increasing temperatures on antimicrobial resistance by showing that a 0.5 °C increase in the year-wise temperature would result in a 1.02-fold increase in carbapenem-resistant *P. aeruginosa* prevalence. Moreover, Pärnänen et al. (2019) showed that WWTPs in southern European countries (i.e. Portugal, Spain, and Cyprus) generally released more ARGs than those in northern European countries (i.e. Germany, Norway, and Finland). Knowing the optimal growth temperatures of *E. coli* (37°C), *K. pneumoniae* (30–35°C), *P. aeruginosa* (37°C), and *Acinetobacter* spp. (33–35°C), the authors suggested that this higher resistance dispersion in southern European countries may be partly due to the increased survival of antibiotic-resistant bacteria in these warmer environments. Nevertheless, it is important to keep in mind that an increase in temperature can also promote the development of predators (especially protozoa). Moreover, Czekalski et al. (2015) related some ARG abundances with the level of eutrophication of lakes, which is expected to increase with global change. In addition to the global increase in temperature, the general decrease in precipitation, increase in the precipitation intensity (European Academies Science Advisory Council 2018) and subsequent increase in combined sewer overflows could also accelerate the dissemination of antibiotic resistance in the environment downstream of combined sewage systems (Jalliffier-Verne et al. 2015; Olds et al. 2018). Moreover, the future increase in droughts (European academies' science advisory council 2018) will decrease river flows, and thus the



**Fig. 4.7** General summary scheme. Wastewater treatment plants, agriculture and aquaculture are the main sources of antibiotic resistance in the environment. The environmental antibiotic resistance hazard is limited in high income countries but is highest in summer (i.e. increased exposure) and in low and middle income countries (i.e. increased hazard and exposure) (WWTP wastewater treatment plant)

dilution of ARB released in the receiving water through the treated effluent from WWTPs. Finally, the future increase in the world population will lead to a global increase in antibiotic resistance (Jalliffier-Verne et al. 2015) and its associated risk, *via* both hazard and exposure increases, especially in low and middle income countries.

Overall, the following conclusions can be summarized (Fig. 4.7): (i) the antibiotic resistance exposome (i.e. the sum of an individual's lifetime environmental exposure to antibiotic resistance in water, food and other environmental compartments) can pose a health hazard for human populations; (ii) poultry meat is highly contaminated by antibiotic-resistant bacteria, but the associated hazard is low if the meat is well cooked; (iii) the general environmental exposure associated with antibiotic resistance is limited in high income countries; (iv) few studies have estimated the real risk of contamination of humans by antibiotic-resistant bacteria after exposure to the environment; (v) studies are needed to assess this risk, especially in low and middle income countries; and (vi) climate change and the increase of human population could increase antibiotic resistance in the environment.

## 4.6 What Are the Solutions to Reduce Antibiotic-Resistant Bacteria Dispersion in the Environment and the Associated Likelihood of Exposure?

### 4.6.1 *The Design of Wastewater Sewage Systems*

Combined sewage systems need particular attention. First, wastewater treatment plants that collect wastewater from combined sewage systems should be equipped with retention tanks to limit combined sewer overflows. Moreover, separate sewage systems, when appropriate, need to be prioritized in newly built areas. Obviously, separate sewage systems are not the solution in all contexts, especially in densely anthropized areas where runoff is also highly polluted, and effluent needs to be treated before being discharged into the environment. However, separate sewer systems need to be considered and encouraged in moderately and minimally anthropized areas. A risk assessment of environmental contamination by microorganisms needs to be performed to properly design sewage systems.

### 4.6.2 *Wastewater Treatment Plants*

**Reduction of Antibiotic Resistance in Wastewater by Advanced Wastewater Treatment Methods** Although WWTPs reduce the absolute load of antibiotic-resistant bacteria, they are a major source of the spread of antibiotic-resistant bacteria in the environment. Some studies even reported a relative enrichment of antibiotic-resistant bacteria through WWTP processes (i.e. compared to the total bacterial load). Biological treatments are not designed to reduce antibiotic-resistance. Nevertheless, advanced wastewater treatments can effectively reduce bacterial pollution within WWTPs.

Water chlorination adds chlorine or chlorine compounds to the water. Chlorine is highly toxic to microorganisms and can efficiently clear them from wastewater. Disinfection by chlorination is one of the most commonly used methods to kill microorganisms, including ARB, in the tertiary treatment of WWTPs. However, chlorination does not significantly reduce antibiotic resistance in WWTPs (Iwane et al. 2001; Auerbach et al. 2007; Gao et al. 2012; Munir et al. 2011). Moreover, disinfections based on the chemically aggressive destruction of bacterial cell structures, such as chlorination, can promote residual microbial communities that are increasingly resistant to antibiotics and competitive in natural environments (Dodd 2012; Mao et al. 2015; Di Cesare et al. 2016b).

Conventional advanced oxidation processes include UV radiation, ozone treatment and hydrogen peroxide. Gouliouris et al. (2019) showed that UV radiation reduced environmental contamination with vancomycin-resistant *E. faecium*. In contrast, Auerbach et al. (2007) reported that the UV radiation of wastewater

effluent showed no reduction in 10 tetracycline antibiotic resistance genes (Auerbach et al. 2007). Jäger et al. (2018b) found that the efficiency of ozone treatment depends on the ARGs considered. Among conventional advanced oxidation processes, the authors reported that ozone treatment was the most efficient in the reduction of ARGs. Generally, advanced oxidation processes have been reported to be efficient in reducing antibiotic-resistant bacteria compared to the use of conventional secondary treatment by WWTPs.

The solar photo-Fenton process is an advanced oxidation process that improves solar disinfection by the addition of  $\text{H}_2\text{O}_2$  and  $\text{Fe}^{2+}$  to produce hydroxyl radicals that degrade the external membrane of bacteria and eventually inactivate the cell (Giannakis et al. 2018). Giannakis et al. (2018) tested the effect of this process on a range of antibiotic-resistant *E. coli*, *S. aureus* and *K. pneumoniae*. They found that antibiotic-resistant bacteria reduction depends on the treatment duration. A notable decrease in ARB occurred after 30 min of solar photo-Fenton treatment or after a 3-h exposure to light. *S. aureus* and *E. coli* were totally inactivated by the solar photo-Fenton treatment within 1.5 h, whereas 4 h was required with solar light only (Giannakis et al. 2018).

In low and middle income countries with poor water sanitation infrastructure, the implementation of the costly processes described above is unreasonable. Affordable treatment processes that could limit the contamination of the environment, such as the construction of wetlands or oxidation ponds, could be implemented (Sleytr et al. 2007; Butler et al. 2017).

**Reduction of Antibiotic Resistance in Sewage Sludge** Anaerobic treatments are generally described as more efficient than aerobic treatments in reducing antibiotic resistance in sewage sludge (Diehl and LaPara 2010; Tian et al. 2016; Bürgmann et al. 2018), even if the majority of studies have focused on antibiotic resistance genes rather than antibiotic-resistant bacteria. Several studies have suggested that thermophilic anaerobic digestion ( $48^\circ\text{C}$ – $55^\circ\text{C}$ ) is comparatively more efficient for removing antibiotic resistance genes (Diehl and LaPara 2010; Tian et al. 2016).

Composting is also widely used in the sewage sludge treatment process. The majority of investigations have been conducted on antibiotic resistance genes rather than antibiotic-resistant bacteria. Tien et al. (2017) tested several processes associated with ARG removal before manure spreading (untreated, anaerobically digested, mechanically dewatered or composted) and reported that composted manure displayed the lowest ARG abundances. The ARG loading rates were 10- to 1000-fold lower in soils receiving composted manure than those for raw manure, reflecting the efficiency of composting in lowering the antibiotic resistance dissemination. Nevertheless, composted manure has also been shown to increase the diversity of antibiotic resistance genes (Chen et al. 2019). Tien et al. (2017) recommended a delay of one season between the application of manure and the harvest of fresh produce to minimize the antibiotic resistance hazard.

Aerobic digestion, anaerobic digestion and composting are not considered as stabilization processes; they need to be completed with heat or lime stabilization, for example, to reduce the antibiotic-resistant bacteria load of sewage sludge/manure.

Finally, Lau et al. (2017) observed that with the application of pelletized biosolids, the abundance of antibiotic resistance genes in receiving soils was lowered.

**Reducing the Selective Pressure** It is also possible to control pollutants that exert a selective pressure on ARB, such as antibiotics, to limit the selection of ARB in sewers, WWTPs and receiving environments. Some antibiotics are stable in wastewater (e.g. quinolones, macrolides and sulfamides) and then for a long time in the receiving environments. Briefly, the major removal pathways of antibiotics include adsorption, biodegradation, disinfection and membrane filtration in wastewater treatment processes (Li and Zhang 2010; Berendonk et al. 2015). UV radiation can efficiently reduce the antibiotic concentration in wastewater, therefore reducing the selective pressure within the WWTP and downstream area (Liu et al. 2017; Bürgmann et al. 2018). Composting and anaerobic digestion have also been demonstrated to be efficient in reducing the levels of some antibiotics in sewage sludge (Ezzariai 2018).

Some advanced wastewater treatments are therefore efficient in reducing the dispersion of antibiotic resistance in the environment; their efficiency depends on the antibiotic resistance determinant and the context of the WWTP. Further investigations are needed to better understand the microbial ecological processes that take place at WWTPs, and to test the ability of new advanced wastewater treatments and sewage sludge processes to reduce the ARB and ARG loads (e.g. activated carbon followed by ultrafiltration; Margot et al. 2013).

### ***4.6.3 Other Sources of Antibiotic-Resistant Bacteria***

For hospital, we have previously seen that it is inefficient to separately treat hospital wastewater and urban wastewater (Buelow et al. 2020). Moreover, the cost/benefit ratio of such a pretreatment would not be favourable because it is too costly for a very limited reduction in antibiotic-resistant bacteria when taking into account the entire sewage system. Nevertheless, the appropriate use of antibiotics will reduce the antibiotic concentrations in hospital wastewater, the selective pressure on sewage systems and WWTPs and the abundance of antibiotic-resistant bacteria in WWTP effluents and biosolids.

The reduction of antibiotics in agriculture and fish farms will also limit the contamination of the environment. Furthermore, the spread of biosolids or manure needs to be adapted to consider weather events, such as precipitation and frost (Beattie et al. 2018). Composting can also help in the reduction of antibiotic resistance on agricultural lands. As described above, ‘buffer periods’ could be established between spreading and cereal/vegetable cultivation (Tien et al. 2017).

Finally, the treatment of the effluents of pharmaceutical industries is crucial for reducing the selective pressure in receiving environments. This need is particularly notable in India, where a poor sanitation system and many important pharmaceutical industries exist (Changing Markets and Ecostorm 2016; World Health Organization 2019).

#### 4.6.4 *Education and Practices*

Generally, education, knowledge exchange and antibiotic prescription practices are pillars for reducing the dispersion of antibiotic resistance (Topp et al. 2018; Bürgmann et al. 2018). If not already implemented, policies need to be adopted to better manage the use of antibiotics. These policies must not only be implemented but also monitored over long periods so that they can be adapted to changing situations or local contexts. Policies and research are needed at large scales, but even more at low scales, to limit the dispersion of antibiotic resistance, considering local parameters. At the landscape scale, the factors that control the dissemination of antibiotic-resistant bacteria and the associated risk are poorly understood and need further investigation in environments affected by faecal pollution.

Low and middle income countries are particularly concerned with these recommendations because the contamination of the environment by antibiotic resistance is high, and the populations are therefore often exposed due to poor sanitation infrastructure (World Health Organization 2019). Improving sanitation, increasing access to clean water and good governance are necessary to reduce the spread of global antimicrobial resistance (Collignon et al. 2018). Moreover, knowledge and water treatment techniques/technologies for a limited environmental contamination by antibiotic-resistant bacteria must be fully accessible to local decision makers: this is also true for the implementation of uncontaminated drinking water networks. As in high income countries, populations in low and middle income countries need to be made aware of antibiotic resistance and its possible health consequences in the near future.

Overall, the following conclusions can be summarized: (i) particular importance should be placed on the adequate design adequacy of the sewage systems; when a combined sewage system is already operative, the corresponding WWTP should be equipped with retention tanks to reduce combined sewer overflows; (ii) new processes for wastewater or sewage sludge treatment could reduce ARB and ARG loads but require further investigation; (iii) decreasing of antibiotic use for humans and animals can reduce the spread of antibiotics and antibiotic resistance determinants in the environment; (iv) special attention must be paid to the effluents of pharmaceutical industries; (v) managing the spread of antimicrobial resistance in the environment will require increased public awareness, knowledge exchange and the implementation of local policies; and (vi) ultimately, efforts will have to be concentrated in low and middle income countries, where the environment and populations are the most exposed to antibiotic resistance hazards.

## 4.7 Conclusions

Even if wastewater treatment plants reduce the antibiotic-resistant bacteria load of wastewater, their outputs (treated wastewater and biosolids) are the main sources of antibiotic resistance determinants into the environment in high income countries, especially if wastewater treatment plants are connected to a combined sewage system (Fig. 4.7). Even if hospital wastewater is highly contaminated with antibiotic-resistant bacteria, urban wastewater contributes to most of the antibiotic resistance related to wastewater treatment plants. Agriculture and aquaculture practices also release antibiotic-resistant bacteria in the environment (Table 4.2, Fig. 4.7). Resulting from these multiple sources of release, antibiotic-resistant bacteria are retrieved in many ecosystems, mostly aquatic ones. Logically, contamination with antibiotic-resistant bacteria is related to the level of anthropization. The roles of flora and fauna in the dissemination and survival of human-associated antibiotic resistance determinants are poorly known and require further investigation. The selective pressures exerted by antibiotics, metals and biocides could have little influence on antibiotic-resistant bacteria enrichment in contaminated environments (Table 4.2). However, the situation is different in environments heavily contaminated by antibiotics, especially downstream of pharmaceutical industries. Despite the dispersal of antibiotic resistance in our environment, the associated risk is limited in high income countries (Fig. 4.7). In low and middle income countries with poor water sanitation and hygiene infrastructure, the conclusions need to be nuanced because the majority of human waste goes untreated into the environment. It is clear that antibiotic resistance spread via the environment and throughout the One Health continuum is enormously problematic in these countries.

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### **Transition**

Ce premier chapitre fait la synthèse des données disponibles sur la diffusion environnementale des BRA et des gènes de résistance aux antibiotiques (GRA). Les BRA et GRA sont largement dispersés dans l'environnement, principalement *via* les effluents de STEU. D'autres sources ont également été décrites. Ces BGN-CP atteignent une multitude d'écosystèmes, aquatiques pour la plupart, mais également terrestres agricoles. Les deux prochains chapitres vont étudier plus précisément le devenir des BGN-CP dans des hydrosystèmes tempérés. Plusieurs types d'écosystèmes aquatiques de plaines alluviales seront considérés, avec une attention particulière portée aux zones humides, comme développé plus haut (voir "Méthodologies générales"). Les processus de dissémination environnementale des pathogènes associés à l'Homme restent peu connus. L'origine, la dispersion et la survie de *E. coli*, *K. pneumoniae* et *P. aeruginosa* seront étudiées avec (i) une approche inter-hydrosystèmes (chapitre 2) et (ii) une approche intra-hydrosystème (chapitre 3).



## *CHAPITRE 2*

# APPROCHE INTER-HYDROSYSTÈME : LES PLAINES ALLUVIALES DE L'AIN, DU DOUBS ET DE LA LOUE



## 4. CHAPITRE 2 : APPROCHE INTER-HYDROSYSTÈME : LES PLAINES ALLUVIALES DE L'AIN, DU DOUBS ET DE LA LOUE

### 4.1. Dispersion inter-hydrosystème : abondances bactériennes dans trois plaines alluviales de l'est de la France

**Titre :** Occurrence et déterminants écologiques de la contamination des zones humides des plaines alluviales par *Klebsiella pneumoniae* et *Escherichia coli* pathogène ou résistant aux antibiotiques

**Revue :** FEMS Microbiology Ecology, volume 95

**État :** publié, 2019

#### **Éléments essentiels :**

- Nous avons étudié la dispersion et la survie de BGN-CP dans trois hydrosystèmes karstiques, rangés sur un gradient d'eutrophisation, de l'est de la France ;
- Les trois rivières transportent des bactéries pathogènes et des bactéries résistantes aux antibiotiques vers les zones humides ;
- Les zones humides peu profondes et chaudes semblent permettre à *K. pneumoniae* de prospérer ;
- *E. coli* rencontre également des conditions favorables dans certaines zones humides ;
- La contamination des zones humides ne semble pas seulement dépendre de l'apport fluvial. Par conséquent, d'autres sources de BGN-CP pourraient être impliquées et doivent être prises en compte à l'échelle du bassin versant des zones humides.



## Résumé

La survie et la multiplication des bactéries pathogènes et résistantes aux antibiotiques dans les écosystèmes sont de plus en plus préoccupantes mais ont été peu explorées. Les zones humides peuvent être contaminées par les flux d'eau provenant des rivières et peuvent présenter des conditions environnementales favorisant la survie et la multiplication des bactéries pathogènes. Pour tester cette hypothèse, nous avons échantillonné 16 zones humides situées le long de trois rivières du massif du Jura (France). La contamination bactérienne des eaux des zones humides et des rivières a été mesurée mensuellement sur un cycle d'un an, tout comme les caractéristiques physico-chimiques de l'eau. Nous avons évalué l'abondance de trois espèces pathogènes : *Escherichia coli*, *Klebsiella pneumoniae* et *Pseudomonas aeruginosa*. Les concentrations d'*E. coli* producteurs de BLSE ou appartenant au groupe phylogénétique B2 – le plus virulents – ont également été mesurées. Nous avons constaté que les rivières transportent les *E. coli* totaux, les *E. coli* producteurs de BLSE et les *K. pneumoniae* vers les zones humides. Les *E. coli* producteurs de BLSE survivent peu dans les zones humides, alors que les *E. coli* totaux et *K. pneumoniae* rencontrent probablement des conditions physico-chimiques favorables à leur survie et à leur multiplication dans ces habitats. Les abondances de *K. pneumoniae* culminent en été dans les zones humides chaudes et peu profondes. *E. coli* total et *E. coli* B2 atteignent potentiellement les zones humides par des sources autres que les rivières (eaux souterraines ou lessivage depuis les terres agricoles contaminées).

## RESEARCH ARTICLE

# Occurrence and ecological determinants of the contamination of floodplain wetlands with *Klebsiella pneumoniae* and pathogenic or antibiotic-resistant *Escherichia coli*

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**One sentence summary:** Hydrological connectivity and ecological conditions may favour the dispersal and survival of human-associated bacteria in rivers and their floodplains.

**Editor:** Martin Hahn

## ABSTRACT

The survival and multiplication of human pathogenic and antibiotic-resistant bacteria in ecosystems is of increasing concern but has been little explored. Wetlands can be contaminated by water fluxes from rivers and may present environmental conditions leading to bacterial survival and multiplication. To test this hypothesis, we sampled 16 wetlands located along three rivers of the Jura Massif, France. The bacterial contamination of the wetland and river waters was measured monthly over a one-year cycle together with the water physico-chemical characteristics. We assessed the abundance of three pathogenic species: *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. The concentrations of *E. coli* producing extended-spectrum  $\beta$ -lactamase (ESBL *E. coli*) or belonging to the phylogenetic group B2 (*E. coli* B2–more pathogenic) were also measured. We found that rivers carried total *E. coli*, ESBL *E. coli*, and *K. pneumoniae* to wetlands. ESBL *E. coli* poorly survived in wetlands, whereas total *E. coli* and *K. pneumoniae* possibly met favourable physico-chemical conditions for survival and multiplication in these habitats. *K. pneumoniae* peaked in summer in warm and shallow wetlands. Total *E. coli* and *E. coli* B2 potentially reached wetlands through sources other than rivers (hillslope groundwater or leaching from contaminated fields).

Received: 29 January 2019; Accepted: 20 June 2019

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**Keywords:** antibiotic-resistance; pathogens; ecological niche; public health; wetlands; hydrological connectivity

## INTRODUCTION

Human activities frequently release bacteria into the environment through point sources (e.g. wastewater treatment plants (WWTPs) and farms) and non-point sources (e.g. rainwater runoff, sewage sludge and manure). Some of these bacteria can be pathogenic to humans, and others are resistant to antibiotics, leading to sanitation issues and consequent risk (Gupta, Hooton and Stamm 2001; Rodríguez-Baño et al. 2008).

Pathogenic bacteria generally spread through water contaminated by human or cattle faeces (Cabral 2010). They mostly originate from municipal sewage (Cabral 2010) and agricultural practices that spread manure (Guan and Holley 2003; Cabral 2010) and contaminated sewage sludge (Niu and Phanikumar 2015; Su et al. 2015; Chen et al. 2016) in agricultural lands. Pathogens contaminate most areas of the environment, including agricultural watersheds (Jamieson et al. 2004), reservoirs and lakes (Brookes et al. 2004) and groundwater (John and Rose 2005). The Environmental Protection Agency of the United States of America (USA EPA), which monitors the quality of water bodies, estimated that pathogenic bacteria contaminated more than 480 000 km of rivers and shorelines, and two million ha of lakes in the USA (USA EPA 2010).

Antibiotic-resistant bacteria originate from hospitals (Hocquet, Muller and Bertrand 2016), WWTP effluents (Young, Juhl and O'Mullan 2013; Amos et al. 2014) and sewage sludge and animal slurry in farmland (Byrne-Bailey et al. 2009). Soils, surface waters (e.g. seas and rivers) and sediments are contaminated by these bacteria, which harbour a large array of antibiotic resistance genes (ARGs; Leonard et al. 2015). Given the increasing global spread of antibiotic-resistant bacteria, contaminated ecosystems have to be monitored.

The dispersal of bacteria in floodplains depends upon their survival during vertical (from soil to groundwater), lateral (from rivers to wetlands) and longitudinal water fluxes (via the upstream and downstream flow of river water). Their survival relies on both abiotic (e.g. ecosystem characteristics and pollutants such as antibiotics or chemicals (Lim and Flint 1989; Beaudreau et al. 2001; Joint et al. 2002; Jonsson and Agerberg 2015)) and biotic parameters (e.g. competition with autochthonous microbial communities and predation (Flint 1987; Beaudreau et al. 2001)). Some authors have observed the spread of contamination in rivers (Chu et al. 2014), and others have proposed models of bacterial mobility in streams (Niu and Phanikumar 2015). Thus, freshwater ecosystems, particularly rivers, are suspected to play an important role in the dispersion of human-associated bacteria into the environment and to serve as reservoirs that can potentially lead to human contamination. However, little is known about bacterial dispersion throughout entire hydrologic networks, and riverine annexes have generally been excluded from previous studies; this paper proposes to remedy this.

Floodplain wetlands are particularly vulnerable to bacterial contamination because they are connected to potentially contaminated rivers through overflows and backflows during floods and through seepage water that flows from rivers to wetlands through coarse sediment (Bornette et al. 1998; Amoros and Bornette 2002). The functioning of floodplain wetlands (in terms of the kinetics and intensity of the biological activity from microorganisms to animal and plant macroorganisms as well as the structure and complexity of food webs) is directly governed by the geological and climatic environment in addition to the

hydrogeological, geomorphological and anthropogenic contexts (Bornette et al. 2008). These features control the connectivity of wetlands with their hydrological environment (aquifers, rivers) and the physico-chemical characteristics of the water (Amoros and Bornette 2002). Some studies have outlined the roles of nutrients and temperature in determining the bacterial community structure in riverine lakes (Kalcheva et al. 2016). However, these relationships (especially those concerning temperatures) vary between different studies and according to the bacteria that are considered (Beaudreau et al. 2001; Schulz and Childers 2011; Kalcheva et al. 2016).

Wetlands and rivers support human recreational activities such as fishing and bathing. Floodplain wetlands are abundant along rivers (Yang et al. 2016) and may constitute reservoirs for human-associated bacteria because they are potentially nutrient-rich and connected to contaminated rivers and groundwater. The role of natural environments in the transmission of antibiotic-resistant pathogens is poorly understood. However, contamination of water with antibiotic-resistant pathogens could present a risk for water users (Leonard et al. 2018). The role of such aquatic ecosystems in the dispersion of pathogenic and antibiotic-resistant bacteria into the environment should therefore be assessed.

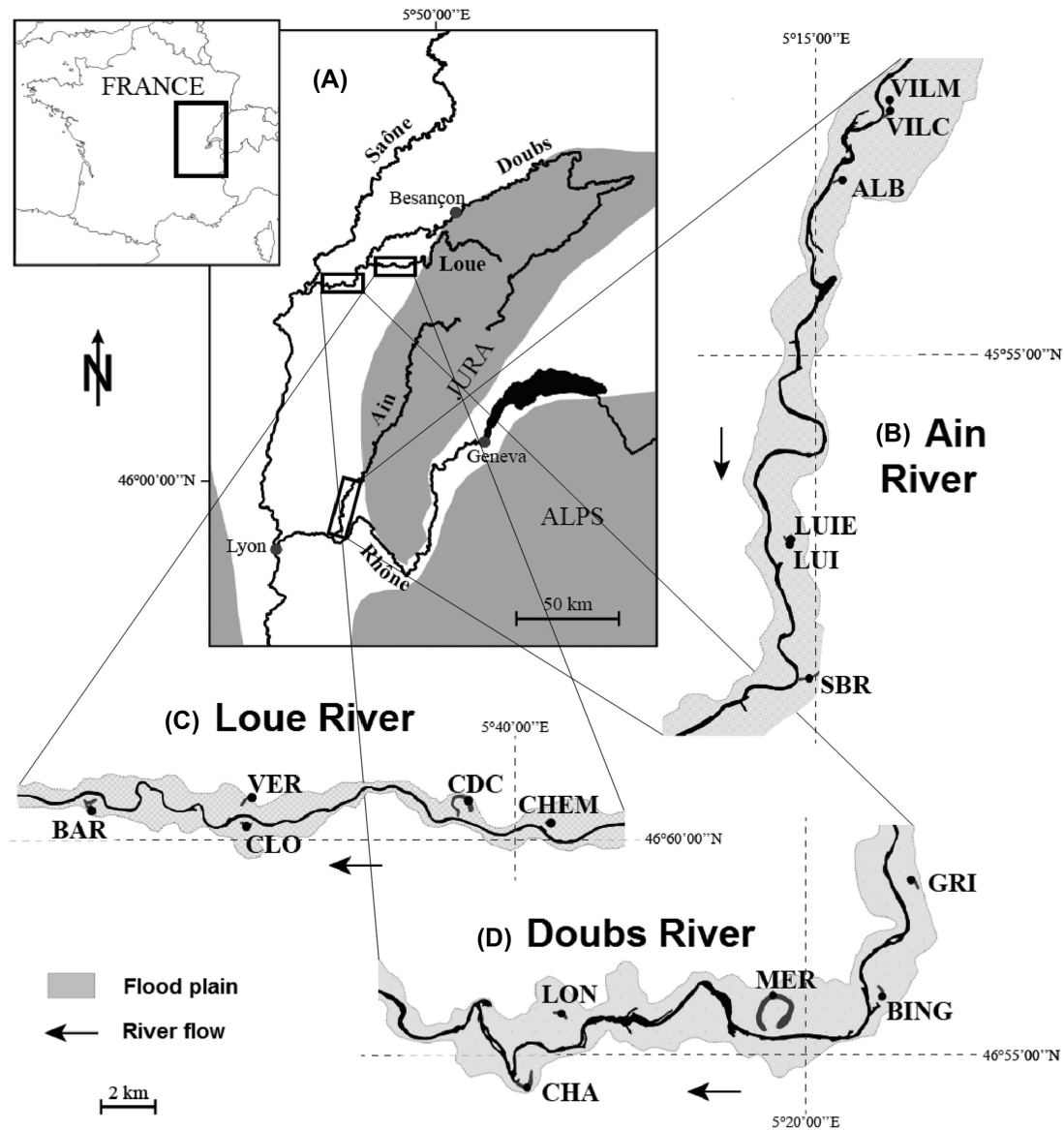
The present study aimed to determine (i) whether river and wetland contamination are similar, which would suggest transversal dispersal between rivers and their riverine aquatic habitats and (ii) whether wetland contamination is related to wetland ecological characteristics in terms of the physico-chemical characteristics of water, including the thermal regime and the nutrient concentrations.

To achieve these objectives, the occurrence and abundance of bacteria and the physico-chemical characteristics of water were measured monthly for a year in 16 wetlands belonging to three river floodplains. This study focused on three bacteria of major concern for humans: *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. *E. coli* is one of the main pathogens responsible for community infections (Gupta, Hooton and Stamm 2001; Cabral 2010). *P. aeruginosa* and *K. pneumoniae* are markers of the contamination of wastewater networks due to hospital activity (Slekovec et al. 2012). *E. coli* and *P. aeruginosa* are also good markers of the overall level of bacterial resistance to antibiotics because they easily accumulate resistance determinants (Lautenbach et al. 2001; Slekovec et al. 2012; Hocquet, Muller and Bertrand 2016).

## MATERIALS AND METHODS

### Studied wetlands

The 16 wetlands studied were distributed along a wide gradient of eutrophication (in terms of nitrogen, phosphorus and organic carbon concentrations in the water) and thermal variability. They were distributed along the lower floodplains of three karstic rivers of the Jura Massif: the Ain, Doubs and Loue Rivers (Fig. 1). To compare the wetlands and rivers, we selected one river sampling station per floodplain that was located as close as possible to the sampled wetlands (Ain River: 45°57'50"N, 05°15'2"E; Doubs River: 46°55'22"N, 05°20'59"E; Loue River: 47°00'53"N, 05°29'39"E). The Ain River has an average monthly discharge of 109.2 m<sup>3</sup>.s<sup>-1</sup>, the Doubs River has an average monthly discharge of 161.8 m<sup>3</sup>.s<sup>-1</sup> and the Loue River has



**Figure 1.** Locations of the 16 studied wetlands. The top left panel represents the locations of the three considered hydrosystems in France. A. Location of the river network under study on the map of France. Boxes indicate the locations of the river sections shown in panels b, c and d. The three other panels indicate the wetland locations along the three river sections. B. The Ain River, from upstream to downstream: VILM (Vilette Amont), VILC (Vilette Centre), ALB (L'Albarine), LUIE (Le Luizard Est), LUI (Le Luizard) and SBR (Sous-Bresse). C. The Loue River, from upstream to downstream: CHEM (Chemillière), CDC (Champs des Creux), CLO (Le Clos), VER (La Verne) and BAR (Le Baraquier). D. The Doubs River, from upstream to downstream: GRI (Grimonts), BING (Bras des Ingles), MER (Mératon), LON (Longepierre) and CHA (Charette).

an average monthly discharge of  $49.0 \text{ m}^3 \cdot \text{s}^{-1}$  ([www.eaufrance.fr](http://www.eaufrance.fr), last consultation: 24 October 2017). The three hydrosystems are located in a temperate climate. All but one wetland remained aquatic during the summer. The substrate is usually fine-grained and ranges from silt to clay and/or peat.

Each wetland and river were sampled monthly over a one-year cycle to take into account seasonal hydrological and climatic variability. The sampling took place from April 2015 to March 2016 for physico-chemical characterisation and bacterial quantification.

### Physico-chemical characteristics of the water

We measured nine parameters that have been demonstrated to be related to ecosystem functioning and/or to potentially

drive bacterial survival and multiplication: water temperature (Sakura 1993; Beaudreau et al. 2001; Bornette et al. 2008; Schulz and Childers 2011; Blaustein et al. 2013), electrical conductivity and pH (Maberly and Spence 1989; Bornette et al. 2008), dissolved oxygen (Doughari et al. 2012; Gu et al. 2012), nitrate, ammonium, phosphate and dissolved organic carbon (DOC) concentrations (Lim and Flint 1989; Coffin, Connolly and Harris 1993; Hong, Qiu and Liang 2010), and chlorophyll-a concentration (Arthaud et al. 2012).

Water temperature was measured daily at each solar hour using immersed probes (HOBO UTBI-001 Tidbit®, Onset Computer Corporation, Bourne, MA, USA). One temperature probe was placed at the bottom of each site (16 wetlands and three rivers) at the beginning of the sampling period that automatically returned the temperature each solar hour during the

one-year cycle sampling period. Both the pH and the electrical conductivity (Robach et al. 1996) were measured *in situ* at a 10-cm water depth using a WTW pH sensor (SenTix® 41, Onset Computer Corporation, Bourne, MA, USA) and a WTW conductivity sensor (TetraCon® 325, Onset Computer Corporation, Bourne, MA, USA), respectively. The dissolved oxygen was measured using a WTW oxygen sensor (CellOx® 325-3, Onset Computer Corporation, Bourne, MA, USA). To determine the concentrations of nitrate, ammonium, phosphate and DOC, 500 ml of water was collected and filtered through 0.2-µm polycarbonate filters and analysed within 48 h of collection. The samples were kept at 4°C throughout the process. The P-phosphate (P-PO<sub>4</sub><sup>3-</sup>) concentration was measured using the molybdenum blue colouration method after acidification. The N-ammonium (N-NH<sub>4</sub><sup>+</sup>) concentration was measured using the indophenol blue method (Berthelot's reaction, Merck Spectroquant 14 752). The N-nitrate (N-NO<sub>3</sub><sup>-</sup>) concentration was measured using a Dionex ICS-1000 ion chromatograph. The DOC concentration was measured using a total organic carbon (TOC) analyser (Vario TOC Cube, Elementar, Stuttgart, Germany). To assess the chlorophyll-a concentration, 3 L of water was collected and then filtered through a 200-µm filter to remove zooplankton and any detritus, after which it was filtered through 0.7 µm filters. 0.7-µm filters were stored at -20°C until extraction (overnight at 4°C in acetone (90%)). The chlorophyll-a concentration was measured the next day using a spectrophotometer (the measurements were conducted at λ = 630, 645 and 663 nm (Yentsch and Menzel 1963)).

### Connectivity between wetlands and rivers

To characterise the connectivity between rivers, groundwater and wetlands, we used the temperature, because it is the only physico-chemical parameter that was easily measurable and allowed comparison of connectivity among rivers (Sakura 1993; Brodie et al. 2007). Groundwater temperature is stable throughout the year, whereas the temperature of surface water varies greatly on a daily and seasonal basis (Kalbus, Reinstorf and Schirmer 2006). We measured the temperatures in rivers and wetlands according to the same protocol, so we were able to compare the values. Consequently, the connectivity between rivers, groundwater and wetlands was estimated based on the thermal contrast between each wetland and its connected river (Kalbus, Reinstorf and Schirmer 2006; Brodie et al. 2007; Kløve et al. 2011). The higher the thermal stability of the wetland compared to the river, the higher the connectivity between the wetland and groundwater was and the lower the connectivity between the wetland and the river was. For each wetland, linear regressions ( $y = a x + b$ ) were calculated between the wetland (y) and river (x) temperatures, which were both taken monthly at 7:00 am (UTC) to avoid biases due to daily warming. The *a* (slope) value directly relates wetland and river temperatures and can thus be considered to be the thermal variation coefficient of the wetlands according to river temperature fluctuations (hereafter referred to as the 'connectivity index'). The *b* (intercept) value represents the thermal inertia of the wetlands. *a* and *b* are inversely related ( $R^2 = 0.95$ ).

### Bacterial sampling

Samples of water (250 ml) were collected in sterile conditions and stored at 4°C before analysis, which occurred within 8 h of collection. The total concentrations of *E. coli*, *K. pneumoniae* and *P. aeruginosa* were measured as previously described (Slekovec et al. 2012; Bréchet et al. 2014). Aliquots of water (100 ml) were

filtered through 0.45-µm membranes, which were then placed onto a Drigalski agar plate to measure the total *E. coli* and *K. pneumoniae* concentrations (Oxoid), a Cetrimide agar plate to measure the *P. aeruginosa* concentration (Bio-Rad) and a chromID ESBL agar plate to measure the concentration of *E. coli* producing extended-spectrum beta-lactamases (ESBL), which are resistant to β-lactam antibiotics. When we suspected high bacterial concentrations, we plated 500 µl an additional of sample on the selective media. The plates were incubated for 24 h at 37°C. Colonies were counted and classified according to their phenotype. Each type of colony was identified using a MALDI-TOF MS spectrometer (Microflex, Bruker Daltonics, Bremen, Germany) according to the manufacturer's recommendations.

For each morphotype of *E. coli* growing on chromID ESBL, the production of ESBL was detected using a synergy test following the recommendations of the Antibiogram Committee of the French Society for Microbiology (CA-SFM, <http://www.sfm-microbiologie.org/>, last consultation: 10 November 2018). We also assessed the activity of 16 antibiotics alone or with inhibitors (amoxicillin (AMX), amoxicillin-clavulanate (AMC), amikacin (AKN), cefalexin (CXN), cefepime (FEP), cefotaxime (COX), ceftazidime (CZD), ciprofloxacin (CIP), cotrimoxazole (SXT), ertapenem (ETP), ofloxacin (OFX), piperacillin-tazobactam (PTZ), ticarcillin (TIC), ticarcillin-clavulanate (TCC) and tobramycin (TMN)). The results were interpreted according to the EUCAST 2017 recommendations ([www.eucast.org](http://www.eucast.org), last consultation: 10 November 2018). For each water sample, we stored an isolate representing each antibiotype in brain heart infusion broth supplemented with 30% glycerol at -80°C until further analysis. *E. coli* forms phylogroups with various pathogenic behaviours; *E. coli* sequence types belonging to the B2 phylogroup are the most pathogenic for humans (Tourret and Denamur 2016). All *E. coli* isolates, regardless of their antibiotype, were typed by phylogrouping as previously described (Clermont et al. 2013).

Consequently, the bacterial data set consisted of the concentrations of total *E. coli* (hereafter 'total *E. coli*'), ESBL-producing *E. coli* (hereafter 'ESBL *E. coli*'), *E. coli* phylogroup B2 (hereafter '*E. coli* B2'), *K. pneumoniae* and *P. aeruginosa* in each water sample. For all bacterial concentrations, the detection limit was 1 bacterium per 100 ml.

### Statistical analyses

The normality and homoscedasticity of the data were tested using the Shapiro-Wilk test and Levene's test, respectively. The bacteriological data set was over-dispersed (variance >> mean).

We first analysed the bacterial abundances using Kruskal-Wallis rank sum tests to highlight significant bacterial variation through space and time. To do so, several potential levels of spatial variation were considered: (i) the total variability (across the 16 wetlands and three rivers), (ii) the contrast between rivers and wetlands, (iii) the variation among the whole set of wetlands (all 16 wetlands), (iv) the variation among the rivers (all three rivers), (v) the variation among the hydrosystems (the 16 wetlands and three rivers grouped according to hydrosystem) and (vi, vii and viii) the variation among wetlands within a given hydrosystem (among the wetlands of the Ain, Doubs and Loue hydrosystems, respectively). Concerning temporal variation, we considered either the individual months or seasons by grouping the months according to season (March to May for spring, June to August for summer, September to November for autumn and December to February for winter).

To identify which sites and dates had significantly lower or higher bacterial values than the others, after the Kruskal–Wallis tests, a pairwise comparison test was performed (Dunn's rank sum test). Dunn's test identifies any significantly different groups but does not order them. Therefore, to do this, we compared the bacterial abundances of the identified groups with the mean bacterial abundance of the other groups.

To determine the physico-chemical determinants of pathogenic bacteria survival in wetlands, we used negative binomial generalised linear models (nbGLMs) to decrease the over-dispersion of the bacterial data set (dispersion parameter  $< 1$  after the application of a nbGLM). A nbGLM was used to successively analyse (i) the whole wetland data set to identify the wetlands and environmental characteristics associated with bacterial occurrence and abundance and (ii) the data from the wetlands where the bacterial abundance was significantly higher than that in the others according to the previously performed Dunn's tests to identify the environmental characteristics that allow bacterial survival and/or multiplication in these wetlands. The connectivity index ( $a$ ) and the river flows were added as explanatory variables in the two nbGLMs. We compared the fitted nbGLMs with the corresponding null model by using an ANOVA function to determine the significance of the model. Finally, we had to take into account the relationships between the explanatory variables and bacterial contamination that are specific to wetlands, independently of an effect related to the river to which the wetland belongs. For example, if a river feeds wetlands with both nutrient-rich water and bacteria, these two parameters will obviously correlate in these wetlands, irrespective of what happens in that potential niche. We thus had to consider any 'river effect' that may affect the wetlands connected to a particular river. To do so, we used Spearman correlation tests. Correlations between the physico-chemical characteristics and bacterial contamination in wetlands that already existed in rivers were not taken into account when assessing which parameters may specifically regulate bacterial occurrence in wetlands, and were removed from the analysis.

The  $\alpha$  value was set to 0.05. All analyses were performed with R 3.4.3 software (R Core Team packages 2017).

## RESULTS

### Connectivity between wetlands and rivers

CHEM was the most connected wetland, with a connectivity index  $> 1$  (Table 1), meaning that its water is heated faster than the river water and that it is likely very poorly connected to the groundwater. VILM and ALB showed low thermal variation (Table 1), suggesting very high connectivity to the groundwater. The other wetlands were distributed between these two extremes (Table 1), illustrating that they are probably supplied by both river water and groundwater.

### Spatio-temporal variability in the bacterial abundances

Over the study period, 224 samples were collected, as one wetland was dry on four sampling dates (LUIE). *E. coli*, ESBL *E. coli*, *E. coli* B2 and *K. pneumoniae* occurred in 150/224 (67.0%), 50/224 (22.3%), 38/224 (17.0%) and 47/224 (21.0%) of the samples, respectively. The spatial bacterial abundances of *E. coli*, ESBL *E. coli*, *E. coli* B2 and *K. pneumoniae* in wetlands and rivers are grouped and shown in Fig. 2. Unexpectedly, *P. aeruginosa* was only detected in four samples among the 224 tested (1.8%): one sample from VILC (1 CFU/100 ml, 28 April 2015), one sample from the Ain River (3

**Table 1.** Connectivity levels between wetlands and rivers based on the thermal contrast between a river and its associated wetlands. 'a' (slope) and 'b' (y-intercept) are indicated for each regression between wetland temperatures and river temperatures. Higher 'a' values indicate a higher thermal proximity between the wetland and its river, and so a higher connexion.

Floodplain	Wetland	a (connectivity index)	b	R <sup>2</sup>
Ain	ALB	0.1828	9.8539	0.63
	LUI	0.528	5.7617	0.98
	LUIE	0.7316	3.0569	0.89
	SBR	0.8154	3.1248	0.95
	VILC	0.5667	5.2124	0.93
Doubs	VILM	0.2156	10.03	0.34
	BING	0.655	3.3996	0.94
	CHA	0.7945	2.3925	0.69
	GRI	0.6499	3.9977	0.85
	LON	0.6949	2.0662	0.87
Loue	MER	0.6784	5.0013	0.94
	BAR	0.577	5.239	0.83
	CDC	0.6218	4.7856	0.92
	CHEM	1.1187	-1.5346	0.95
	CLO	0.5815	4.4202	0.94
	VER	0.7669	2.2217	0.94

CFU/100 ml, 28 April 2015) and two samples from CHA (1 CFU/100 ml, 2 February 2016, and 1000 CFU/100 ml, 2 March 2016). Thus, *P. aeruginosa* was not considered in the statistical analyses.

The abundances of the bacterial groups (all groups but *P. aeruginosa*) did not differ among the rivers or the hydrosystems (a given river and its wetlands; Table 2).

### Total *E. coli*

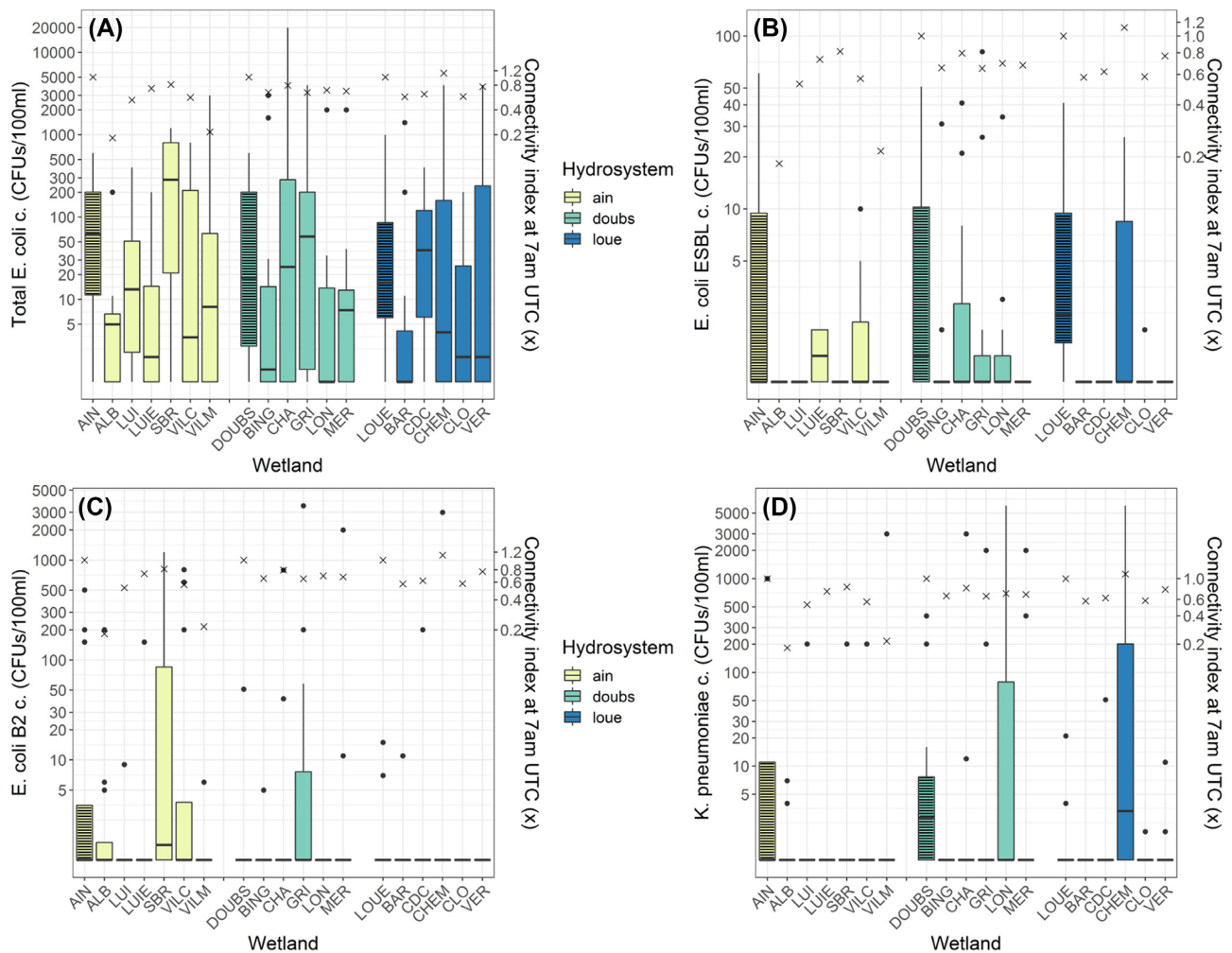
Spatially, the rivers were significantly more contaminated than the wetlands by total *E. coli* (Table 2). However, the total *E. coli* concentration did not differ among the wetlands (Table 2), although the wetlands differed in their connectivity to the rivers. Moreover, certain wetlands were more contaminated than their corresponding river on some dates (Fig. 2(A)); this was particularly true for SBR, which was more contaminated by total *E. coli* than the other wetlands in the Ain hydrosystem.

Total *E. coli* abundance did not increase during winter in wetlands, in contrast with its increase in rivers during this high-flow period (Table 2). We measured a peak in the total *E. coli* contamination in wetlands in September (Table 2 and Fig. 3(A)) during a peak of precipitation after a dry period in the three studied floodplains (Météo France, stations of Ambérieu-en-Bugey (station 01089001), Chamblanc (station 21131001) and Tavaux (station 39526003)).

### ESBL *E. coli*

Regarding its spatial distribution, ESBL *E. coli* was always more abundant in rivers than in wetlands (Table 2 and Fig. 2(B)), even when the three hydrosystems were considered separately (Kruskal–Wallis *P*-values: Ain:  $< 0.01$ , Doubs:  $< 0.05$ , Loue:  $< 0.001$ ). Moreover, ESBL *E. coli* concentrations were higher in CHA (the most connected wetland of the Doubs hydrosystem), GRI, CHEM (the most connected wetland of the Loue hydrosystem), LUI and VILC (the two most connected wetlands of the Ain hydrosystem; Table 2).

Figure S1 shows the distribution of the bacterial concentrations in each wetland on each sampling date according to



**Figure 2.** Concentrations (‘c.’) of pathogenic bacterial species in rivers and their wetlands. A. Total *E. coli*, B. ESBL *E. coli*, C. *E. coli* B2, and D. *K. pneumoniae* across space (sites) and according to the connectivity between wetlands and their associated rivers (connectivity index, crossed points on the graph). Logarithmic scale. Yellow: Ain sites, green: Doubs sites, blue: Loue sites. Cross-hatched boxplots represent the bacterial concentration of rivers. Dots represent boxplot outliers. Crosses represent the connectivity index of each site.

the temperature contrast between the wetland and its associated river on the same date. ESBL *E. coli* was more abundant in the water samples from wetlands that were thermally similar to their associated river, which highlighted their connection (Kruskal-Wallis test,  $P$ -value < 0.001, and Dunn’s test significant for  $|\Delta T| \leq 1^\circ\text{C}$ ,  $\Delta T = T_{\text{wetland}} - T_{\text{river}}$ ).

Regarding the temporal dispersion, the ESBL *E. coli* abundance decreased during summer and increased during winter, specifically in February and March, when the whole data set and the wetland data set were considered (Table 2 and Fig. 3(B)).

#### *E. coli* phylogroup B2

In this study, the *E. coli* B2 concentrations did not vary spatially (Table 2).

Within the three rivers, we measured higher abundances of *E. coli* B2 in September (Table 2). In wetlands, *E. coli* B2 abundance did not vary according to months or seasons. In SBR (the wetland showing the highest *E. coli* B2 concentrations; Fig. 2(C)), contamination by *E. coli* B2 increased in September (without reaching a significant level; Kruskal-Wallis test,  $P$ -value = 0.069, and Dunn’s test), despite its low connectivity to the river.

#### Total *K. pneumoniae*

Spatially, *K. pneumoniae* was more abundant in rivers than in wetlands (Table 2). Moreover, CHEM and LON (2 wetlands highly connected to their river) contained more *K. pneumoniae* than the other wetlands (Table 2 and Fig. 2(D)).

Considering all wetlands of the three hydrosystems, *K. pneumoniae* was more abundant in August and September (Table 2, Fig. 3(D)).

#### Antibiotic resistance profiles of ESBL *E. coli*

The antibiotic resistance profiles of ESBL *E. coli* are shown in Table S1. Of the ESBL *E. coli* that were found, 50/50 (100%) were resistant to amoxicillin, 11/50 (22%) to amoxicillin-clavulanate, 47/50 (94%) to cefalexin, 15/50 (30%) to cefepime, 46/50 (92%) to cefotaxime, 3/50 (6%) to cefoxitin, 20/50 (40%) to ceftazidime, 26/50 (42%) to ciprofloxacin, 30/50 (60%) to cotrimoxazole, 26/50 (52%) to ofloxacin, 3/50 (6%) to piperacillin-tazobactam, 50/50 (100%) to ticarcillin, 29/50 (58%) to ticarcillin-clavulanate and 13/50 (26%) to tobramycin. The ESBL *E. coli* were fully susceptible to amikacin and ertapenem.

**Table 2.** Spatio-temporal variability in bacterial abundances. Significance of the Kruskal-Wallis test performed on bacterial abundances ( $P$ -value:  $x > 0.05 > * > 0.01 > ** > 0.001 > ***$ ) followed by Dunn's tests if significant. In the table, the groups identified by Dunn's test are followed by their position according to the rest of the data (significantly higher ( $\uparrow$ ) or lower ( $\downarrow$ ) bacterial abundance).

Bacteria considered	Contrast between rivers and wetlands	Total variability	Variation among wetlands	Variation among rivers	Variation among hydrosystems	Variation among wetlands of the Ain hydrosystem	Variation among wetlands of the Doubs hydrosystem	Variation among wetlands of the Loue hydrosystem
<b>Total <i>E. coli</i></b>	* river, $\uparrow$	x	x	x	x	x	* SBR, $\uparrow$	x
<b>ESBL <i>E. coli</i></b>	*** river, $\uparrow$	*** Ain, $\uparrow$ ; Doubs, $\uparrow$ ; Loue, $\uparrow$ ; CHA, $\uparrow$ ; CHEM, $\uparrow$ ; LUIE, $\uparrow$	*** CHA, $\uparrow$ ; CHEM, $\uparrow$ ; GRI, $\uparrow$ ; LUIE, $\uparrow$ ; VILC, $\uparrow$	x	x	x	x LUIE, $\uparrow$ ; VILC, $\uparrow$	* CHEM, $\uparrow$
<b><i>E. coli</i> B2</b>	x	x	x	x	x	x	x	x
<b><i>K. pneumoniae</i></b>	** river, $\uparrow$	** Ain, $\uparrow$ ; Doubs, $\uparrow$ ; CHEM, $\uparrow$ ; LON, $\uparrow$	* CHEM, $\uparrow$ ; LON, $\uparrow$	x	x	x	x	** CHEM, $\uparrow$
TEMPORAL VARIABILITY – SEASONS								
<b>Total <i>E. coli</i></b>	-	** summer, $\downarrow$	* summer, $\downarrow$	*** summer, $\downarrow$ ; winter, $\uparrow$	-	x	** summer, $\downarrow$	* autumn, $\uparrow$
<b>ESBL <i>E. coli</i></b>	-	*** summer, $\downarrow$ ; winter, $\uparrow$	*** summer, $\downarrow$ ; winter, $\uparrow$	*** summer, $\downarrow$ ; winter, $\uparrow$	-	x	*** winter, $\uparrow$	* winter, $\uparrow$
<b><i>E. coli</i> B2</b>	-	x	x	x	-	x	x	x
<b><i>K. pneumoniae</i></b>	-	x	x	x	-	* autumn, $\uparrow$	x	x
TEMPORAL VARIABILITY – MONTHS								
<b>Total <i>E. coli</i></b>	-	* September, $\uparrow$	* September, $\uparrow$	* August, $\downarrow$ ; November, $\downarrow$	-	x	x	x
<b>ESBL <i>E. coli</i></b>	-	*** February, $\uparrow$ ; March, $\uparrow$	** February, $\uparrow$ ; March, $\uparrow$	* February, $\uparrow$	-	x	* December, $\uparrow$ ; January, $\uparrow$ ; February, $\uparrow$	x
<b><i>E. coli</i> B2</b>	-	x	x	* September, $\uparrow$	-	x	x	x
<b><i>K. pneumoniae</i></b>	-	x	** August, $\uparrow$ ; September, $\uparrow$	x	-	** August, $\uparrow$ ; September, $\uparrow$	x	x

### Physico-chemical determinants of pathogenic bacteria survival in wetlands

In the three rivers, the total *E. coli* abundances were negatively correlated with the river temperature ( $-0.41$ ,  $P < 0.05$ ). The abundance of ESBL *E. coli* in the rivers was positively correlated with the phosphate ( $0.77$ ,  $P < 0.001$ ), ammonium ( $0.35$ ,  $0.01 < P < 0.05$ ), nitrate ( $0.41$ ,  $P < 0.05$ ) and chlorophyll-a ( $0.48$ ,  $P < 0.01$ ) concentrations and negatively correlated with the temperature ( $-0.46$ ,  $P < 0.01$ ) and pH ( $-0.38$ ,  $P < 0.05$ ). The *E. coli* B2 concentrations in the rivers did not correlate with any of the river water physico-chemical parameters. The *K. pneumoniae* concentrations in the river water were positively correlated with both the phosphate ( $0.92$ ,  $P < 0.001$ ) and ammonium ( $0.46$ ,  $P < 0.01$ ) concentrations.

The following results and discussions take into account the 'river effect' (from previous Spearman correlations obtained for the rivers; see Materials and Methods).

Within the 16 wetlands, the total *E. coli* concentration had positive regression coefficients with pH and the nitrate and phosphate concentrations (Table 3).

The ESBL *E. coli* concentration had a significant regression coefficient solely with the connectivity index in the 16 wetlands (Table 3). Among the wetlands highlighted previously based on the results of Dunn's test (Table 2), the ESBL *E. coli* concentrations were still highly related to connectivity in CHA, CHEM and GRI (Table 3).

The *E. coli* B2 concentration was not related to wetland connectivity with the river or water characteristics (Table 3). The corresponding nbGLM was not significant (Table 3).

The *K. pneumoniae* concentration had positive regression coefficients with both temperature and pH and a negative coefficient with dissolved oxygen in the 16 wetlands (Table 3). The

data from CHEM and LON (highlighted previously in Table 2) seemed to confirm these regression coefficients and additionally produced a significant and positive regression coefficient between the *K. pneumoniae* concentration and the nitrate concentration (Table 3). However, the nbGLM obtained for CHEM and LON was not significant (Table 3).

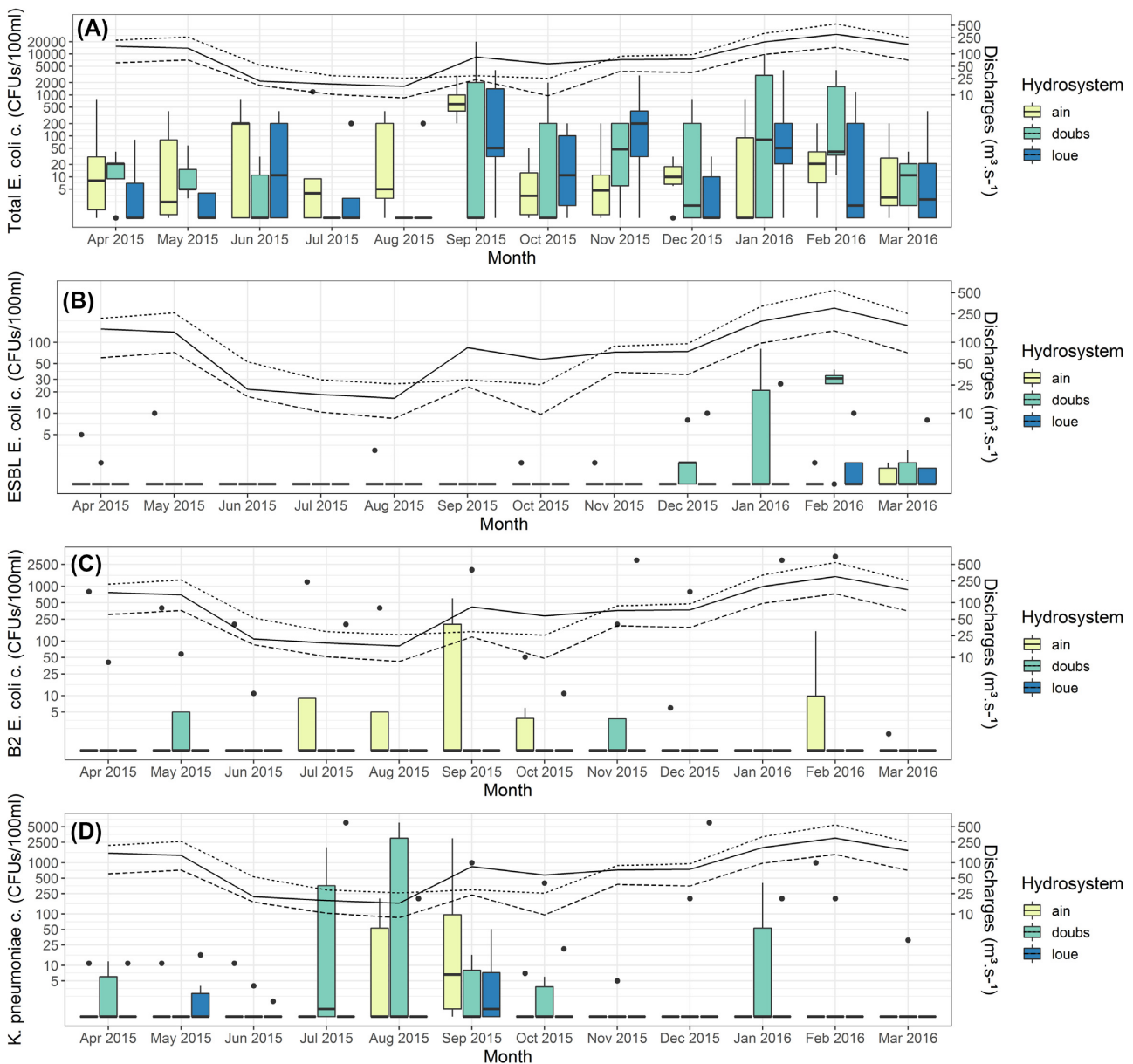
## DISCUSSION

### Connectivity between wetlands and rivers

The assessment of the connectivity between wetlands and rivers based on the thermal variability were generally congruent with the field observations. Only the connectivity of SBR with its river may have been over-estimated because this wetland is chemically similar to the groundwater (Bornette et al. 1998) but was partly dug out by farmers, leading to a strong increase in the hydraulic capacity of its upper section and favouring water stagnation and high temperature. Moreover, the assessment of the connectivity based on the thermal variability is also highly correlated (Pearson correlation) with the physical characteristics of the wetland waters such as pH ( $r = 0.63$ ) and dissolved oxygen ( $r = 0.57$ ). Indeed, pH is generally lower in groundwater than in rivers in the karstic environment of our study, and dissolved oxygen is generally lower, but more stable, in groundwater than in rivers. In contrast, the connectivity index was not correlated with the water conductivity.

Among the three hydrosystems, the Doubs wetlands were rather homogeneous in terms of connectivity, while the Ain wetlands were more heterogeneous and generally had low river connectivity, which is potentially due to the incision process taking place in the Ain River that increases connectivity between





**Figure 3.** Temporal variation in the concentrations ('c.') of pathogenic bacterial species in wetlands. **A.** Total *E. coli*, **B.** ESBL *E. coli*, **C.** *E. coli* B2, and **D.** *K. pneumoniae* in the 16 wetlands over time (months) and according to river flows. Logarithmic scale. Yellow: Ain wetlands, green: Doubs wetlands, blue: Loue wetlands. Lines indicate the temporal change in the water flows (monthly averaged values) of the three rivers. Solid lines: Ain River, dotted lines: Doubs River, dashed lines: Loue River. Dots represent boxplot outliers.

wetlands and hillslope aquifers (Bravard et al. 1997) and consequently decreases wetland temperature.

### The specific case of *P. aeruginosa*

*P. aeruginosa* is highly concentrated in hospital effluents (Kerr and Snelling 2009; Slekovec et al. 2012; Hocquet, Muller and Bertrand 2016) and can survive in multiple environments (Wheater et al. 1980; Pirnay et al. 2005). It is thus considered to be an anthropogenic marker in natural environments (Wheater et al. 1980). Our data suggest either a high capability of WWTPs to remove *P. aeruginosa* from the water, or a low capacity of *P. aeruginosa* to survive in the Ain, Doubs and Loue Rivers. As we only quantified culturable bacteria, quiescent bacteria may not have been detected.

### River contamination

We first hypothesised that the Doubs River was more contaminated than the other two rivers because the studied section was more populated and includes the cities of Besançon (120 000 inhabitants) and Dole (25 000 inhabitants) upstream, both of which contain a hospital. The studied section of the Ain River is located downstream from a city with 15 000 inhabitant and a hospital (Ambérieu-en-Bugey). The studied section of the Loue River is the only section that is not located downstream from such a source of contamination. Despite these contrasting situations, the abundances of the bacterial groups did not differ among the rivers or the hydrosystems (a river and its wetland). The lack of differences in river contamination can be attributed to the fact that we only sampled one river sampling station

**Table 3.** Results of the nbGLMs performed on the bacteriological data and physico-chemical characteristics of 1) all wetlands and 2) the wetlands identified by Dunn's tests (Table 2). The 'Model P-value' is the P-value of the ANOVA performed between each pair of nbGLM and null model. Only correlations that may not relate to river-wetland connectivity (i.e. the 'river effect', see Materials and Methods) were considered (bolded values). P-value: \* <0.05 <\*\* <0.01 <\*\*\* <0.001.

	1)				2)			
	Total <i>E. coli</i>	ESBL <i>E. coli</i>	<i>E. coli</i> B2	<i>K.</i> <i>pneumoniae</i>	Total <i>E. coli</i>	ESBL <i>E. coli</i>	<i>E. coli</i> B2	<i>K.</i> <i>pneumoniae</i>
Considered wetlands	all	all	all	all	-	CHA, CHEM, GRI	-	CHEM, LON
Model p-value	<b>7.91.10<sup>-8</sup></b>	<b>1.88.10<sup>-10</sup></b>	0.256	<b>0.00230</b>	-	<b>1.09.10<sup>-8</sup></b>	-	1
Temperature	-0.04	-0.39 **	-0.04	<b>0.51 *</b>	-	-0.44 *	-	<b>1.34 *</b>
pH	<b>4.69 ***</b>	2.51	4.51	<b>7.06 **</b>	-	0.83	-	<b>6.95 *</b>
Saturated O <sub>2</sub>	-0.01	-0.03	-0.01	<b>-0.10 ***</b>	-	-0.003	-	<b>-0.31 *</b>
Conductivity	-0.001	-0.002	0.004	0.02	-	<b>0.006 *</b>	-	-0.01
DOC	0.03	-0.005	0.09	0.0006	-	0.02	-	-0.28
P-PO <sub>4</sub>	<b>14.48 ***</b>	13.01 *	13.0	51.0	-	23.13 **	-	4.68
N-NH <sub>4</sub>	-1.67	-13.78	-23.1	-11.2	-	8.61	-	-0.01
N-NO <sub>3</sub>	<b>0.57 ***</b>	-0.03	0.56	-1.01	-	-0.42	-	<b>4.21 ***</b>
Chlorophyll-a	<b>0.03 *</b>	-0.04	0.04	0.006	-	-0.08	-	-0.16
Water flow	-0.001	0.004	0.00	0.0003	-	<b>0.01 **</b>	-	-0.00001
Connectivity index	-0.45	<b>2.96 *</b>	2.07	4.53	-	<b>9.42 **</b>	-	4.59

located close to the wetlands of interest (to ensure our data reflected the physico-chemical characteristics of the river water close to wetlands) and rather far from the sources of bacterial pathogens (i.e. WWTP effluents). We could also attribute the lack of differences in hydrosystem contamination to the large disparity in wetlands within hydrosystems in terms of the physico-chemical characteristics, the sources of the water and the connectivity to the river.

### Wetland contamination: pathogenic and antibiotic-resistant bacteria dispersal in floodplains

#### Total *E. coli*

As rivers were significantly more contaminated than wetlands, we expected that they would serve as a dispersal mechanism for *E. coli*, as previously suggested (Vermeulen and Hofstra 2014; Jonsson and Agerberg 2015). However, we did not find any differences in wetland contamination within a given hydrosystem although the wetlands differ in their connectivity to the rivers. This may be explained by other sources of contamination, as discussed below.

Surprisingly, the total *E. coli* abundance did not significantly increase with the highest river flows during winter, as previously demonstrated (Chu et al. 2014). This may suggest the resilience of wetlands to total *E. coli* contamination. However, certain wetlands were more contaminated than their corresponding rivers on some dates. Either total *E. coli* are dispersed through other sources, such as runoff from agricultural and potentially contaminated lands (Guan and Holley 2003; Jamieson et al. 2004; Vermeulen and Hofstra 2014), or they may proliferate in some wetlands. We showed that total *E. coli* contaminates wetlands during high-precipitation periods (runoff), rather than high-flow periods (river overflows and backflows). This suggests that non-point sources of contamination occurred in the watershed (e.g.

contaminated fields and cattle) and indirectly contributed to the contamination of wetlands, as suggested by Jamieson et al. (2004) and Vermeulen and Hofstra (2014). Moreover, our data support the survival of total *E. coli* in nutrient-rich wetlands. The temperature did not correlate with the bacterial concentrations, which is in contrast with the conclusions of previous studies (Beaudeau et al. 2001; Schulz and Childers 2011; Blaustein et al. 2013). Biotic interactions could also influence bacterial abundances: Joint et al. (2002) suggested that bacterioplankton exert competitive pressure on phytoplankton in terms of nutrients, and Beaudeau et al. (2001) observed the predation of *E. coli* by benthic micro-grazers. However, the absence of data on other biotic compartments prevents us from drawing conclusions on this point. The contribution of wild animal populations (e.g. coy-pus and beavers) can also not be neglected.

#### ESBL *E. coli*

ESBL *E. coli* exhibited the typical antibiotic resistance profiles, most of which were presumably due to the production of CTX-M-type enzymes, as already reported (Bouxom et al. 2018).

ESBL *E. coli* was always more abundant in rivers than in wetlands. Moreover, the ESBL *E. coli* concentrations were higher in highly connected wetlands. These results identify rivers as the major vector of dispersal for this antibiotic-resistant pathogen in the floodplains and the source of wetland contamination.

Regarding the temporal dispersion, the ESBL *E. coli* abundance in wetlands was lower during the summer (low-flow) and higher during the winter (high-flow), which confirms the relationship between ESBL *E. coli* river contamination and ESBL *E. coli* wetland contamination. The same conclusion can be drawn from the study of the physico-chemical determinants of bacterial survival in wetlands (we shown that the ESBL *E. coli* abundance was only related with the connectivity index). These results demonstrated that the most connected wetlands had the

highest contamination levels during the periods with the highest river flows. This higher contamination in winter is likely due to the outflow of raw wastewater into the river after peaks in rainfall when the WWTPs are saturated. Several studies have demonstrated the key role of WWTPs in the release of antibiotic-resistant bacteria into the environment, especially during high discharge periods (Storteboom *et al.* 2010; Marti, Jofre and Balcazar 2013; Rizzo *et al.* 2013). These studies proposed that the transport of ARGs from specific sources was likely the dominant mechanism of ARG proliferation in riverine environments rather than the selection of ARGs from among native bacteria by antibiotics and other pollutants. Manure application to agricultural fields has also been identified as a source of the spread of antibiotic resistance genes (Heuer, Schmitt and Smalla 2011), but we cannot confirm this here. This study demonstrates the pivotal role of rivers in the spread of ESBL *E. coli* in floodplains, particularly in wetlands, and the contamination of wetlands with this antibiotic-resistant pathogen. Overall, our data suggest that ESBL *E. coli* poorly survive in wetlands (no regression with the physico-chemical characteristics of wetland waters was shown), where they were mostly present during high-flow periods and probably originated from upstream WWTPs.

#### *E. coli* phylogroup B2

*E. coli* B2 concentrations did not vary spatially, and were not higher in rivers, suggesting that rivers poorly influence the abundance of pathogenic *E. coli* in wetlands. In addition, the physico-chemical characteristics of water do not seem to influence the concentration of *E. coli* B2 in wetlands. The lack of statistical spatial variation could be attributed to the low number of samples containing *E. coli* B2 (17%).

The observation of the highest *E. coli* B2 concentrations in September suggest that precipitation rather than river flow contributed to the increase in *E. coli* B2 abundance in the rivers. The special case of SBR, which was the single Ain wetland that was directly bordered by maize fields and was contaminated in September, suggests that precipitation leads to the leaching of contaminants from fields to this wetland (Jamieson *et al.* 2004; Vermeulen and Hofstra 2014). Indeed, sewage sludge is spread on crops in large quantities in the Ain lower valley (e.g. 335 t of dry matter from the WWTP of Ambérieu-en-Bugey in 2016 [Ministry of Ecological and Solidary Transition]; based on the synthesis of the 2015 and 2016 spreading registers of the Chamber of Agriculture of the Ain department), which has a floodplain with a very coarse substrate that is composed of cobblestones overlaid with a shallow soil layer. We hypothesise that the spreading of sewage sludge in floodplains may contribute to an increase in the concentrations of *E. coli* B2 in wetlands.

#### Total *K. pneumoniae*

As it was the case for ESBL *E. coli*, because rivers were significantly more contaminated than wetlands and connected wetlands were more contaminated than other wetlands, this likely indicates the river-driven contamination of wetlands by *K. pneumoniae*.

The high concentrations of *K. pneumoniae* in August and September suggest that *K. pneumoniae* reach wetlands during periods of high river flow (as suggested by the spatial approach), survive, and thrive during summer in these eutrophic and warm wetlands. One can also hypothesise that summer storms bring this species to wetlands. However, the low concentrations of ESBL *E. coli* (which are also highly dispersed by rivers) in wetlands in summer rule out this possibility. The study of the

physico-chemical determinants of bacterial survival in wetlands suggests that alkaline, warm and stagnant wetlands allow *K. pneumoniae* to thrive. In contrast, the connectivity with the river and the nutrient concentrations apparently did not play a role in the distribution of this species, even though *K. pneumoniae* seems to be nitrogen-dependent as it is able to undergo nitrification and denitrification to satisfy its nitrogen requirements (Padhi *et al.* 2013; Pal, Khardenavis and Purohit 2015). The two highly contaminated wetlands (LON and CHEM) were very shallow during summer (~20 cm), which this may favour the transfer of bacteria from the sediment to the water column. We did not sample surficial sediments in this study, but it has already been shown that sediments are a reservoir of bacteria in rivers (Chu *et al.* 2014; Abia, Ubomba-Jaswa and Momba 2015), and this is also probably the case in wetlands.

#### Limitations and implications of the study

We sampled 19 sites (16 wetlands and 3 river stations) monthly over a one-year cycle to determine both the hydrological and the meteorological fluctuations that may affect the sites, resulting in a high number of water samples. However, processes affecting human-associated bacteria dispersal at the floodplain scale could have been explored over a longer period to cope with interannual fluctuations in hydrology and climatic conditions. Indeed, local weather events may have influenced the data and seasonal effects would have been smoothed out by several years of data. Furthermore, the sometimes-small number of positive samples, as discussed above for *E. coli* B2, may have influenced the statistical results. Finally, our data did not allow us to know precisely the pathways of bacteria at the floodplain scale, but we were able to hypothesise the sources of the bacterial contamination of wetlands.

Despite these limitations, this study provides a rather new and important data set necessary to understand the dispersion and the survival of human-associated bacteria in the environment. The survival of the studied human-associated bacteria during the dispersal process within the floodplains is a key issue in terms of public health, as recreational activities are frequent along these rivers and wetlands and involve contact with contaminated water through fishing, bathing, or accidental drinking. In view of the multiple human activities in floodplains and the potential damage caused by human-associated bacteria in the environment, this study emphasises the urgent need to implement measures in all components of water management that could limit the dispersion of human-associated bacteria into the environment. Additional water treatment in WWTP and prudent spread of WWTP sludge could be worth considering.

Future investigations must clarify the contribution of agricultural practices at the floodplain scale, particularly in groundwater and wetlands where bacteria can potentially meet favourable conditions for their survival and/or multiplication, as demonstrated in this study. Once they find their way to wetlands, little is known about the direct and indirect interactions of these bacteria with their environment (competition and/or predation by/on endemic species, gene transfer, etc.). To take into account all the dimensions of these aquatic environments, sediments, biofilms and aquatic fauna should also be studied. In addition, the examination of the genetic structure of the bacterial population at the floodplain scale will help to decipher the fluxes of pathogens and resistance determinants within water bodies.

## CONCLUSIONS

This study aimed to examine the dispersal and survival of pathogenic and antibiotic-resistant human-associated bacteria in floodplains and provides new results:

- Rivers convey bacterial and antibiotic-resistant pathogens (among which ESBL *E. coli* and *K. pneumoniae*) to wetlands, most probably originating from upstream WWTP outflows.
- Shallow and warm wetlands allow *K. pneumoniae* to thrive. Total *E. coli* also find favourable conditions in some wetlands.
- Wetland contamination does not fully depend on river input. Therefore, other sources of pathogenic bacteria in the wetland watershed should be considered (e.g. sewage sludge and manure spreading that could directly contaminate wetlands through rainwater runoff and infiltration), as suggested previously.

This study emphasises the need to better understand the dispersion processes and the fate of pathogenic and antibiotic-resistant bacteria in the environment, in order to prevent risks to humans and their environment. This work also recalls the need to control and reduce as much as possible the anthropogenic bacterial input into the environment.

## ACKNOWLEDGEMENTS

Tom Battin is greatly acknowledged for his beneficial comments on the previous version of this manuscript. Marion Richard is acknowledged for her technical assistance. The data on river flows were provided by EauFrance (Doubs River), the Syndicat Mixte du Val d'Amour (Loue River) and the Syndicat Mixte de la Basse Vallée de l'Ain (Ain River). The meteorological data were provided by Météo France. The partial data on the spreading of WWTP sludge were provided by the Chamber of Agriculture of the Ain department (Ain River), the Chamber of Agriculture of the Rhône-et-Loire department (Doubs River) and the Departmental Direction of the Territory of the Jura department (Loue River).

## FUNDING

This work was supported by the French National Centre for Scientific Research (CNRS, Observatory of Universe Sciences (OSU Theta), Structuring Initiative Continental and Coastal Ecosphere (EC2CO, EC2CO2018) program).

**Conflicts of interest.** None declared.

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### **Transition**

L'étude des abondances de *E. coli*, *E. coli* producteurs de BLSE et de *K. pneumoniae* dans différents hydrosystèmes montre que la rivière transporte et disperse des BGN-CP dans les écosystèmes aquatiques des plaines alluviales. Toutefois, d'autres sources de contamination que les STEU sont supposées, notamment avec le lessivage et/ou le ruissellement de flux contaminés depuis le bassin versant. Les BGN-CP peuvent trouver des conditions favorables qui leur permettent de survivre dans les écosystèmes alluviaux. Les *E. coli* du phylogroupe B2 – les plus virulents – ne sont pas défavorisés dans l'environnement. En se basant sur les isolats collectés ici, la prochaine étude va étudier la structure de population des BGN-CP dans les écosystèmes alluviaux. Elle a pour buts principaux (i) de confirmer l'origine humaine des BGN-CP environnementaux et (ii) de comparer leur structure de population avec celles d'autres environnements.



## 4.2. Dispersion inter-hydrosystème : structure des populations bactériennes dans trois plaines alluviales de l'est de la France

**Titre** : Forte prévalence d'*Escherichia coli* d'origine humaine dans des zones humides de l'est de la France

**Revue** : Frontiers in Microbiology, volume 11

**État** : publié, 2020

### **Éléments essentiels :**

- Nous avons étudié la structure de population des isolats d'*E. coli* retrouvés dans trois hydrosystèmes de l'est de la France ;
- Une forte proportion des isolats d'*E. coli* retrouvés dans les zones humides alluviales est associée à l'Homme ;
- Les isolats résistants aux antibiotiques peuvent survivre dans les plaines alluviales ;
- Les structures de population similaires observées en milieu clinique, dans les effluents de STEU et dans l'environnement suggèrent (i) qu'aucune sous-population spécifique d'*E. coli* n'est adaptée à un environnement donné, (ii) que la contamination de l'environnement résulte principalement de la dilution des effluents de STEU et (iii) que les *E. coli* libérés dans l'environnement peuvent atteindre des écosystèmes spécifiques comme les zones humides et y survivre.



## Résumé

Les *E. coli* présents dans les rivières sont principalement apportés par les excréments humains et animaux. La contamination se fait principalement par les rejets des STEU et par l'amendement des champs avec les boues d'épuration ou les produits résiduaux organiques. Cependant, la survie de ces isolats dans les zones humides alluviales associées reste inconnue. Ici, nous avons évalué la structure de la population d'*E. coli* dans des zones humides peu anthropisées situées le long de trois plaines alluviales afin d'identifier la principale source de contamination des zones humides, dont le fonctionnement est différent de celui des rivières. Nous avons retrouvé 179 isolats d'*E. coli* dans des échantillons d'eau prélevés mensuellement dans 19 sites situés dans l'est de la France pendant un an. Les phylogroupes B1 et B2 dominaient dans la population d'*E. coli*, tandis que le phylogroupe A dominait dans les isolats résistants aux céphalosporines de 3<sup>ème</sup> génération, dont le génome abritait les gènes codant pour les  $\beta$ -lactamases à spectre étendu *bla*<sub>CTX-M-15</sub> ou *bla*<sub>CTX-M-27</sub> dans la moitié des cas. La forte proportion d'isolats d'origine humaine peut être attribuée aux effluents des STEU et, dans une moindre mesure, à l'épandage des boues d'épuration. Nous avons visualisé la distribution des isolats appartenant aux phylogroupes les plus associés à l'Homme (B2 et D) sur un arbre phylogénétique de l'ensemble de l'espèce et l'avons comparée à celle des isolats prélevés chez les patients et dans les eaux usées. La distribution des trois populations d'*E. coli* était similaire, ce qui suggère l'absence d'une population spécifique dans l'environnement. Nos résultats suggèrent qu'une forte proportion d'isolats d'*E. coli* qui atteignent et survivent dans des environnements faiblement anthropisés, tels que les zones humides, sont d'origine humaine. À notre connaissance, il s'agit de la première étude évaluant la contamination par *E. coli* et les *bla*<sub>ESBL</sub> dans les zones humides naturelles d'eau douce.



# High Prevalence of Human-Associated *Escherichia coli* in Wetlands Located in Eastern France

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## OPEN ACCESS

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### Specialty section:

This article was submitted to  
Antimicrobials, Resistance  
and Chemotherapy,  
a section of the journal  
Frontiers in Microbiology

**Received:** 16 April 2020

**Accepted:** 18 August 2020

**Published:** 04 September 2020

### Citation:

Martak D, Henriot CP,  
Broussier M, Couchoud C, Valot B,  
Richard M, Couchot J, Bornette G,  
Hocquet D and Bertrand X (2020)  
High Prevalence  
of Human-Associated *Escherichia coli*  
in Wetlands Located in Eastern  
France. *Front. Microbiol.* 11:552566.  
doi: 10.3389/fmicb.2020.552566

*Escherichia coli* that are present in the rivers are mostly brought by human and animal feces. Contamination occurs mostly through wastewater treatment plant (WWTP) outflows and field amendment with sewage sludge or manure. However, the survival of these isolates in river-associated wetlands remains unknown. Here, we assessed *E. coli* population structure in low-anthropized wetlands located along three floodplains to identify the major source of contamination of wetlands, whose functioning is different from the rivers. We retrieved 179 *E. coli* in water samples collected monthly from 19 sites located in eastern France over 1 year. Phylogroups B1 and B2 were dominant in the *E. coli* population, while phylogroup A was dominant in isolates resistant to third-generation cephalosporins, which harbored the extended-spectrum  $\beta$ -lactamase (ESBL) encoding genes *bla*<sub>CTX-M-15</sub> and *bla*<sub>CTX-M-27</sub> in half of the cases. The high proportion of isolates from human source can be attributed to WWTP outflows and the spread of sewage sludge. We analyzed the distribution of the isolates belonging to the most human-associated phylogroups (B2 and D) on a phylogenetic tree of the whole species and compared it with that of isolates retrieved from patients and from WWTP outflows. The distribution of the three *E. coli* populations was similar, suggesting the absence of a specific population in the environment. Our results suggest that a high proportion of *E. coli* isolates that reach and survive in low-anthropized environments such as wetlands are from human source. To the best of our knowledge, this is the first study assessing *E. coli* contamination and resistance genes in natural freshwater wetlands.

**Keywords:** floodplains, wetlands, *E. coli*, human-associated *E. coli*, wastewater treatment plant

## INTRODUCTION

*Escherichia coli* is a facultative anaerobic organism commensal of the vertebrate gut microbiota. Paradoxically, it can also be responsible of infections and is one of the main pathogens responsible for hospital- and community-acquired infections (Tenailon et al., 2010). Pathogenic strains of *E. coli* are divided into intestinal pathogens causing diarrhea and extraintestinal *E. coli* (ExPEC)

causing a variety of infections in both humans and animals including urinary tract infections, meningitis, and septicemia (Johnson et al., 2003; Riley, 2014). Although the ExPEC major clone sequence type (ST) 131 became predominant and have spread worldwide, other clones are also frequent (i.e., ST10, ST38, ST69, ST73, and ST405) (Nicolas-Chanoine et al., 2014; Manges et al., 2019). In contrast, diarrheagenic *E. coli* have been clustered into different pathovars according to their virulence gene arsenal. Humans widely contribute to the dissemination of *E. coli* in the environment through wastewaters. In developed countries, these wastewaters are treated in wastewater treatment plants (WWTPs). However, WWTP outflows contain *E. coli* that are released to the rivers (Br chet et al., 2014). Agriculture also contributes to *E. coli* dissemination through practices such as spreading manure or sewage sludge (Cabral, 2010; Niu and Phanikumar, 2015; Hocquet et al., 2016). Cattle also play an important role in the dissemination of *E. coli* in the environment through their feces (Cabral, 2010). Overall, *E. coli* contaminates nearly all environments, with both human-associated or not human-associated *E. coli* present in rivers, lakes, groundwater, plants, and soils (Brookes et al., 2004; John and Rose, 2005; Park et al., 2016).

*E. coli* clusters into phylogroups A, B1, B2, C, D, E, F, and G (Clermont et al., 2013, 2019). Strains belonging to phylogroups A and B1 are ubiquitous and adapted to humans or vertebrate animals. Phylogroup A strains are predominant in humans while B1 strains are predominant in animals (Berthe et al., 2013). The rare phylogroup C is closely related to phylogroup B1 (Moissenet et al., 2010). Strains belonging to phylogroups B2, D, and F are the most frequent phylogroups found in human ExPEC infections (Clermont et al., 2013).

Extended-spectrum  $\beta$ -lactamase (ESBL) is the major cause of resistance to cephalosporins in *E. coli*. In recent decades, ESBL-producing *E. coli* have spread worldwide, becoming a serious public health threat (Pitout and Laupland, 2008). These resistant strains have been widely isolated from humans but also from animals and food sources (Mughini-Gras et al., 2019; P rn nen et al., 2019). In a One-Health perspective, many recent studies assessed the relationships between *E. coli* from humans, meat, livestock, and WWTPs to understand the role played by humans in the spread of *E. coli* (Day et al., 2019; Ludden et al., 2019). Here, we wanted to understand the fate of *E. coli* in low-anthropized environments by determining the *E. coli* population structure in three rivers and their associated wetlands. We further compared their population structure with those from clinical settings and of WWTP outflows to understand the dynamics of the *E. coli* phylogroups from the human source to the wetlands.

## MATERIALS AND METHODS

### Bacterial Sampling

We sampled 16 wetlands distributed along the lower floodplains of the Ain, Doubs and Loue Rivers, three karstic rivers of the Jura Massif in eastern France (Supplementary Figure 1). One sampling point per river was chosen to compare the wetlands

and the rivers. Each river and its associated wetlands were sampled monthly for 1 year (from March 2015 to March 2016). Samples of 250 mL of water were collected and 100 mL of water were filtered by 0.45  $\mu$ m membranes and deposited on Drigalski agar plates (DRIGs) (Oxoid) and on ESBL-producing bacteria selection plates (chromID ESBL plates; bioM rieux, Marcy l'Etoile, France). After an overnight incubation at 37 C, colonies were counted; each lactose positive bacteria cultured on DRIGs and colonies growing on ESBL-specific plates were identified by MALDI-TOF MS (Microflex 100 LT; Bruker Daltonik GmbH, Bremen, Germany). All *E. coli* identified with a log value  $\geq 2$  according to the manufacturer's recommendations and with different morphotypes were stored at  $-80^{\circ}\text{C}$  until further analysis.

### Identification of *bla*<sub>ESBL</sub> in Isolates Resistant to Third-Generation Cephalosporins (3GC-R)

For each morphotype of *E. coli* growing on chromID ESBL plates, we confirmed the production of ESBL with the synergy test recommended by the Antibiogram Committee of the French Society for Microbiology (CA-SFM<sup>1</sup>, last consultation: 10 November 2018). We used *E. coli* reference strain ATCC 25922 as a control. Isolates with a negative synergy test were considered as producer of plasmid-mediated cephalosporinases. Hence, isolates that grow on ESBL selective plates (i.e., resistant to 3GC) were sorted into ESBL or plasmid-mediated cephalosporinase producers. For all ESBL-producing isolates, we identified *bla*<sub>ESBL</sub> genes by PCR and sequencing. We first screened the isolates carrying *bla*<sub>CTX-M</sub> with consensus primers and we then targeted the different groups of CTX-M with specific primers (*bla*<sub>CTX-M-group 1</sub>, *bla*<sub>CTX-M-group 9</sub>, *bla*<sub>CTX-M-group 2</sub>). *bla*<sub>CTX-M</sub> negative isolates were then tested for the presence of *bla*<sub>SHV</sub> and *bla*<sub>TEM</sub> genes (Mabilat and Courvalin, 1990; Cao et al., 2002; Coque et al., 2008). The nucleotide sequences of all PCR products were determined.

### Molecular Typing

Bacterial DNA was extracted from an overnight culture on Mueller-Hinton agar (Bio-Rad, Marne la Coquette, France) using the boiling method. We used the Clermont method to assign all the isolates to one of the eight existing phylogroups (A, B1, B2, C, D, E, F, G) (Clermont et al., 2013, 2019). Phylogroups were assigned depending on the presence/absence of four genes (*arpA*, *chuA*, *yjaA*, and *TspE4.C2*) using a quadruplex PCR. Isolates with identical profiles were further discriminated by additional PCRs. Hence, we amplified *trpA* to differentiate the isolates in phylogroups A and C, *arpA* to differentiate the isolates in phylogroups D and E, and *cfaB* and *ybgD* to differentiate isolates of the phylogroups B2, F, and G. Particular profiles allowed us to differentiate *E. coli* and *Escherichia* cryptic clades (Clermont et al., 2013). Isolates belonging to phylogroups B2 or D were typed by multi-locus sequence typing (MLST) using a previously described method (Wirth et al., 2006).

<sup>1</sup><http://www.sfm-microbiologie.org/>

## Sequence Types Distribution

The distribution of STs of interest was assessed with an SNP-based analysis of the concatenation of the seven alleles of 4195 STs extracted from EnteroBase (Alikhan et al., 2018). We built a phylogenetic tree with FastTree v2.1.9 (Price et al., 2009, 2010), with a GTR-G model to visualize the links between the different STs. Subtrees were then extracted using the R package ggtree (Yu et al., 2017, 2018) to precisely visualize the population structure of phylogroups B2 and D. STs found in clinical settings or in treated wastewater were extracted from previously published results (Gibreel et al., 2012; Br chet et al., 2014; Kallonen et al., 2017). Their distribution on the trees was compared with that of the isolates retrieved from the floodplains.

## Statistical Analysis

The independence of the explanatory variables of the whole dataset (i.e., spatial – floodplain and site variables) was tested using Pearson's  $\chi^2$ -tests on the contingency tables of each pair of explanatory variables. The explanatory variables were all independent of each other. Therefore, the independence of (i) the phylogroups and (ii) the relationships between phylogroups mostly associated to human (B2, D, F) and other (A, B1, C, E) *E. coli* strains with respect to each explanatory variable (one by one) was tested. We first separately constructed contingency tables for (i) the phylogroups and each explanatory variable and (ii) the association or not of the isolates with humans and each explanatory variable for *E. coli* cultured on DRIGs or ESBL selective plates. We then performed Pearson's  $\chi^2$ -tests on each contingency tables. The  $\alpha$  value was set to 0.05. All analyses were performed with R 3.5.1 software (R Core Team Package 2019).

## RESULTS

We retrieved 179 isolates of *E. coli*. We cultured 125 isolates on DRIGs; these isolates represented the unselected *E. coli* population present in the different rivers and their associated wetlands. We also cultured water samples on selective plates and identified 54 3GC-R isolates of which 49 and 5 produced an ESBL and a plasmid-mediated cephalosporinase, respectively. We also found 29 isolates belonging to the *Escherichia* cryptic clades (Clermont et al., 2013).

### Phylogroup Distribution of the Unselected *E. coli* Population

The phylogroup distribution of the unselected *E. coli* population (cultured on DRIGs) showed that isolates belonging to phylogroups B1 (32%) and B2 (29.6%) were predominant (Figure 1A), with a high proportion of B2 isolates in the Ain floodplain ( $p = 0.048$ ). Isolates belonging to phylogroups A (19.2%), C (6.4%), and D (12%) were identified in each floodplain, whereas isolates from phylogroup E (0.8%) were identified from only the Ain floodplain.

### 3GC-R *E. coli*

Compared to the other phylogroups, phylogroup A (39.6%) was dominant in 3GC-R *E. coli* (Figure 1B;  $p = 0.002$ ). Phylogroups

C (18.9%) and D (20.7%) were more frequent than phylogroups B2 (11.3%) and B1 (7.6%). Phylogroup B1 was found only in the Ain and Loue floodplains, whereas phylogroup F (1.8%) was only found in the Doubs floodplain. There were more isolates belonging to phylogroup A in the Loue and Doubs floodplains than in the Ain floodplain ( $p = 0.018$ ). Isolates belonging to the phylogroups mostly associated to human (B2, D, and F) were less frequent than the other phylogroups ( $p = 0.019$ ).

*bla*<sub>ESBL</sub> in 54 3GC-R isolates were mostly *bla*<sub>CTX-M-15</sub> (43%) and *bla*<sub>CTX-M-1</sub> (24%). *bla*<sub>CTX-M-14</sub> and *bla*<sub>CTX-M-27</sub> represented 17 and 7% of the *bla*<sub>ESBL</sub> genes, respectively. The remaining 3-GC-R isolates (9%) presumably produced a plasmid-mediated cephalosporinase. Among the isolates belonging to the phylogroups mostly associated to human (B2, D;  $n = 17$ ), 47, 29, 12, and 12% produced CTX-M-15, CTX-M-14, CTX-M-1, and plasmid-mediated cephalosporinase, respectively.

### MLST Typing of *E. coli* Belonging to Phylogroups B2 and D

All phylogroup B2 and D isolates ( $n = 69$ ) were typed by MLST. We obtained 31 different STs, nine of which were new STs (Supplementary Table 1). Twenty-six isolates belonged to STs often associated with humans (ST10, ST12, ST38, ST69, ST73, ST95, ST131, ST372, ST405).

A phylogenetic tree comprising 4195 STs extracted from EnteroBase (Alikhan et al., 2018) was built. Then, we extracted the phylogroup B2 and phylogroup D subtrees (Figures 3A,B, respectively). The subtrees showed that the isolates from clinical or environmental collections (WWTPs or floodplains) were scattered throughout the trees, indicating (i) that the structure of these populations was comparable and (ii) the absence of a specific population in the environment.

Three phylogroup B2 STs (ST95, ST131, and ST372) and four phylogroup D STs (ST38, ST69, ST362, ST405) were shared among the three different collection groups.

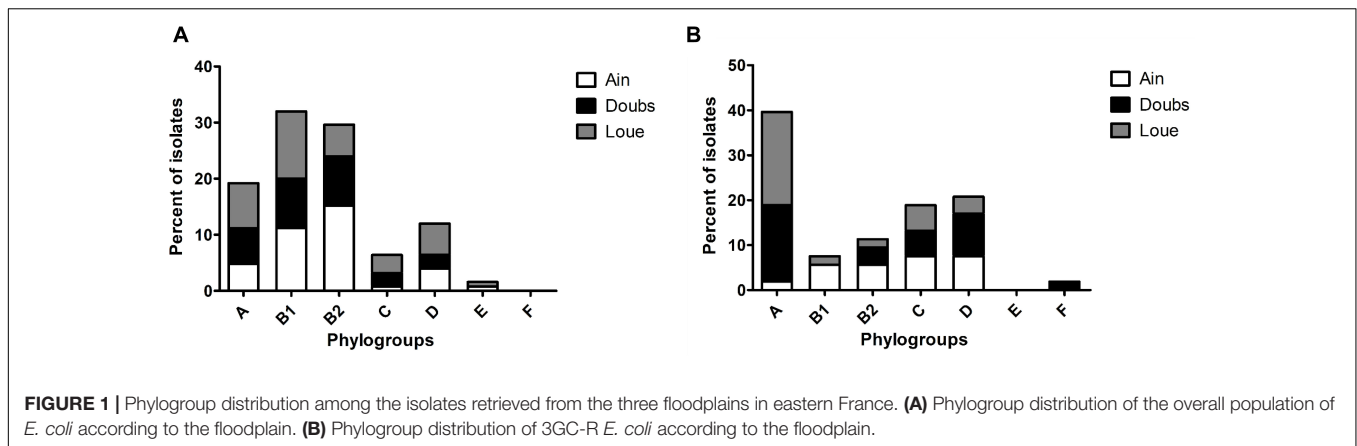
### *Escherichia* Cryptic Clades

We retrieved 29 isolates belonging to *Escherichia* cryptic clades, which represents 14% of the isolates retrieved in this study. The *Escherichia* cryptic clades were predominant in wetlands not connected to rivers ( $p = 0.026$ ).

## DISCUSSION

### Phylogroup Distribution of the Unselected *E. coli* Population

We previously determined that the concentration of total *E. coli* was higher in the rivers than in the wetlands with no significant differences in concentrations between wetlands (Henriot et al., 2019). We then divided 125 *E. coli* isolates into phylogroups and found a high proportion of isolates belonging to phylogroups A and B2, representing half of the isolates retrieved (48.8%). It was unexpected to find mostly human-associated phylogroups in low-anthropized wetlands (Escobar-P ramo et al., 2004; Massot et al., 2016). Previous studies identified WWTPs, sewage sludge and



manure as sources of *E. coli* contamination in the environment (Michael et al., 2013; Balière et al., 2015). The contamination of the wetlands by isolates belonging to phylogroups A and B2 probably resulted from the release of WWTP outflows and the spread of sewage sludge. Phylogroup B1 is the main phylogroup found in animals (Gordon and Cowling, 2003). The high proportion of isolates belonging to this phylogroup (32%) retrieved in the floodplains can be explained by direct contamination of the water by animals (wild animals or cattle, via superficial or groundwater flow) or by the spread of manure. Interestingly, the Ain floodplain was contaminated by a higher proportion of isolates belonging to phylogroup B2 than the Doubs and Loue floodplains (Figure 1A). This difference could be attributed to two concurrent factors in the Ain lower floodplain: a very coarse substrate (cobblestones) overlaid with a shallow soil layer and the spread of large amounts of sewage sludge. One can speculate that the spread of sewage sludge is a major source of contamination of the Ain River and wetlands by phylogroup B2 isolates, which are of human origin. However, this hypothesis needs to be confirmed by precise follow-up regarding the spread of sewage sludge and manure in each floodplain near the studied wetlands.

## Resistance and Phylogroup Distribution of 3GC-R *E. coli*

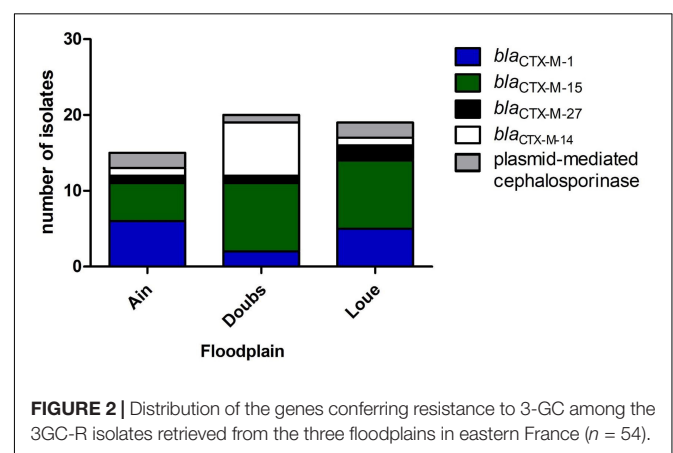
Phylogroup A was the main phylogroup found in 3GC-R *E. coli* (39.6%). These data are in line with the proportion of phylogroup A isolates recovered from humans (Tenailon et al., 2010). This high proportion of isolates belonging to phylogroups A (39.6%) and B2 (11.3%) and the lower proportion of phylogroup B1 isolates (7.6%) suggest that the contamination of rivers and wetlands by 3GC-R *E. coli* could be caused more by human contamination rather than cattle contamination. The identification of *bla*<sub>ESBL</sub> genes showed that *bla*<sub>CTX-M-15</sub> was predominant, accounting for 43% of the *bla*<sub>ESBL</sub> identified in the 3GC-R isolates (Figure 2). *bla*<sub>CTX-M-27</sub> was present in 7% of the isolates. Together, *bla*<sub>CTX-M-15</sub> and *bla*<sub>CTX-M-27</sub> represented half of the *bla*<sub>ESBL</sub> found. These *bla*<sub>ESBL</sub> are highly related to *E. coli* found in humans (Day et al., 2019; Ludden et al., 2019). *bla*<sub>CTX-M-14</sub> has previously been documented as associated to

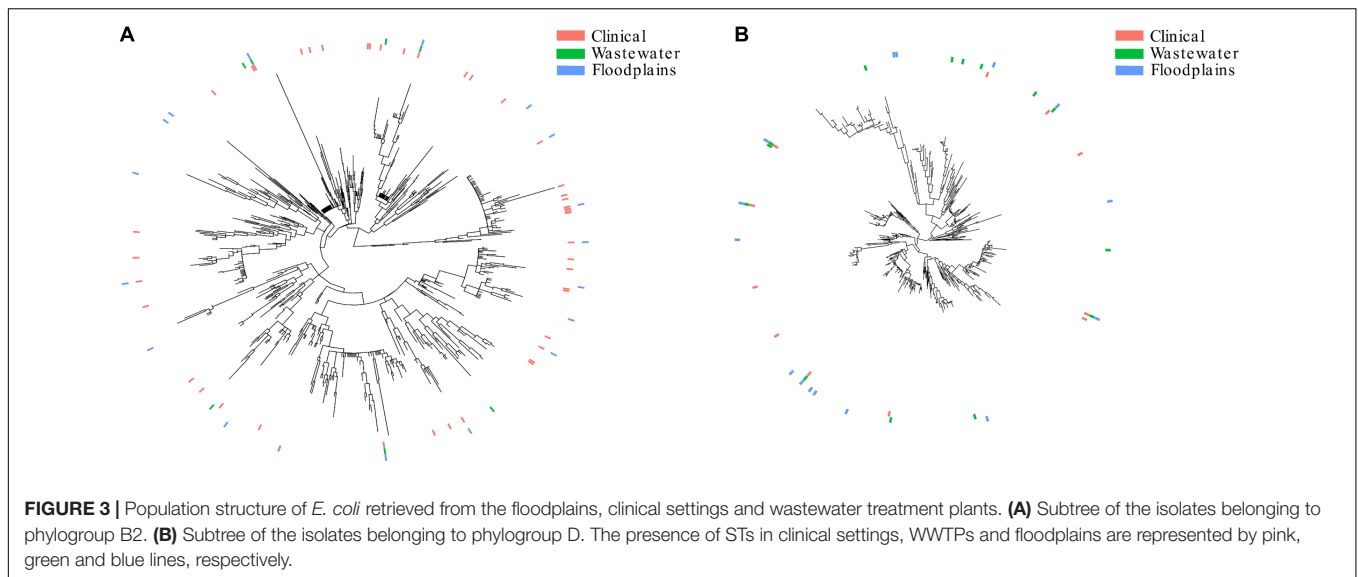
humans (Mughini-Gras et al., 2019), but this ESBL-encoding gene was also found in animals, especially in chicken (Hayashi et al., 2018). In contrast, *bla*<sub>CTX-M-1</sub> is more often related to animals and meat (Day et al., 2019). This indicated that 3GC-R *E. coli* that contaminate the floodplains mostly originated from human effluents released from WWTP outflows into the rivers (Oberlé et al., 2012; Bréchet et al., 2014). Many studies identified multidrug-resistant *E. coli* in rivers downstream large cities (Dhanji et al., 2011; Jang et al., 2013). Here, we found multidrug-resistant *E. coli* in areas that were far from any direct contamination by humans (i.e., up to 20 km downstream from the WWTP outflows in our case), suggesting that these isolates can survive in rivers despite long-distance dispersal by water.

## *Escherichia* Cryptic Clades

The cryptic clades of *E. coli* comprise five different groups. Clade I is considered a divergent *E. coli* and has been reported in human infections, while the other clades are less virulent than clade I (Clermont et al., 2013). These cryptic clades are phenotypically indistinguishable from *E. coli* but genetically different.

The 29 isolates belonging to the cryptic clades retrieved in our study represented 14% of the isolates found. Previous studies found similar proportions of these isolates in environmental samples in Australia, France, and Italy (Walk, 2015). We found





that these clades were frequent in unconnected wetlands which are supplied by groundwater, nutrient-poor, and cold (Bornette and Large, 1995; Bornette et al., 1996). In fact, low chlorophyll-*a* content, which directly relates to lower phytoplankton abundance and nutrient availability (Arthaud et al., 2013), and low temperature were nearly significantly associated with the presence of cryptic clades in wetlands of our study (data not shown). These cryptic clades may be genetically adapted to the environment by the presence of genes promoting their survival in this ecological niche (Walk, 2015).

### MLST Typing

Among the B2 and D isolates, we found ST73, ST131, ST95, ST69, and ST12. These STs are predominant in isolates retrieved in the patient blood samples (Kallonen et al., 2017), further suggesting the human origin of the contamination.

The population structure of the present collection of *E. coli* was compared to that of collections from WWTP outflows and clinical settings (Figures 3A,B). Although not fully representative of all STs present in clinics or WWTPs, we found a comparable distribution of STs in the three collections that were distributed throughout the trees, suggesting that no specific sub-population of *E. coli* is adapted to a given environment. The similar distribution of STs in the WWTP outflows and in the environment strongly suggests that the contamination of the environment mostly results from the dilution of WWTP outflows. It also suggests that these isolates can reach and survive in remote areas like wetlands.

### *E. coli* Survival in Water

Our results showed that low-anthropized wetlands contain *E. coli* isolates of human origin. Although *E. coli* contamination of rivers or lakes has been well-documented (Michael et al., 2013), that of wetlands has been less investigated. Indeed, the studied wetlands have varying degrees of connection with the river, can be supplied in water either by the river or by groundwater, and

the physical and chemical parameters can vary from those of the river (Henriot et al., 2019). It has been reported that *E. coli* can survive and grow in the environment (Jang et al., 2017). However, its survival is influenced by many factors, such as the carbon sources, temperature, pH, and availability of water (van Elsas et al., 2011). Here, we collected human-associated and 3GC-R isolates from a wide range of conditions (see Henriot et al., 2019 for details). Continuous discharge from WWTPs could explain the high proportion of human-associated *E. coli* isolates retrieved in low-anthropized environments. Previous studies suggested the existence of environmentally-adapted *E. coli* populations (van Elsas et al., 2011; Jang et al., 2017). Multi-drug resistant *E. coli* have been found in mangrove estuaries (Ghaderpour et al., 2015). The contamination of this low-anthropized environment was attributed to antibiotic release and human activities. Petit et al. (2017) found different distributions of *E. coli* population depending on the presence of cattle farm near the river. Overall, *E. coli* from human and animal effluents do not appear to be disadvantaged in environments and specific areas such as wetlands or mangrove estuaries which features drastically differ from those in the vertebrate intestine. In other words, the *E. coli* population in wetlands seems to be a dilution of the *E. coli* population found in human and animal guts. It reflects either the survival of *E. coli* of mammal origin in the environment and/or the permanent release of these contaminants in the environment. As the phylogrouping does not fully overlap the isolate origin, we cannot track the sources of the bacterial contamination with certainty.

### CONCLUSION

We found that a high proportion of the *E. coli* isolates retrieved in low-anthropized wetlands were probably human-associated. The similar population structures found in clinical settings, WWTPs and the environment suggest that *E. coli* released in the environment can reach and survive in specific areas like wetlands.

## DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/Supplementary Material.

## AUTHOR CONTRIBUTIONS

DM, CH, GB, XB, and DH conceived and designed the experiments and wrote the manuscript. DM, MB, JC, and MR

carried out the experiments. DM, CH, CC, and BV analyzed the data. All authors contributed to the article and approved the submitted version.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2020.552566/full#supplementary-material>

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**Conflict of Interest:** 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.

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### **Transition**

L'étude de la structure des populations des BGN-CP retrouvés dans l'environnement montre que leur origine est majoritairement humaine. La similarité des structures de population entre les isolats des effluents de STEU et des écosystèmes aquatiques suppose une dispersion directe des BGN-CP des eaux usées jusqu'aux hydrosystèmes, *via* les flux hydrologiques. Ce chapitre nous a permis de mettre en évidence des processus de dispersion et, dans une moindre mesure, de survie des BGN-CP dans les hydrosystèmes. Afin de mieux appréhender le rôle de chacun de ces deux processus dans le devenir des BGN-CP dans l'environnement, le prochain chapitre se focalisera sur une unique plaine alluviale, avec un échantillonnage plus dense réalisé sur plusieurs types d'écosystèmes (rivière principale, affluents, zones humides et nappes phréatiques). La forte proportion d'*E. coli* du phylogroupe B2 – les plus virulents – ainsi que l'hypothèse d'origines de contamination humaines et agricoles, en plus d'autres raisons évoquées plus haut (voir "Méthodologies générales"), nous a encouragé à préférer la plaine alluviale de l'Ain pour modéliser la dissémination des BGN-CP dans les écosystèmes alluviaux.

## *CHAPITRE 3*

# APPROCHE INTRA-HYDROSYSTÈME : LE MODÈLE DE LA PLAINE ALLUVIALE DE L'AIN



## 5. CHAPITRE 3 : APPROCHE INTRA-HYDROSYSTÈME : LE MODÈLE DE LA PLAINE ALLUVIALE DE L'AIN

**Titre** : Origines, flux et réservoirs des bactéries associées à l'Homme dans les écosystèmes alluviaux

**Revue visée** : Water Research

**État** : en révision

**Éléments essentiels** :

- Nous avons caractérisé l'origine, la dispersion et la survie de BGN-CP dans un hydrosystème peu anthropisé de l'est de la France ;
- L'étude de la structure des populations d'*E. coli* suggère que les BGN-CP retrouvés dans les écosystèmes alluviaux sont majoritairement d'origine humaine ;
- En période de hautes eaux, la contamination par les BGN-CP des écosystèmes alluviaux résulte en grande partie de la dispersion par les flux hydrologiques ;
- En période d'étiage, certaines zones humides alluviales oligotrophes déconnectées de la rivière principale et avec une forte disponibilité en nutriments constituent des réservoirs environnementaux où les BGN-CP semblent capable de survivre ;
- Aucune différence dans le devenir des isolats d'*E. coli* sensibles et producteurs de BLSE dans les cours d'eau n'a été observée.

## Résumé

La libération et la propagation de bactéries associées à l'Homme (BAH) (dont certaines sont résistantes aux antibiotiques) dans l'environnement est un défi majeur de santé publique dans le monde entier. Dans cette étude, nous avons cherché à caractériser l'origine, la dispersion et la survie éventuelle des BAH dans les écosystèmes alluviaux afin de mieux comprendre le devenir de ces pathogènes dans l'environnement. En utilisant une méthode de culture, nous avons déterminé les abondances de *Escherichia coli*, *E. coli* producteur de BLSE, *Klebsiella pneumoniae*, *K. pneumoniae* producteur de BLSE et *Pseudomonas aeruginosa* dans une plaine alluviale pauvre en nutriments de l'est de la France. Grâce à une approche basée sur la PCR, nous avons également caractérisé la structure de population des isolats d'*E. coli*, leur contenu plasmidique et la nature des gènes *bla*<sub>BLSE</sub>. Les principaux écosystèmes aquatiques de la plaine alluviale – rivière principale, affluents, zones humides alluviales et eaux souterraines - ont été échantillonnés chaque mois sur un cycle d'un an. Nous avons démontré que la contamination des écosystèmes de la plaine alluviale par les BAH résulte principalement des flux hydrologiques en période de hautes eaux, suggérant que la dispersion et la dilution sont prédominantes. En période d'étiage, les BAH semblent survivre plusieurs mois dans certains écosystèmes isolés, où elles peuvent trouver des conditions favorables à leur prolifération. Les zones humides alluviales riches en nutriments et isolées de la rivière principale sont de potentiels réservoirs de pathogènes. La production de BLSE ne semble pas conférer de désavantage dans les écosystèmes alluviaux faiblement anthropisés.

## **Origin, fluxes, and reservoirs of human-associated bacteria in floodplain ecosystems**

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**Keywords:** Pathogens, antibiotic-resistance, *Escherichia coli*, ecological niches, floodplain ecosystems, public health

## **Abstract**

The release and spread of human-associated bacteria (HAB) (some of which being resistant to antibiotics) in the environment is a major public health challenge worldwide. In this study, we characterized the origin, dispersal, and survival of HAB in floodplain ecosystems to understand the fate of these pathogens in the environment. Using a culture-based method, we determined the abundances of *Escherichia coli*, extended-spectrum  $\beta$ -lactamases (ESBL)-producing *E. coli*, *Klebsiella pneumoniae*, ESBL-producing *K. pneumoniae*, and *Pseudomonas aeruginosa* in a nutrient-poor floodplain of Eastern France. Using a PCR sequencing approach, we assessed the population structure of *E. coli* isolates, their plasmid content, and the nature of the *bla*ESBL genes. The main aquatic ecosystems of the floodplain – river, tributaries, riverine wetlands, and groundwater – were sampled monthly over a year cycle. We demonstrated that the contamination of floodplain ecosystems by HAB mainly results from hydrological fluxes during high-flow periods, suggesting that dispersal and dilution predominated. During low-flow periods, HAB could survive several months in isolated ecosystems where they may find favourable conditions to thrive. The most nutrient-rich and isolated wetlands are consequently potential reservoirs of pathogens. The production of ESBL did not disadvantage to HAB in low-anthropized floodplain ecosystems.

## Introduction

The intestinal microbiome is the primary reservoir of human-associated bacteria (HAB) engaged in a symbiotic relationship with their host. However, some HAB strains are responsible for hospital- and community-acquired infections. Among HAB, *Escherichia coli* is the most known and studied species. *E. coli* pathogenic strains can cause a variety of intestinal (diarrhoeagenic *E. coli*) and extraintestinal (extraintestinal *E. coli* - ExPEC) diseases, and are frequently reported in community- and hospital-acquired infections (Croxen and Finlay, 2010). *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* are major opportunistic pathogens, and are leading pathogens associated with hospital-acquired infections (Weiner-Lastinger et al., 2020). The resistance to the third-generation cephalosporins of *E. coli* and *K. pneumoniae* is mostly due to the production of extended-spectrum  $\beta$ -lactamases (ESBL) and is responsible for the highest burden in humans (Cassini et al., 2018).

HAB are released in the environment mainly through faeces. The main sources of HAB are community wastewater and agriculture (Cabral, 2010). First, community wastewater of high- and intermediate-income countries is treated in wastewater treatment plants (WWTPs), which release effluents still containing HAB in the receiving surface water, mostly rivers (Bréchet et al., 2014; Marti et al., 2014). Second, grazing livestock directly releases pathogenic and antibiotic-resistant bacteria through faeces (Hansen et al., 2020). Third, the spreading of organic waste products (*i.e.* manure, slurry, sewage sludge) contaminate the agricultural lands with HAB (Reinthal et al., 2003; Semenov et al., 2009), which can survive and leach towards the hydrological network (Semenov et al., 2009; Hansen et al., 2020).

As a consequence, floodplains are contaminated with pathogenic and antibiotic-resistant bacteria. Floodplains support aquatic ecosystems whose origin of water, water retention time, dewatering frequency, physicochemical characteristics, and vegetation are very heterogeneous (Bornette et al., 2008). Although the contamination of streams, riverine wetlands, and groundwater with HAB has been documented (Schilling et al., 2009; Servais and Passerat, 2009; Laroche et al., 2010; Chu et al., 2014; Petit et al., 2018; Henriot et al., 2019; Martak et al., 2020), no study has allowed to understand the ecological processes that govern the abundances of HAB in the floodplain ecosystems. Does the contamination with HAB result from their dispersal through hydrologic fluxes, or from the survival and growth of a specific bacterial population in some ecosystems of the floodplain?

To answer this question, we first made the hypothesis that the dispersal of HAB in floodplain ecosystems depends on hydrological fluxes, and therefore on the degree of



hydrological connectivity with the most contaminated ecosystems (*i.e.* generally the rivers, as they receive WWTP outflows (Bréchet et al., 2014; Marti et al., 2014)). Beyond the dispersal, HAB have been reported to survive in some floodplain ecosystems (He et al., 2007; Henriot et al., 2019). Nutrients and temperature are two physicochemical parameters influencing the survival and growth of HAB (Fagerbakke et al., 1996; Blaustein et al., 2013). We thus made the hypothesis that the survival of HAB may be favoured in cool and nutrient-rich floodplain ecosystems. Furthermore, the shelter role of sediment and the development of biofilms have been reported to favour the survival of HAB (Smith et al., 2008; Garzio-Hadzick et al., 2010). HAB may consequently be more abundant in sediment compared to the water column. Finally, as the fitness cost of resistance to antibiotics has been demonstrated in *in vitro* experiments (Andersson and Hughes, 2010), antibiotic-resistant HAB may thus be disadvantaged compared to their susceptible parents in floodplain ecosystems with low selection pressure.

For testing these hypotheses, we quantified and characterized the populations of *E. coli*, *K. pneumoniae*, and *P. aeruginosa* in the aquatic ecosystems (streams, riverine wetlands, and groundwater) of an oligo-mesotrophic floodplain of Eastern France over a year cycle. We modelled the different processes involved, and further related the bacterial data with the characteristics of the floodplain ecosystems. We paid a particular attention to the bacterial sub-population resistant to third-generation cephalosporins.

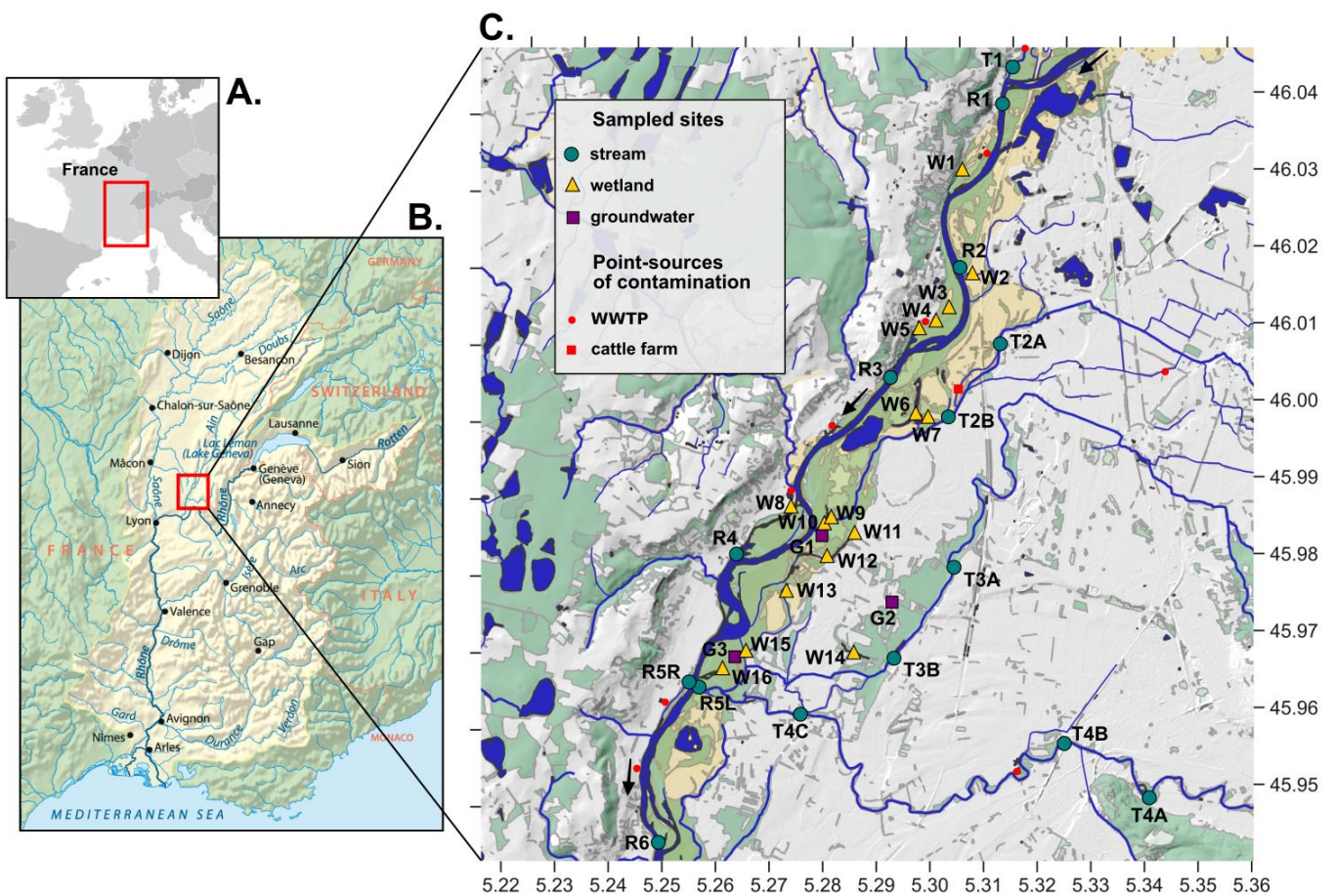
## Materials and Methods

### 1.1. Sampled sites

All sampled ecosystems were distributed along the floodplain of the Ain River, a karstic and rather nutrient-poor river of the French Jura Massif, with a high slope and a coarse river grain-size (pebbles and gravels) (Piégay et al., 2000; Fig. 1). The Ain floodplain is poorly anthropized, and the main activity is the intensive irrigated cultivation of corn. The 100 year floodplain is still naturally colonised by a mosaic of vegetation units including wetlands, alluvial forest, and dry grasslands and shrublands on coarse substrates (Marston et al., 1995).

We sampled 34 sites monthly over a one-year cycle (from March 2019 to February 2020) to consider seasonal hydrological and meteorological variabilities. First, 15 sampled sites were distributed along streams, *i.e.* the Ain River (seven sampled sites distributed along the river upstream-downstream continuum), and its tributaries (eight sampled sites in the four tributaries, named T1 to T4) (see Fig. 1 legend for details). The Ain River had in 2019 a daily average discharge of 101.8 and 122.1 m<sup>3</sup>.s<sup>-1</sup> upstream and downstream the sampled sites, respectively. Tributaries T1 and T4 had in 2019 daily average discharges of 6.5 and 5.7 m<sup>3</sup>.s<sup>-1</sup>, respectively.

No discharge data were available for the tributaries T2 and T3. Six low-capacity WWTPs (< 2000 population equivalent - PE) were located along the Ain River in the studied area (Fig. 1). The tributary T4 was sampled upstream (T4B) and 5 km downstream (due to poor accessibility; T4C) an effluent of a high-capacity WWTP (33 000 PE). Two low-capacity WWTP (3,500 and < 2,000 PE) discharged their effluents in tributaries T1 and T2, respectively (Fig. 1). A cattle farm is located between T2A and T2B (Fig. 1). Second, sixteen sampling sites were located in riverine wetlands (belonging to 12 different wetlands; Fig. 1). All wetlands with an area greater than 1000 m<sup>2</sup> were sampled within a 20 km<sup>2</sup> area, leading to a representative sample of wetlands in the studied hydrosystem in terms of temperature, nutrients, and connectivity to the Ain River. Third, we sampled the groundwater in three sites (Fig. 1). Several sampled points dried out in summer: T2A, T2B, T3A, T4A, T4B, T4C, W3, W8, W11, W12, W13 and W15.



**Fig. 1. Location of the 34 sampled sites in the Ain River floodplain, France.** (A) Location of the Rhône River watershed in Western Europe. (B) Location of the Ain River floodplain in the Rhône River watershed. (C) Location of the 34 sampled sites in the Ain River floodplain. Sample points have R, T, W, and G prefixes for the Ain River, tributaries, wetlands, and groundwater, respectively. They are located on the map according to the colour legend in the panel (C). Sampled sites beginning with T1, T2, T3, and T4 were located on the Suran stream, the Cozance stream, the Seynard stream, and the Albarine stream, respectively (all these streams being tributaries of the Ain River). Reference coordinate system: WGS84.

Water samples were collected in sterile conditions, stored at 4°C, and analysed for bacterial abundances and physicochemical characteristics within 8 h and 24 h of collection, respectively. Surface sediments were collected in sterile conditions when present (T2A, T2B, T3A, T3B, and all wetland points) in July, October, November and December 2019, stored at 4°C, and analysed for physicochemical characteristics within 24 h of collection. To quantify the abundances of HAB in the sediment, 10 g of sediment with 100 ml of ultrapure water were stirred for 5 min. Forty ml were centrifuged at 3000 g for 10 min at 4°C, as suggested by dos Santos Furtado and Casper (2000). The supernatants were collected, stored at 4°C, and analysed for bacterial abundances within 24 h of collection.

### ***1.2. Physicochemical characteristics of water***

We measured 16 parameters related to ecosystem functioning and/or bacterial survival and thriving: water temperature (Blaustein et al., 2013; Lax et al., 2020), electric conductivity and pH (Bornette et al., 2008), saturated and dissolved oxygen (Doughari et al., 2012), total and dissolved organic carbon (TOC and DOC), total and dissolved nitrogen, and ions concentrations: nitrate, nitrite, ammonium, phosphate, sulphate (Fagerbakke et al., 1996), fluoride and chloride. The methods used to assess these physicochemical characteristics are detailed in the Supplementary Text.

### ***1.3. Chemical characteristics of the sediment***

We measured the percentages of total nitrogen, total sulphur, and total carbon of the sediment according to methods detailed in the Supplementary Text.

### ***1.4. Quantification and genotypic characterization human-associated bacteria***

The concentrations of total *E. coli*, ESBL-producing *E. coli*, total *K. pneumoniae*, ESBL-producing *K. pneumoniae*, and *P. aeruginosa* were measured by culturing 0.45-µm filters onto specific growing media as previously described (Henriot et al., 2019) (Supplementary Text).

In order to (i) estimate the origin of the isolates retrieved in the floodplain ecosystems, and to (ii) compare the contamination in space and time, we determined the phylogroups and the plasmid contents of all *E. coli* isolates (n=536) and identified the *bla*<sub>ESBL</sub> in ESBL-producing *E. coli* (n=187). Bacterial DNA was extracted from an overnight culture. We used the Clermont method to assign all the isolates to one of the eight existing phylogroups (A, B1, B2, C, D, E, F, and G) as described before (Clermont et al., 2013 and 2019) (Supplementary Text). We identified IncA/C, IncFIA, IncFIB, IncFIC, IncFrep, IncI1, and IncY plasmid incompatibility

groups and the MOBp12 family (including IncB/O, IncI1, IncK) using PCR-based methods (Tymensen et al., 2019) (Supplementary Text). Using previously described methods (Bréchet et al., 2014), we identified *bla*<sub>ESBL</sub> belonging to *bla*<sub>CTX-M-1</sub>, *bla*<sub>CTX-M-9</sub>, *bla*<sub>CTX-M-2</sub>, *bla*<sub>SHV</sub>, and *bla*<sub>TEM</sub> groups in ESBL-producing isolates by PCR and sequencing (Supplementary Text).

### 1.5. Water isotopy

The natural variations of oxygen and hydrogen isotopes ( $\delta^{18}\text{O}$  ( $^{18}\text{O}/^{16}\text{O}$ ) and  $\delta^2\text{H}$  ( $^2\text{H}/^1\text{H}$ )) in the water make it possible to trace the water cycle (Craig, 1961). To understand the hydrological functioning of the studied floodplain, the  $\delta^{18}\text{O}$  and  $\delta^2\text{H}$  isotopic ratios of the water were determined in all sampled sites during a high-flow period (Picarro Continuous Water Sampler, Picarro, Santa Clara, CA, USA), and compared to the global meteoric water line (GMWL) and the Western Mediterranean meteoric water line (WMMWL). We also compared the  $\delta^{18}\text{O}$  and  $\delta^2\text{H}$  of streams, groundwater and wetlands according to Paran et al. (2015) to estimate the contribution of the different water bodies to each wetland, and therefore precise the origin of the water for each wetland. This allowed us to estimate the connectivity between wetlands and the Ain River, as described in the Results and Discussion section.

### 1.6. Statistical analyses

The normality and homoscedasticity of the data were tested using the Shapiro-Wilk test and Levene's test, respectively. The bacterial dataset was over-dispersed (variance  $\gg$  mean). To study the dispersal and the habitat issue of HAB in floodplains, we performed generalized linear models (GLM) on bacterial abundances data. We approximated the distribution of the bacterial abundances data by a negative binomial law (Lindén and Mäntyniemi, 2011). On the first hand, for the dispersal issue, we used (i) the sampling campaign and the connectivity between wetlands and the Ain River, and (ii) the Ain River discharge and the connectivity between wetlands and the Ain River as explanatory variables of total *E. coli* abundances in wetlands. Because bacterial sampling and discharge data were not acquired at the same time of the day, we chose to use the average Ain River discharge over the two days preceding the sampling. On the second hand, for the habitat issue, we used the physicochemical parameters as explanatory variables of HAB abundances in floodplain ecosystems. The *imputePCA()* function was used to estimate the few missing physicochemical measurements due to equipment malfunction ( $n=57$ , *i.e.* 0.86 % of all physicochemical data) to allow the modelling on the entire dataset. The  $\alpha$  value was set to 0.05. All analyses were performed with R 3.6.2 software (R. Core Team, 2020).

## Results and Discussion

### *2.1. Surface-groundwater connectivity of sampled wetlands*

Plotting  $\delta^{18}\text{O}$  versus  $\delta^2\text{H}$ , all sampled sites were distributed along the GMWL and the WMMWL (Fig. S1). From the  $\delta^{18}\text{O}$  ratios, we calculated the contribution of the Ain River water and groundwater for each sampled wetland (Paran et al., 2015) (Table S1). Because the isotopic analysis was only performed once, we correlated the contribution of the river to each studied wetland (Table S1) with the physicochemical parameters measured at the same date in order to obtain a proxy of the connectivity between water bodies that is valid throughout the sampling period. Clearly, the conductivity was negatively correlated with the Ain River contributions, as exposed in Table S1 ( $\rho = -0.55$ , Pearson correlation, p-value  $< 0.05$ ). We then used the conductivity to qualify the connectivity between a given wetland and the Ain River throughout the sampling period.

### *2.2. Distribution of human-associated bacteria in the floodplain*

#### *2.2.1. Prevalence and origin of the contamination*

Total *E. coli*, ESBL-producing *E. coli*, total *K. pneumoniae*, ESBL-producing *K. pneumoniae* and *P. aeruginosa* were retrieved in 280/361 (77.6 %), 115/361 (31.9 %), 48/361 (13.3 %), 6/361 (1.7 %) and 112/361 (31.0 %) water samples, respectively. HAB widely occur in low-anthropized floodplains, as already reported (Schilling et al., 2009; Servais and Passerat, 2009; Laroche et al., 2010; Henriot et al., 2019). ESBL-producing *K. pneumoniae* only occurred in six samples (all sampled in tributaries at high-flow periods), probably because of its low prevalence in the human community and in agricultural farms (Hu et al., 2021). In the view of its low abundance, ESBL-producing *K. pneumoniae* were not included in the following analyses.

In total *E. coli* retrieved in water, phylogroup A (28.4 %) dominated, followed by phylogroups B1 (22.3 %), B2 (18.6 %) and D (14.9 %) (Table S2). In ESBL-producing *E. coli* retrieved in water, phylogroups B2 (30.6 %) and A (28.4 %) dominated (Tables S2). Strains belonging to phylogroups A and B2 are predominant in humans, while those belonging to phylogroup B1 predominate in animals (Escobar-Páramo et al., 2006). Strains belonging to the B2, D, and F phylogroups are the most virulent (Tourret and Denamur, 2016), and B2 and G strains are frequently implicated in extra-intestinal infections (Escobar-Páramo et al., 2006). The

phylogroups identified, and the surprising high prevalence of phylogroup B2 isolates indicate that floodplains are contaminated by bacteria whose origin is mainly human.

All investigated plasmid incompatibility groups have been identified in *E. coli* isolates retrieved in water samples of the floodplain (Table S3). We found that 47.9 % of total *E. coli* isolates and 84.0 % of ESBL-producing *E. coli* isolates had an IncF plasmid. IncF plasmids are prevalent in human *E. coli* isolates (Carattoli, 2009), also suggesting a human origin of the bacterial contamination of floodplain ecosystems. Nevertheless, IncI1 plasmids, retrieved in approximately 10 % and 25 % of total *E. coli* and ESBL-producing *E. coli* isolates, respectively (Table S3), have been identified in human and agricultural *E. coli* strains (Carattoli, 2009). This indicated that agriculture probably also contributes to the contamination of floodplain ecosystems by HAB.

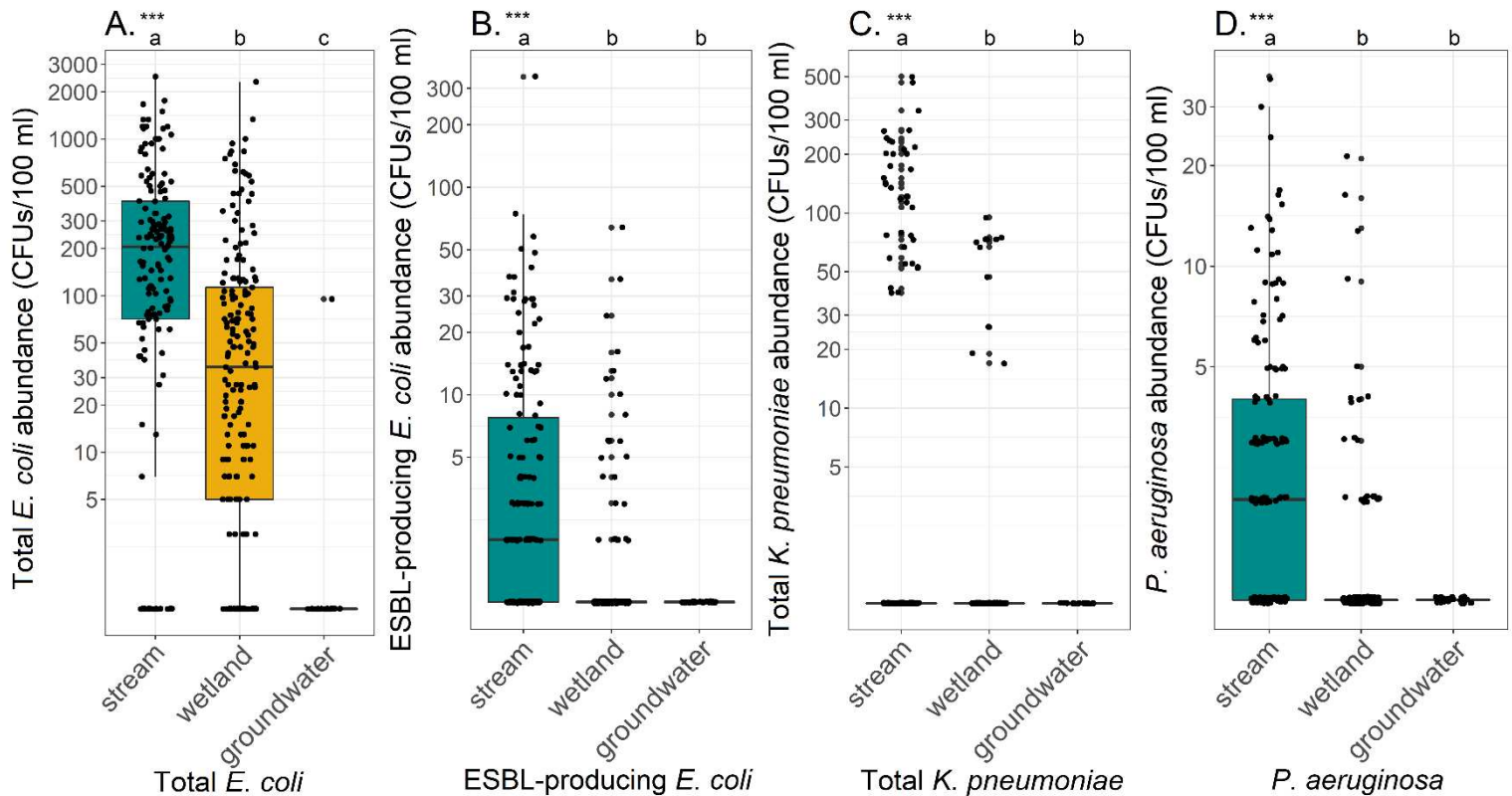
The most common *bla*<sub>ESBL</sub> found in ESBL-producing *E. coli* isolates retrieved in the floodplain were *bla*<sub>CTX-M-15</sub> (43.3 %), *bla*<sub>CTX-M-14</sub> (16.6 %), *bla*<sub>CTX-M-27</sub> (13.4 %) and *bla*<sub>CTX-M-1</sub> (10.2 %) (Table S4). They have been retrieved in very similar proportions in *E. coli* found in human (van Duijkeren et al., 2018), supporting the human origin of most of ESBL-producing *E. coli* isolates retrieved in floodplain ecosystems. *bla*<sub>CTX-M-1</sub> is also the most frequent *bla*<sub>ESBL</sub> retrieved in antibiotic-resistant *E. coli* strains from livestock (Ceccarelli et al., 2019) and wildlife (Guenther et al., 2011). Although the majority of contamination of the floodplain appears to have a human origin, non-human sources cannot be excluded.

### 2.2.2. Distribution of human-associated bacteria in floodplain ecosystems

To understand the HAB contamination at the floodplain scale, we compared the contamination of different ecosystems. Streams were more contaminated than wetlands and groundwater by total *E. coli*, ESBL-producing *E. coli*, total *K. pneumoniae*, and *P. aeruginosa* (Fig. 2, Kruskal-Wallis test, p-values =  $<2.2 \times 10^{-16}$ ,  $<2.2 \times 10^{-16}$ ,  $1.00 \times 10^{-6}$  and  $<2.2 \times 10^{-16}$ ; followed by a Dunn's test; Fig. S2), probably because they receive the major part of continental WWTP outflows, known to be a main source of HAB contamination in floodplains (Bréchet et al., 2014; Marti et al., 2014). However, high abundances of HAB were reported in some wetlands, suggesting processes of dispersal and/or survival of HAB in low-anthropized environments, as already reported (Laroche et al., 2010; Henriot et al., 2019).

We never cultured ESBL-producing *E. coli*, *K. pneumoniae*, or *P. aeruginosa* from groundwater samples (Fig. 2, Table S5). *E. coli* was only retrieved once in a groundwater sample point during a high-flow period (*i.e.* in January 2020, Fig. 2, Table S5). This groundwater sample may have been contaminated during river overflow that covered the

piezometer (Table S5). The rarity of HAB in groundwater was surprising since their contamination is likely. Hence, the very coarse grain size of the substrate in the studied floodplain allowed surface-groundwater fluxes in both directions. Either HAB are filtered as the water percolates through the sedimentary substrate of the floodplain (Reinoso et al., 2008), or HAB survive in groundwater, but are no longer culturable because of the harsh physicochemical conditions of these ecosystems (Petit et al., 2018).



**Fig. 2. Abundances of total *E. coli* (A), ESBL-producing *E. coli* (B), total *K. pneumoniae* (C), and *P. aeruginosa* (D) in water in the Ain River floodplain, France.** Samples were taken between March 2019 and February 2020. The data are presented according to the type of sampled sites (streams, n=153; wetlands, n=181; and groundwater, n=26). Boxplots are presented on a logarithmic scale. Bacterial abundances were determined using a culture-based method. For each panel, asterisks and letters represent the p-value (0.05>\*>0.01>\*\*\*>0.001>\*\*\*\*) of the Kruskal-Wallis test, and the groups highlighted by the Dunn's test, respectively. Streams are more contaminated by HAB than wetlands and groundwater. Wetlands are more contaminated by HAB than groundwater by total *E. coli*.

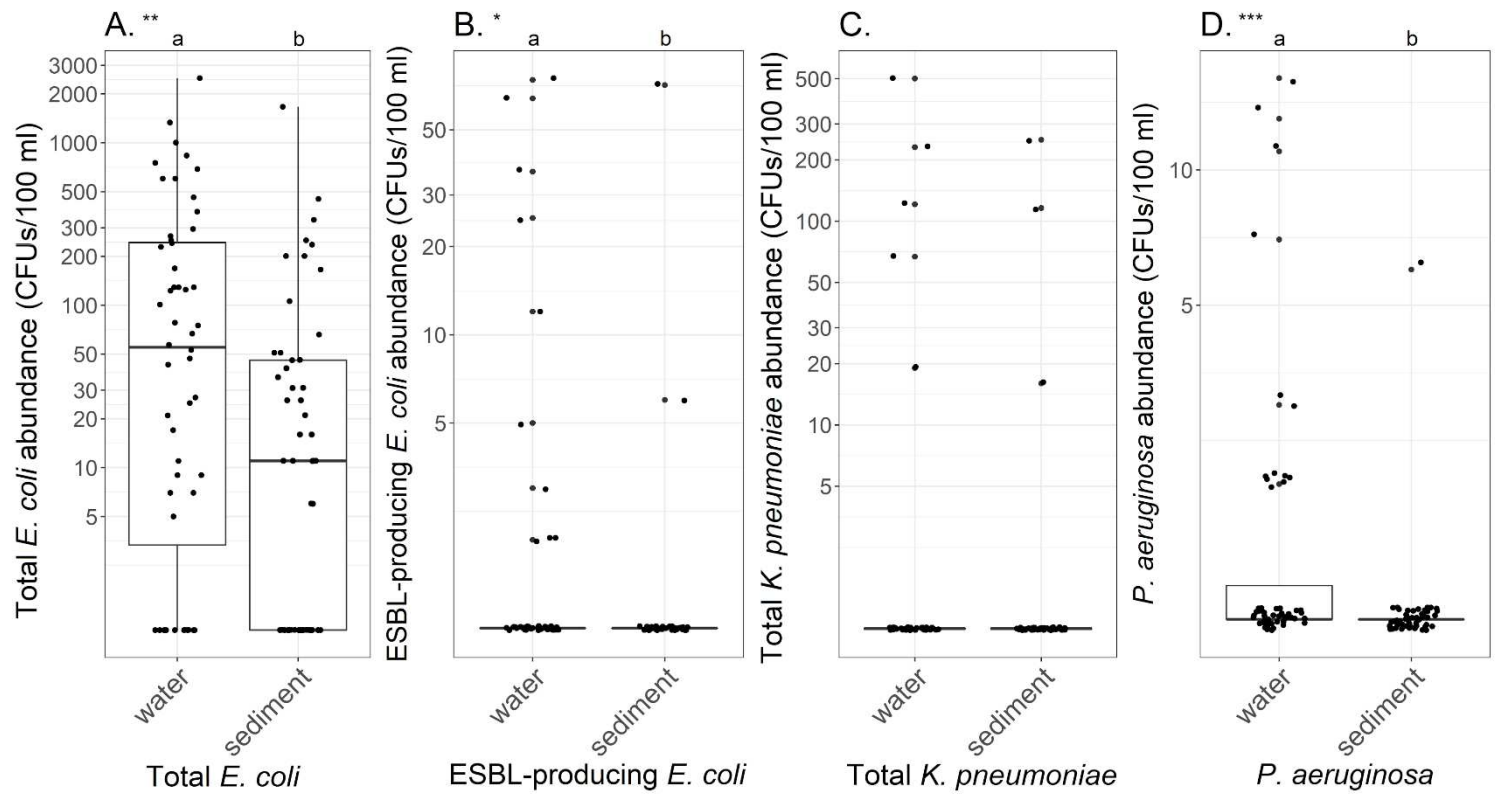
To study the origin of contamination according to the ecosystems, we compared the populations of total *E. coli* and ESBL-producing *E. coli* (i.e. phylogroups, plasmid incompatibility groups, and *bla*<sub>ESBL</sub> identity) in streams and wetlands. We found the same number of plasmids in total *E. coli* isolates from streams and wetlands (Student's t-test, p-value > 0.05). The distribution of

plasmid incompatibility groups detected in total *E. coli* isolates was similar in streams and wetlands (test of equal proportions, p-value > 0.05 for all plasmid incompatibility groups). In ESBL-producing *E. coli*, the distribution of *bla*<sub>ESBL</sub> between streams and wetlands was similar (test of equal proportions assessed on *bla*<sub>CTX-M-1</sub>, *bla*<sub>CTX-M-14</sub>, *bla*<sub>CTX-M-15</sub>, *bla*<sub>CTX-M-27</sub> and *bla*<sub>SHV12</sub> genes, p-value > 0.05). HAB populations therefore seems homogeneous between the different ecosystems of the floodplain, suggesting a common origin of the contamination in floodplain ecosystems. Nevertheless, among total *E. coli*, isolates of phylogroup A were more frequently retrieved in streams than in wetlands (test of equal proportions, p-value =  $2.22 \times 10^{-3}$ ), whereas isolates of phylogroup B2 were more frequently retrieved in wetlands than in streams (test of equal proportions, p-value =  $2.92 \times 10^{-3}$ ). Both A and B2 isolates have a human origin (Escobar-Páramo et al., 2006), but B2 isolates are more virulent than A isolates (Tourret and Denamur, 2016). Pathogenicity islands often carry operons encoding metabolic pathways: their acquisition could provide bacteria, such as *E. coli* isolates belonging to the B2 phylogroup, with metabolic capabilities allowing the colonization of new ecological niches, such as wetlands (Rohmer et al., 2011).

### 2.2.3. Human-associated bacteria in the sediment

HAB are reported to survive longer in sediment than in the water column mainly due to the shelter role of the substrate and the development of biofilms (Smith et al., 2008; Garzio-Hadzick et al., 2010). We therefore tested the hypothesis that sediments act as a reservoir for HAB in floodplain ecosystems. Total *E. coli*, ESBL-producing *E. coli*, total *K. pneumoniae* and *P. aeruginosa* were retrieved in 30/52 (57.7 %), 2/52 (3.8 %), 3/52 (5.8 %), and 1/52 (1.9 %) sediment samples, respectively. Sediment samples were less contaminated than water samples by total *E. coli*, ESBL-producing *E. coli* and *P. aeruginosa* (Wilcoxon-Mann-Whitney test, p-value =  $6.06 \times 10^{-3}$ ,  $1.71 \times 10^{-2}$ , and  $7.05 \times 10^{-4}$ , respectively; Figure 3, Table S6). Moreover, we found no correlation between the contamination in water and sediment, therefore invalidating the tested hypothesis. Other studies also found lower abundances of HAB in sediments compared to the water column (Berthe et al., 2008; Heß et al., 2018). The reservoir role of sediment can be variable and depending on several parameters, as hydrology (Smith et al., 2008), and the nature of the sediment (*e.g.* grain size, organic carbon content; Garzio-Hadzick et al., 2010). Here, sediment were mostly sampled in wetlands (because the bed of the Ain River is composed of cobblestones), *i.e.* far from contamination point-sources and in mostly nutrient-poor ecosystems, which can explain the low abundances of HAB retrieved in sediments.





**Fig. 3.** Abundances of total *E. coli* (A), ESBL-producing *E. coli* (B), total *K. pneumoniae* (C) and *P. aeruginosa* (D) in water and sediment in the Ain River floodplain. 52 samples of each matrix were taken between July, October, November, and December 2019. Boxplots are presented on a logarithmic scale. Only sites where sediment was present are included (*i.e.* T2A, T2B, T3A, T3B, and all wetland points; Fig. 1). Bacterial abundances were determined using a culture-based method. For each panel, asterisks and letters represent the p-value ( $0.05 > * > 0.01 > ** > 0.001 > ***$ ) of the Kruskal-Wallis test, and the groups highlighted by the Dunn's test, performed on bacterial abundances according to the matrix, respectively. Water samples are generally more contaminated than sediment samples by human-associated bacteria.

### 2.3. Dispersal of human-associated bacteria in the floodplain

#### 2.3.1. Point-sources of contamination

The discharge of HAB from WWTPs is largely associated with periods of heavy rainfall (Di Cesare et al., 2017; Honda et al., 2020). To confirm the point-source role of WWTPs in the studied floodplain, we investigated the temporality of contaminations in streams. Total *E. coli*, ESBL-producing *E. coli* and *P. aeruginosa* were more abundant in February 2020 (*i.e.* the sampling campaign during which the discharge of the Ain River was the highest; Fig. S3; Dunn's Test). Furthermore total *E. coli* and ESBL-producing *E. coli* abundances in the Ain River were correlated with the river discharge ( $\rho = 0.58$  and  $0.82$  respectively; Pearson correlation; p-value  $< 0.001$  and  $< 0.01$ , respectively), confirming the contamination of floodplains by WWTP effluents (Bréchet et al., 2014; Marti et al., 2014).

Secondly, in order to explore the potential role of farms as point-sources of contamination at the scale of the floodplain, we compared the contamination with HAB upstream (T2A) and downstream (T2B) the cattle farm (Fig. 1) and found that total *E. coli* and ESBL-producing *E. coli* were more concentrated in T2B than in T2A (Wilcoxon-Mann-Whitney test, p-value =  $2.46 \times 10^{-2}$ , and  $9.87 \times 10^{-3}$ , respectively). This confirms that agriculture is also a source of HAB in floodplains (Laroche et al., 2010; Hansen et al., 2020).

### 2.3.2. *Dispersal of human-associated bacteria in streams*

We then explored the impact of the WWTP outflow on the contamination of streams and found that HAB abundances were similar upstream (T4B) and 5 km downstream a WWTP outflow (T4C). Similarly, the higher bacterial abundances observed just downstream the farm (T2B) were no longer observed 3 km downstream (W10; Wilcoxon-Mann-Whitney test, p-value > 0.05). These results suggest a generally poor survival of the culturable forms of human- and cattle-associated bacteria in streams, as already reported (Garcia-Armisen and Servais, 2004), and a subsequent poor dispersal of HAB in floodplains over long distances.

### 2.3.3. *Dilution of bacterial contaminations*

The tributaries T1, T2, and T4 were more contaminated than the Ain River by total *E. coli*, ESBL-producing *E. coli*, total *K. pneumoniae*, and *P. aeruginosa* (Fig. S4; Kruskal-Wallis test, p-value =  $3.11 \times 10^{-6}$ ,  $3.19 \times 10^{-9}$ ,  $5.10 \times 10^{-3}$ , and  $6.28 \times 10^{-8}$ , respectively; followed by a Dunn's test). When we compared the contaminations of the seven sample points distributed along the Ain River, we did not find any significant change along the upstream-downstream continuum (Kruskal-Wallis test, p-value > 0.05). Consequently, even if the tributaries were more contaminated than the Ain River, their contribution to the contamination of the river was largely counterbalanced by the dilution effect.

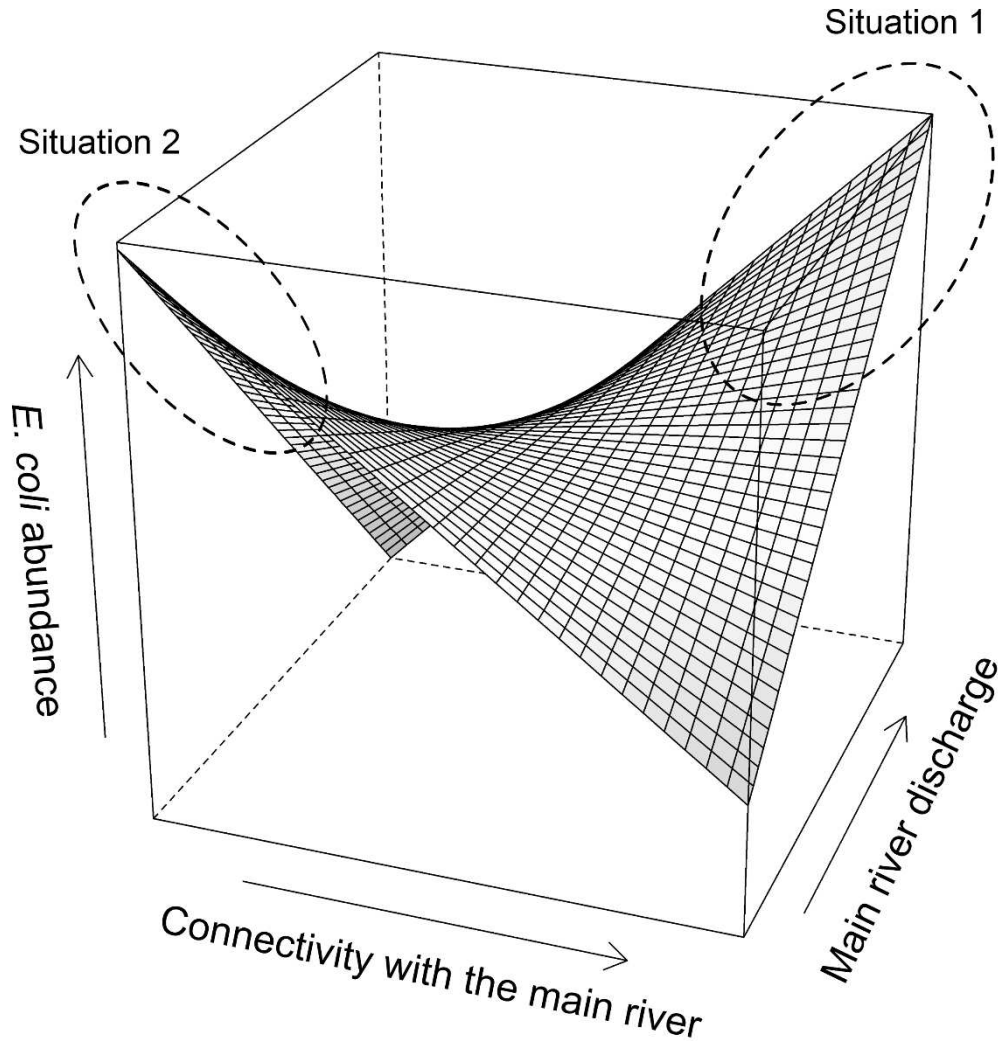
### 2.3.4. *The contamination of riverine wetlands*

Riverine wetlands were less contaminated by total *E. coli* than streams, but more contaminated than groundwater (Fig 2; Kruskal-Wallis test, p-value =  $< 2.2 \times 10^{-16}$ ; followed by a Dunn's test). As in streams, total *E. coli* and ESBL-producing *E. coli* were more abundant in February 2020 in wetlands (*i.e.* sampling month with the highest discharges; Fig. S5). These ecosystems are variously connected to the Ain River (Table S1). We thus hypothesized that the contamination of riverine wetlands resulted from the dispersal of HAB from the Ain River. To test this hypothesis, we modelled the total *E. coli* abundances retrieved in wetlands through the

connectivity between wetlands and the Ain River, the sampling campaign, and the interaction between them. The model had a pseudo  $R^2$  of 0.464. This result suggests that the bacterial contamination of floodplains is time-dependent and/or river discharge-dependent. We therefore constructed a second model based on the connectivity between wetlands and the Ain River, and the Ain River discharge. This model had a pseudo- $R^2$  of 0.183. Total *E. coli* abundances in wetlands were linked to the connectivity between wetlands and the Ain River, the Ain River discharge, and also to the interaction between both connectivity and river discharge (regression coefficients:  $1.05 \times 10^{-2}$ ,  $5.86 \times 10^{-2}$ , and  $-1.22 \times 10^{-4}$  respectively; p-value:  $7.24 \times 10^{-3}$ ,  $2.11 \times 10^{-5}$ , and  $3.94 \times 10^{-5}$ , respectively) (Fig. 4). Two situations emerged from this second model (Fig. 4).

**Situation 1.** During high-flow periods, total *E. coli* abundances were related to the connectivity between wetlands and the Ain River: highly-connected wetlands were more contaminated than poorly-connected ones (Fig. 4, Fig. S6A,C,D). In this case, the bacterial contamination of the floodplain results from hydrological fluxes, as reported by Martak et al. (2020).

**Situation 2.** During low-flow periods, total *E. coli* abundances were reversely related to the connectivity between wetlands and the Ain River: some wetlands poorly connected to the Ain River were more contaminated than highly-connected ones (Fig 4, Fig. S6B,C,D). In this situation, either precipitations – and the favoured leaching during drought periods – may disperse bacteria from the floodplain agricultural soils to the poorly-connected wetlands (Laroche et al., 2010; Hansen et al., 2020), and/or HAB can survive in certain poorly-connected wetlands when they are not washed away by hydrological fluxes ("flush effect"). HAB could therefore find more stable physicochemical characteristics in these poorly-connected and stagnant ecosystems (Van Elsas et al., 2011; Henriot et al., 2019).



**Fig. 4. Total *E. coli* abundances in wetlands according to the connectivity between wetlands and the Ain River, the Ain River discharge, and the interaction between both last parameters in the Ain River floodplain, France.** Total *E. coli* abundances were determined in wetland water of the Ain floodplain between March 2019 and February 2020 (n=181) using a culture-based method. The connectivity between wetlands and the Ain River was approximated by the conductivity of the water (section 2.1). This graph is a visualisation of a negative binomial generalized linear model. The model has a pseudo- $R^2$  of 0.183. The regression coefficients between total *E. coli* abundances and the connectivity, the Ain River discharge, and the interaction between both connectivity and river discharge were  $1.05 \times 10^{-2}$ ,  $5.86 \times 10^{-2}$  and  $-1.22 \times 10^{-4}$ , respectively (p-value= $7.24 \times 10^{-3}$ ,  $2.11 \times 10^{-5}$  and  $3.94 \times 10^{-5}$ , respectively). **Situation 1:** during high-flow periods, total *E. coli* abundances are related to the connectivity between wetlands and the Ain River: highly-connected wetlands are more contaminated than poorly-connected ones. **Situation 2:** during low-flow periods, total *E. coli* abundances are reversely related to the connectivity between wetlands and the Ain River: certain wetlands poorly connected to the Ain River are more contaminated than highly-connected ones.

## 2.4. Survival of human-associated bacteria the floodplain

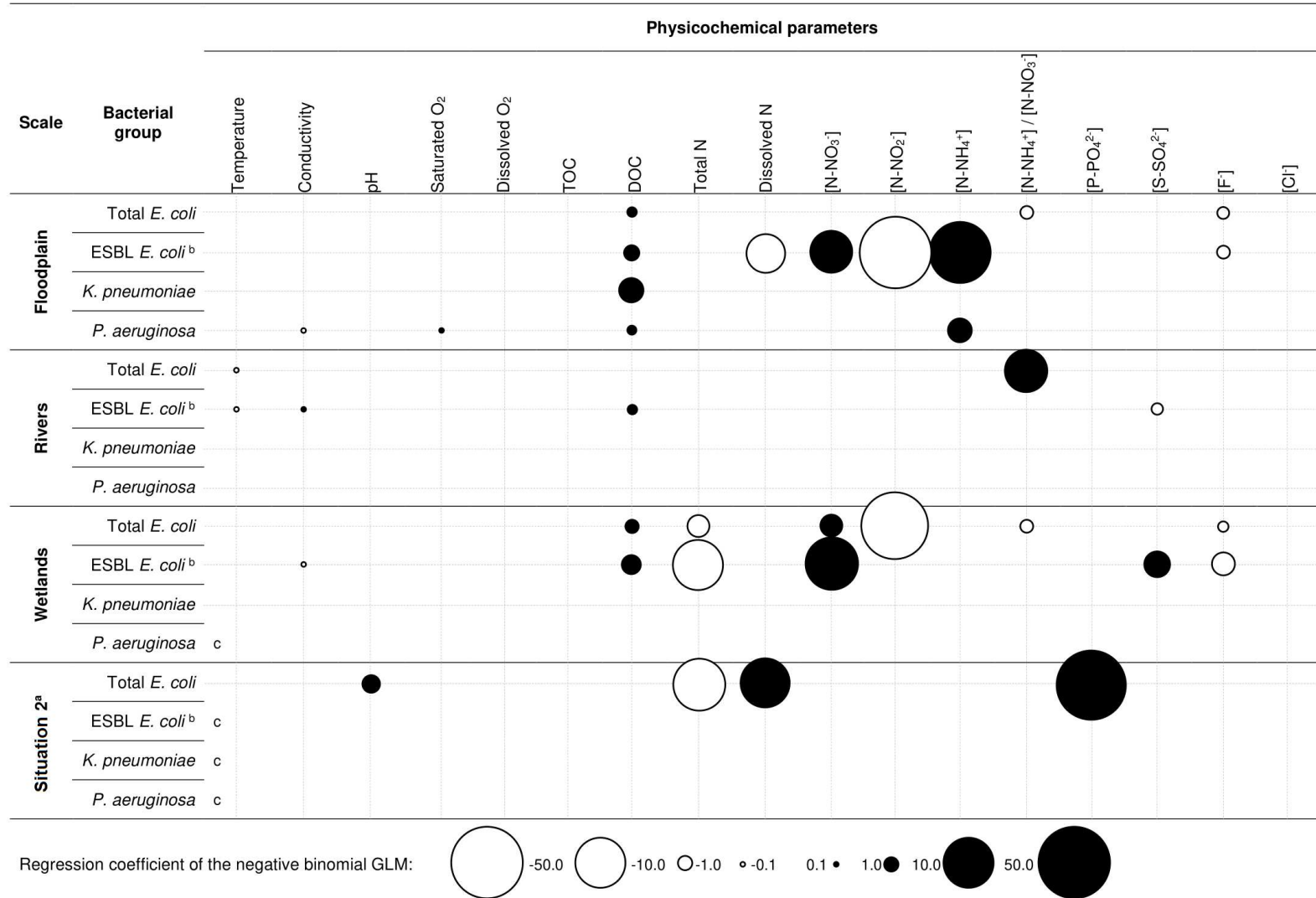
### 2.4.1. Distinct populations in riverine wetlands?

We compared the phylogroup distribution of total *E. coli* and the plasmid incompatibility groups they harboured in the situations 1 and 2. Overall, no difference was observed between the structure of population of total *E. coli* in situation 1 and situation 2; the higher proportion of isolates with an IncFrep plasmid in situation 1 than in situation 2 was the only exception (equality of proportions test,  $p$ -value =  $1.17 \times 10^{-3}$ ). This homogeneity between total *E. coli* populations showed that wetlands are contaminated by the same source during high- (situation 1) and low-flow periods (situation 2), ruling out the hypothetical agricultural contamination during low-flow periods (section 2.3.4). In other words, the Ain River carried HAB from the WWTP outflow to the wetlands during high-flow periods, and these bacteria can survive several months and thrive in poorly-connected wetlands during the low-flow periods. Although our data need to be confirmed in more eutrophic ecosystems, the survival and thriving of HAB populations in certain poorly-connected wetlands suggest that wetlands can be environmental reservoirs for bacterial pathogens of human origin. These bacterial populations could be dispersed when the water level rises again, contaminating the floodplain downstream.

### 2.4.2. Physicochemical characteristics and survival of human-associated bacteria in floodplain ecosystems

Beyond hydro-morphological factors, we made the hypothesis that physicochemical parameters of the water influenced the survival of HAB in floodplain aquatic ecosystems. We therefore constructed models at different scales: (i) the whole floodplain, (ii) the streams, (iii) the wetlands and (iv) the poorly-connected wetlands during low-flow periods (*i.e.* situation 2) (Fig. 5, Table S7). Total *E. coli* and ESBL-producing *E. coli* abundances were negatively related to temperature in streams (Fig. 5) suggesting either that HAB were less dispersed in floodplains during warm periods (usually occurring during low-flow periods) (Schilling et al., 2009; Honda et al., 2020) and/or, that warmer temperatures favoured the growth of slower-growing bacterial species capable of competing with HAB (Lax et al., 2020). Moreover, abundances of HAB were positively related to DOC and N-nitrate (Fig. 5). This may be observed because bacteria, nitrate, and DOC have the same source (*i.e.* WWTP outflows) or because nitrogen and carbon can favour the survival of HAB (Vital et al., 2008; Garzio-Hadzick et al., 2010; Martín-Rodríguez et al., 2020). Finally, abundances of HAB were also negatively related to N-nitrite, which has been reported to be toxic for bacteria (Philips et al., 2002).

In the specific case of poorly-connected wetlands during low-flow periods (*i.e.* situation 2), total *E. coli* abundances were highly related to physicochemical parameters of the water (pseudo- $R^2$  of 0.709, Table S7). Total *E. coli* abundances were positively and highly related to P-phosphate and dissolved nitrogen (Fig. 5), two essential nutrients for the metabolism of bacteria (Fagerbakke et al., 1996). Although we have found that agriculture unlikely contaminates poorly-connected wetlands (section 2.4.1), it is nevertheless possible that agriculture plays an indirect role through the enrichment of groundwater in nutrients that may favour the survival of HAB in isolated groundwater-supplied wetlands during low-flow periods. Total *E. coli* was negatively linked to total nitrogen, which includes nitrogen from microorganisms (*i.e.* bacteria, phytoplankton, and protozoa) (Fig. 5). This suggests that HAB are disfavoured in microorganism-rich ecosystems, probably due to predation and competition processes (Vital et al., 2012). Nutrient-poor ecosystems could thus be more favourable than eutrophic ones for HAB (Johnson and Carpenter, 2010). This hypothesis needs to be tested on a wider range of eutrophication.



**Fig. 5. Abundances of total *E. coli*, ESBL-producing *E. coli*, total *K. pneumoniae* and *P. aeruginosa* related to the physicochemical parameters in the Ain River floodplain, France.** Water samples were taken between March 2019 and February 2020. Significant regression coefficients of negative binomial generalized linear models are represented. Four scales are considered: the whole floodplain (n=360), the streams (n=153), the wetlands (n=181) and the wetlands corresponding to the situation 2<sup>a</sup> (n=29). Regression coefficients, p-values and the pseudo-R<sup>2</sup> of each model are provided in Table S7.

<sup>a</sup>: *i.e.* situation 2 in section 2.3.4: we selected bacterial abundances from poorly-connected wetlands (*i.e.* W1, W3, W6, W11, W12, W13 and W16) during low-flow periods (*i.e.* from May to October 2019).

<sup>b</sup>: ESBL-producing *E. coli*

<sup>c</sup>: Not enough non-zero bacterial abundances: the model did not converge

### 2.4.3. Fate of ESBL-producing strains

The cost of resistance to antibiotics has been demonstrated in *in vitro* experiments (Andersson and Hughes, 2010). We thus hypothesized that antibiotic-resistant HAB may be disadvantaged compared to their susceptible parents in floodplain ecosystems. However, the fitness cost of antibiotic-resistance is hard to evaluate in complex environments that are possibly contaminated with a mixture of selecting agents that can compensate the disadvantage of resistant bacteria over their susceptible parents (Dickinson et al., 2019). To that aim, we tested the correlation of the ratio of ESBL-producing and total *E. coli* with the distance between the point-source of contamination and the point of sampling in the streams. Our data suggest that the concentration of total *E. coli* in streams provided a good estimation of that distance. We found that the above-mentioned ratio was not correlated to the total *E. coli* abundances (Spearman correlation, p-value > 0.05) suggesting a similar behaviour (*i.e.* dispersal, survival) of *E. coli* strains in heterogeneous environments, whatever the production of ESBL (Laroche et al., 2009).

## Conclusions

In summary, this study characterized the origin, the fluxes, and the reservoirs of HAB in a low-anthropized floodplain. The culture-based approach did not allow us to assess the importance of viable but non-culturable HAB in the environment. Moreover, processes highlighted in this study are based on a single floodplain and cannot be extended to any hydrosystem, especially highly-anthropized and eutrophic ones. Despite these limitations, we showed a high prevalence of pathogens of human origin in poorly-anthropized floodplain ecosystems. We found that the production of ESBL does not confer a disadvantage to HAB in streams. Furthermore, some patterns of dispersal and survival of HAB were identified. During high-flow periods, the contamination of floodplain ecosystems by HAB results from water fluxes. Water dispersal, and dilution were the two key parameters that govern the HAB contamination of streams. However, HAB concentrated in certain poorly-connected wetlands even in low-flow situations, suggesting that these bacteria can survive several months in these ecosystems, where they later find favourable environmental conditions to thrive. Our findings demonstrate that some ecosystems of nutrient-poor floodplains may be reservoirs of HAB. In the context of climate change with rainfall events more intense (*i.e.* isolated ecosystems connected during floods) but less frequent (*i.e.* spacing the "flush effects") in temperate regions, the reservoir role of isolated wetlands could be strengthened.



## **Funding**

This project was funded by the French National Centre for Scientific Research (CNRS) through the MITI interdisciplinary program “Osez l’Interdisciplinarité” (OSEZ 2018), the Structuring Initiative “Continental and Coastal Ecosphere” (EC2CO 2016) program), and through a support from the Observatory of Universe Sciences (OSU Theta 2015).

## **Acknowledgements**

The authors warmly thank the PEA<sup>2t</sup> platform (Chrono-environment, University of Bourgogne Franche-Comté, France), which manages and maintains the analytical equipment used in this study. Dr. Nadia Crini and Christophe Loup conducted the physicochemical analyses. Olivier Araspin (Commissariat à l'énergie atomique et aux énergies alternatives, Arpajon, France) conducted the isotopic analysis. Dr. Catherine Bertrand and Pr. Hélène Celle (UMR6249 Chrono-environnement, Besançon, France) helped to the interpretation of the isotopic results. Dr. Pascal Cholley and Justine Maroilley participated in the analysis of the ESBL-encoding genes. Dr. Lisa Tymensen is thanked for sharing the protocol of identification of plasmid incompatibility groups. Alexandre Meunier, Emilia Chiapponi and Vanessa Stefani are warmly thanked for their help in sampling. The SR3A (Syndicat de la Rivière d'Ain et ses Affluents) has guaranteed access to the piezometers. The streams flows data were provided by the HYDRO database (Ministère de l'Ecologie, de l'Energie, du Développement Durable et de l'Aménagement du Territoire / Direction Générale de la Prévention des Risques / Service des Risques Naturels et Hydrauliques; data collected by the Direction Régionale de l'Environnement, de l'Aménagement et du Logement de Rhône-Alpes). The meteorological data were provided by Météo France.

***Conflict of interest.*** None declared.

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## Supplementary data

### Supplementary text

#### Measurement of the physicochemical characteristics of the water

Water temperature was measured using immersed probes (Onset HOBO UTBI-001 TidbiT<sup>®</sup>, Prosensor, Armanvillers, France). One temperature probe was placed at the bottom of each site at the beginning of the sampling period and automatically returned the temperature each hour during the one-year cycle sampling period. The pH and the electrical conductivity were measured *in situ* at a 10 cm water depth using a WTW pH sensor (SenTix<sup>®</sup> 41, WTW, Weilheim, Germany) and a WTW conductivity sensor (TetraCon<sup>®</sup> 325, WTW, Weilheim, Germany), respectively. The saturated and dissolved oxygen were measured using a WTW oxygen sensor (CellOx<sup>®</sup> 325-3, WTW, Weilheim, Germany). To determine the concentrations of TOC, DOC, total nitrogen, dissolved nitrogen, nitrate, nitrite, ammonium, phosphate, sulphate, fluoride and chloride, 500 ml of water were collected and filtered through 0.2 mm polycarbonate filters and analysed within 48 h of collection. The samples were kept at 4°C throughout the process. TOC, DOC, total nitrogen and dissolved nitrogen concentrations were measured using a TOC analyser (Vario TOC Cube, Elementar, Hanau, Germany). The nitrate, nitrite, sulphate, fluoride and chloride concentrations were measured using an ion chromatograph (ICS-1000, Dionex, USA). The N-ammonium concentration was measured using the indophenol blue method (Berthelot's reaction, Merck Spectroquant). The phosphate concentration was measured using the molybdenum blue coloration method.

#### Chemical characteristics of the sediments

Sediments have been stored at 4°C before analysis, which occurred 24 h after sampling. The sediment samples were crushed, then burned to obtain the total nitrogen, sulfur and carbon contents, via the gaseous releases of N<sub>2</sub>, SO<sub>2</sub> and CO<sub>2</sub> using a Vario MAX CNS (Elementar, Hanau, Germany). Then, by adding hydrochloric acid (3.7 %) to the sample then heated to 50°C overnight to remove carbonates (inorganic carbon), we were able to obtain the TOC by using a Vario TOC Cube (Elementar, Hanau, Germany).

#### Bacterial sampling and identification

Aliquots of water (100 ml) or leachate (25-50 ml) were filtered through 0.45-µm membranes, which were then placed onto a Drigalski agar plate to measure the total *E. coli* and total *K. pneumoniae* abundances (Oxoid), a Cetrimide agar plate to measure the *P. aeruginosa*

abundances (Bio-Rad, Marne la Coquette, France) and a chromID ESBL agar plate (bioMérieux, Marcy l'Etoile, France) to measure the abundances of ESBL-producing *E. coli* and ESBL-producing *K. pneumoniae*. When we suspected high bacterial abundances, we only plated 50 ml of water on the selective media. The plates were incubated for 24 h at 37°C. Colonies were counted and classified according to their phenotype. Each type of colony was identified using a MALDI-TOF MS spectrometer (Microflex 100 LT, Bruker Daltonics GmbH, Bremen, Germany) according to the manufacturer's recommendations. For all bacterial abundances, the detection limit was 1 bacterium per 100 ml.

For each morphotype of *E. coli* and *K. pneumoniae* growing on chromID ESBL, the production of ESBL was detected using a synergy test following the recommendations of the Antibiogram Committee of the French Society for Microbiology.

We assessed the activity of 7 antibiotics (amoxicillin-clavulanate (AMC), cefepime (FEP), cotrimoxazole (SXT), ertapenem (ETP), gentamicin (GMN), ticarcillin (TIC) and tobramycin (TMN)) to differentiate the strains of a same sampling. We stored the isolates in brain heart infusion broth supplemented with 30% glycerol at -80°C until further analysis.

### **Genetic structure of *E. coli* isolates**

Phylogroup typing. Briefly, phylogroups were assigned depending on the presence/absence of four genes (*arpA*, *chuA*, *yjaA*, and *tspE4.C2*) using a quadruplex PCR (Clermont et al., 2013; Clermont et al., 2019). Isolates with identical profiles were further discriminated by additional PCRs. Hence, we amplified *trpA* to differentiate the isolates in phylogroups A and C, *arpA* to differentiate the isolates in phylogroups D and E, and *cfaB* and *ybgD* to differentiate isolates of the phylogroups B2, F, and G. Particular profiles allowed us to differentiate *E. coli* and *Escherichia* cryptic clades (Clermont et al., 2013).

Plasmid incompatibility group typing. For each isolate, associated plasmids were assigned to incompatibility (Inc) groups by PCR-based replicon typing (PBRT) (Carattoli et al., 2005; Tymensen et al., 2019) with slight modification (Table SMM1). IncA/C, IncFIA, IncFIB, IncFIC, IncFrep, IncI1, and IncY have been amplified through two multiplex PCRs (amplifying IncA/C, IncFIB, IncFIC, and IncFIA, IncI1, IncY) and one simplex PCR (amplifying IncFrep). Due to nonspecific binding with the PBRT IncB/O primer set, we conducted degenerate primer MOB typing (DPMT) for the MOBp12 family (Alvarado et al., 2012) with a simplex PCR which amplifies plasmids belonging to IncI1 group, including IncB/O, IncI1, IncK (Tymensen et al., 2019). IncFIA, IncFIB, IncFIC and IncFrep belong to the IncF plasmid incompatibility group.



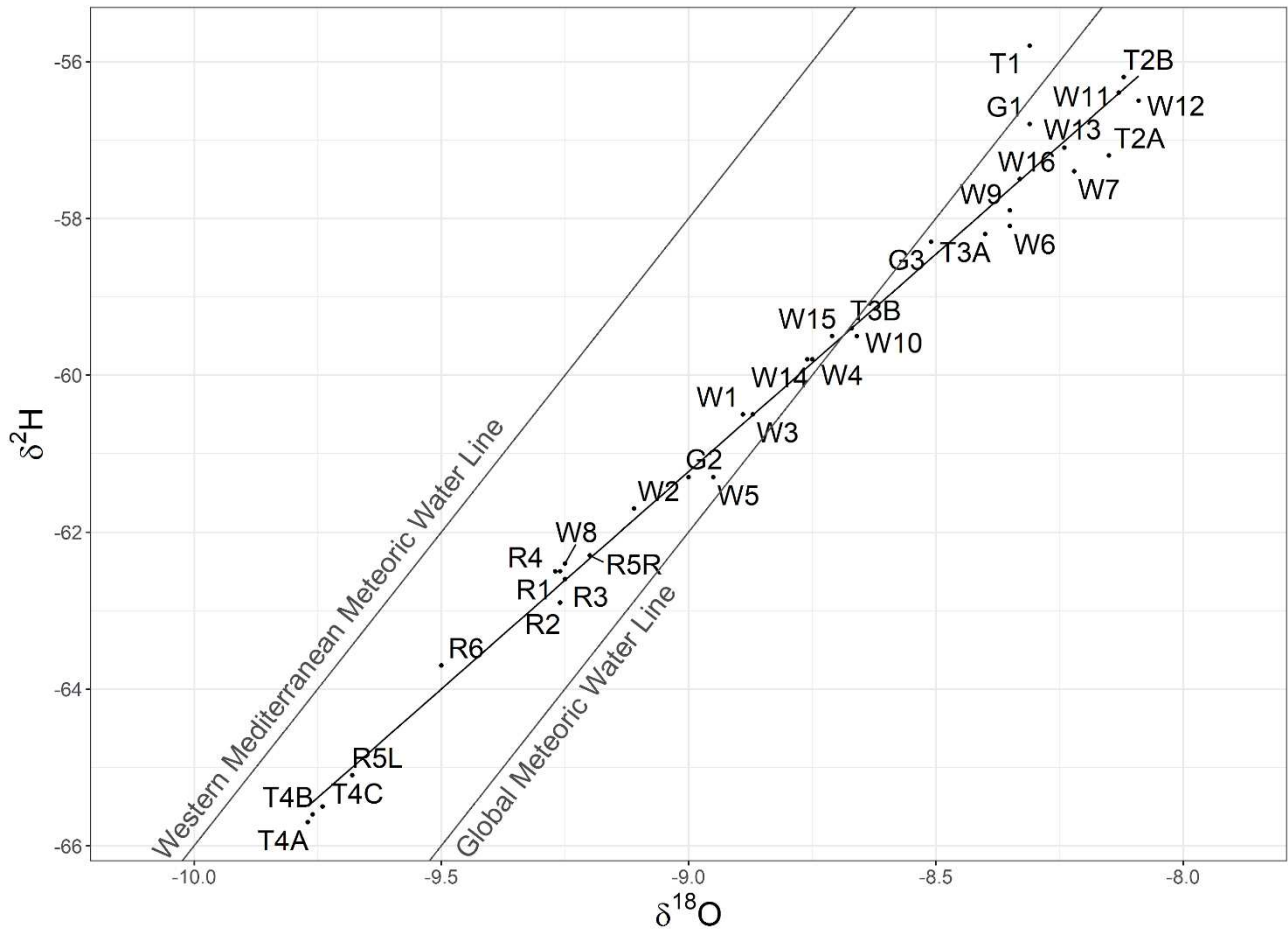
*bla*<sub>ESBL</sub> resistance genes. We first screened the isolates carrying *bla*<sub>CTX-M</sub> with consensus primers (Table SMM1) and we then targeted the different groups of CTX-M with specific primers (*bla*<sub>CTX-M-group 1</sub>, *bla*<sub>CTX-M-group 9</sub>, *bla*<sub>CTX-M-group 2</sub>) (Bréchet et al., 2014). *bla*<sub>CTX-M</sub> negative isolates were then tested for the presence of *bla*<sub>SHV</sub> and *bla*<sub>TEM</sub> genes (Cao et al., 2002; Mabilat and Courvalin, 1990). The nucleotide sequences of all PCR products were determined (Microsynth Seqlab, Göttingen, Germany).

**Table SMM1.** Sequences of primers for the detection of plasmid incompatibility groups, and for *bla*<sub>ESBL</sub> resistance genes.

Target	Primer sequence (5'-3') <sup>a</sup>	PCR type	Denaturation	Annealing <sup>b</sup>	Elongation <sup>b</sup>	Amplicon size (bp)	Reference
<i>Plasmid incompatibility groups</i>							
<b>IncA/C</b>	F: GAGAACCAAAGACAAAGACCTGGA R: ACGACAAACCTGAATTGCCTCCTT	Multiplex 1	94°C, 30 sec	60°C, 60 sec	72°C, 60 sec	465	Carattoli et al., 2005
<b>IncFIB</b>	F: GGAGTTCTGACACACGATTTTCTG R: CTCCCGTCGCTTCAGGGCATT	Multiplex 1	94°C, 30 sec	60°C, 60 sec	72°C, 60 sec	702	Carattoli et al., 2005
<b>IncFIC</b>	F: GTGAAGTGGCAGATGAGGAAGG R: TTCTCCTCGTCGCCAAACTAGAT	Multiplex 1	94°C, 30 sec	60°C, 60 sec	72°C, 60 sec	262	Carattoli et al., 2005
<b>IncFIA</b>	F: CCATGCTGGTTCTAGAGAAGGTG R: GTATATCCTTACTGGCTTCCGCAG	Multiplex 2	94°C, 30 sec	56°C, 60 sec	72°C, 60 sec	462	Carattoli et al., 2005
<b>IncII</b>	F: CGAAAGCCGGACGGCAGAA R: TCGTCGTTCCGCCAAGTTCGT	Multiplex 2	94°C, 30 sec	56°C, 60 sec	72°C, 60 sec	139	Carattoli et al., 2005
<b>IncY</b>	F: AATTCAAACAACACTGTGCAGCCTG R: GCGAGAATGGACGATTACAAAACCTT	Multiplex 2	94°C, 30 sec	56°C, 60 sec	72°C, 60 sec	765	Carattoli et al., 2005
<b>IncFrep</b>	F: TGATCGTTTAAGGAATTTTG R: GAAGATCAGTCACACCATCC	Simplex	94°C, 30 sec	56°C, 60 sec	72°C, 60 sec	270	Carattoli et al., 2005
<b>MOBp12</b>	F: GCACACTATGCAAAAGATGATACTGAYCCYGT R: AGCGATGTGGATGTGAAGGTTTRTCNGTRTC	Simplex	94°C, 30 sec	50°C, 60 sec	72°C, 60 sec	189	Alvarado et al., 2012
<i>bla</i> <sub>ESBL</sub> resistance genes							
<i>bla</i> <sub>CTX-M-group 1</sub>	F: CTTCCAGAATAAGGAATC R: CCGTTTCCGCTATTACAA	Simplex	94°C, 30 sec	50°C, 30 sec	72°C, 30 sec	907	Bréchet et al., 2014
<i>bla</i> <sub>CTX-M-group 9</sub>	F: TGGTGACAAAGAGARTGCAACGG R: ACAGCCCYTYGGCGATGATTCT	Simplex	94°C, 30 sec	57.5°C, 30 sec	72°C, 30 sec	873	Bréchet et al., 2014
<i>bla</i> <sub>CTX-M-group 2</sub>	F: TGAAGTCAAGCATTSGCCGCT R: CGYGGTTACGATTTTCGCGYCG	Simplex	94°C, 30 sec	61°C, 30 sec	72°C, 30 sec	864	Bréchet et al., 2014
<i>bla</i> <sub>SHV</sub>	F: TTATCTCCCTGTTAGCCACC R: GATTGCTGATTTCCGCTCGG	Simplex	94°C, 30 sec	52°C, 30 sec	72°C, 30 sec	785	Cao et al., 2002
<i>bla</i> <sub>TEM</sub>	F: TAAAATCTTGAAGACG R: TTACCAATGCTTAATCA	Simplex	94°C, 30 sec	51°C, 30 sec	72°C, 30 sec	516	Mabilat and Courvalin, 1990

<sup>a</sup>: R: G, A; S: G, C.<sup>b</sup>: annealing and elongation temperatures and times have been adapted from the original references.

## Supplementary tables and figures



**Fig. S1. Meteoric water line of the studied sites of the Ain River floodplain.**  $\delta^{18}\text{O}$  ( $^{18}\text{O}/^{16}\text{O}$ ) and  $\delta^2\text{H}$  ( $^2\text{H}/^1\text{H}$ ) isotopic ratios have been obtained from water sampled during a high-flow period (*i.e.* February 3<sup>rd</sup> 2020). The global meteoric water line (GMWL; Craig, 1961), and the Western Mediterranean meteoric water line (WMMWL; Celle-Jeanton et al., 2001) are represented in grey. The line according to which our sites are distributed has a lower slope than the GMWL and the WMMWL, revealing evaporation processes taking place for the most  $\delta^{18}\text{O}$ -enriched sites. All sites are aligned showing a mixture between a “streams” end-member (main river and tributary T4 points; lower left) and an “evaporated water” one (mainly wetland and groundwater points; upper right).

**Table S1. Contribution of the main river and groundwater to each studied wetland of the Ain River floodplain (%).** These percentages were obtained from  $\delta^{18}\text{O}$  ( $^{18}\text{O}/^{16}\text{O}$ ) isotopic ratios obtained from waters sampled during a high-flow period (*i.e.* February 3<sup>rd</sup> 2020) and from Paran et al. (2015). The two springs W3 and W11 have been set as groundwater references for the right and the left bank of the studied main river, respectively, because they were located in sectors where the main river is especially incised (*i.e.* groundwater table higher than the alluvial water table).

<b>Wetland</b>	<b>Contribution of the main river water (%)</b>	<b>Contribution of groundwater (%)</b>
<b>W1</b>	5.1	94.9
<b>W2</b>	86.7	13.3
<b>W3</b>	0	100
<b>W4</b>	0	100
<b>W5</b>	20.5	79.5
<b>W6</b>	19.6	80.4
<b>W7</b>	8	92
<b>W8</b>	100	0
<b>W9</b>	19.6	80.4
<b>W10</b>	47.3	52.7
<b>W11</b>	0	100
<b>W12</b>	0	100
<b>W13</b>	9.8	90.2
<b>W14</b>	55.3	44.7
<b>W15</b>	50.9	49.1
<b>W16</b>	17.5	82.5

**Table S2. Phylogroups of total *E. coli* and ESBL-producing *E. coli* retrieved in water samples of the Ain River floodplain between March 2019 and February 2020.**

<i>E. coli</i> isolates	Phylogroup									
	A	B1	B2	C	D	E	F	G	Unknown	<i>E. clades</i>
<b>Total <i>E. coli</i> (n=349)</b>	99 (28.4 %)	78 (22.3 %)	65 (18.6 %)	10 (2.9 %)	52 (14.9 %)	12 (3.4 %)	2 (0.6 %)	4 (1.2 %)	0 (0.0 %)	27 (7.7 %)
<b>ESBL-producing <i>E. coli</i> (n=187)</b>	52 (27.8 %)	20 (10.7 %)	57 (30.6 %)	15 (8.0 %)	35 (18.7 %)	3 (1.6 %)	3 (1.6 %)	1 (0.5 %)	1* (0.5 %)	0 (0.0 %)

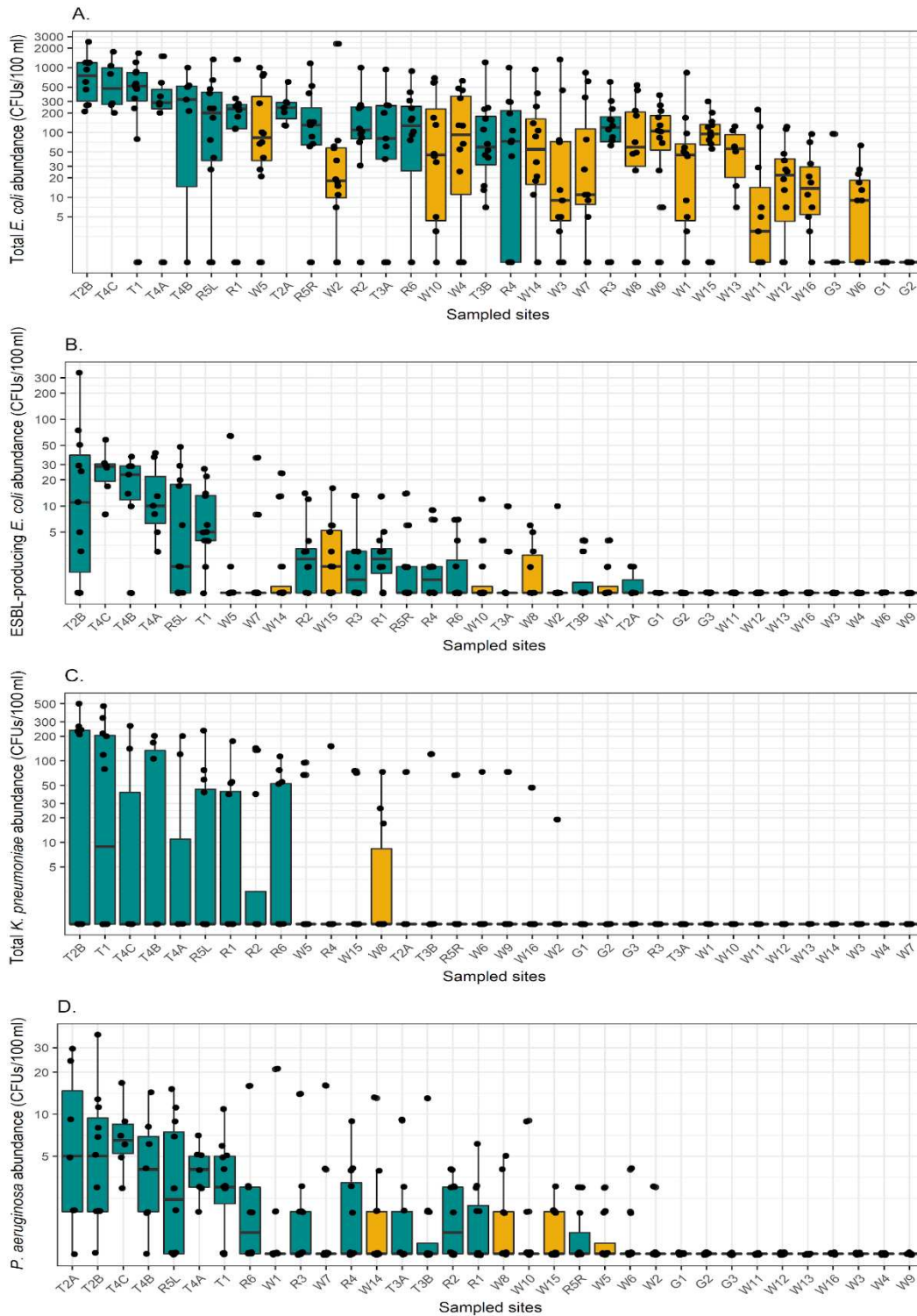
\* one ESBL-producing *E. coli* isolate had an unknown phylogroup profile corresponding to *arpA* -, *chuA* -, *yjaA* +, TspE4.C2 +.

**Table S3. Plasmid incompatibility groups retrieved in total *E. coli* and ESBL-producing *E. coli* retrieved in water samples of the Ain River floodplain between March 2019 and February 2020.** The sum of percentages is greater than 100% since a single isolate can carry several plasmids.

<i>E. coli</i> isolates	Plasmid incompatibility group							
	IncA/C	IncFIA	IncFIB	IncFIC	IncFrep	IncI1	IncY	MOBp12 family
<b>Total <i>E. coli</i> (n=349)</b>	1 (0.3 %)	26 (7.4 %)	89 (25.5 %)	10 (2.9 %)	124 (35.5 %)	34 (9.7 %)	19 (5.4 %)	56 (16.0 %)
<b>ESBL-producing <i>E. coli</i> (n=187)</b>	2 (1.1%)	45 (24.1 %)	91 (48.7 %)	0 (0.0 %)	140 (74.9 %)	48 (25.7 %)	11 (5.9 %)	62 (33.2 %)

**Table S4. ESBL-encoding genes found in the 187 ESBL-producing *E. coli* isolates retrieved in water samples of the Ain River floodplain between March 2019 and February 2020. Each isolate harboured only one *bla*<sub>ESBL</sub>.**

	<b>n</b>	<b>%</b>
<i>bla</i> CTX-M-1	19	10.2
<i>bla</i> CTX-M-3	5	2.7
<i>bla</i> CTX-M-15	81	43.3
<i>bla</i> CTX-M-32	4	2.1
<i>bla</i> CTX-M-55	4	2.1
<i>bla</i> CTX-M-9	4	2.1
<i>bla</i> CTX-M-14	31	16.6
<i>bla</i> CTX-M-24	2	1.1
<i>bla</i> CTX-M-27	25	13.4
<i>bla</i> CTX-M-65	1	0.5
<i>bla</i> CTX-M-2	1	0.5
<i>bla</i> SHV-12	7	3.8
<i>bla</i> TEM-24	1	0.5
<b>Unknown</b>	2	1.1



**Fig. S2.** Abundances of total *E. coli* (A), ESBL-producing *E. coli* (B), total *K. pneumoniae* (C) and *P. aeruginosa* (D) retrieved in water sampled in the Ain River floodplain between March 2019 and February 2020, according to the sampling sites. Boxplots are presented on a logarithmic scale. Each boxplot represent 12 measurements (*i.e.* one by sampling campaign; except for sites that dried out in summer). Bacterial abundances were determined using a culture-based method. We sorted the sampling sites according to their average level of contamination (from the highest on the left, to the lowest on the right). Sampling sites are coloured by type (streams in blue, wetlands in yellow; and groundwater in purple). Purple boxplots were invisible because the bacterial abundances retrieved in the three groundwater sites are very low.

**Table S5. Abundances of human-associated bacteria and ratio between ESBL-producing *E. coli* and total *E. coli* in the Ain River floodplain between March 2019 and February 2020, according to the ecosystem type (*i.e.* groundwater, streams, and wetlands). The means  $\pm$  standard deviation are given.**

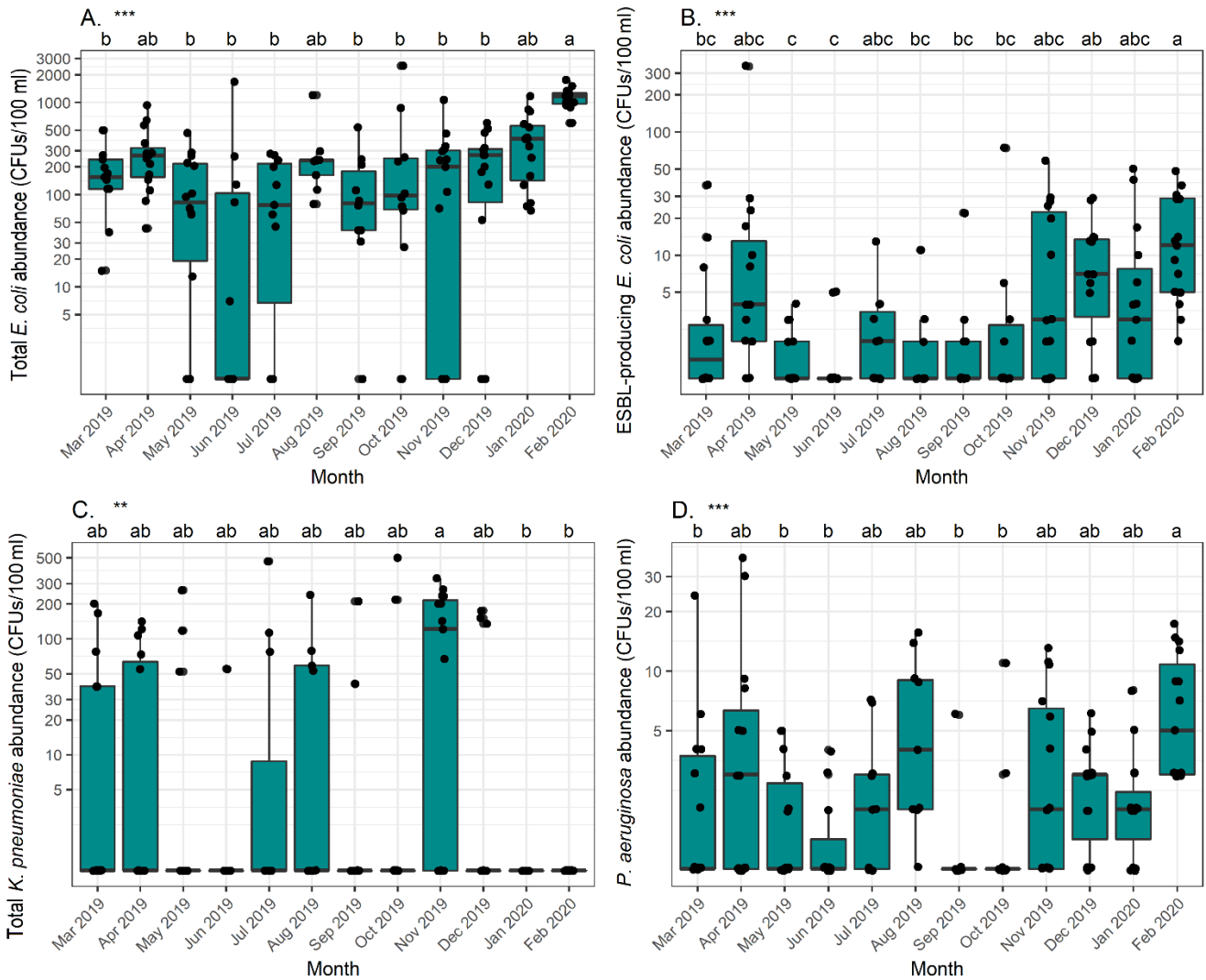
Type of ecosystem	Number of samples	Bacterial abundances (CFUs/100 ml)				Ratio ESBL-producing <i>E. coli</i> /total <i>E. coli</i>
		Total <i>E. coli</i>	ESBL-producing <i>E. coli</i>	Total <i>K. pneumoniae</i>	<i>P. aeruginosa</i>	
<b>Groundwater</b>	26	3.62 $\pm$ 18.4 <sup>a</sup>	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00
<b>Streams</b>	154	331.1 $\pm$ 416.6	8.60 $\pm$ 29.5	37.6 $\pm$ 85.8	2.78 $\pm$ 5.13	0.016 $\pm$ 0.036
<b>Wetlands</b>	181	130.4 $\pm$ 270.6	1.19 $\pm$ 5.88	3.45 $\pm$ 15.0	0.497 $\pm$ 2.19	0.003 $\pm$ 0.017

<sup>a</sup>: Only one groundwater sample was positive to total *E. coli*: during the high-flow sampling of January 2020, we found 94 *E. coli* CFUs/100 ml in G3. In this date, the surrounding wetland (W15) overflowed and the piezometer (G3) was full of water: we retrieved 120 *E. coli* CFU/100 ml in W15, which probably contaminated G3.

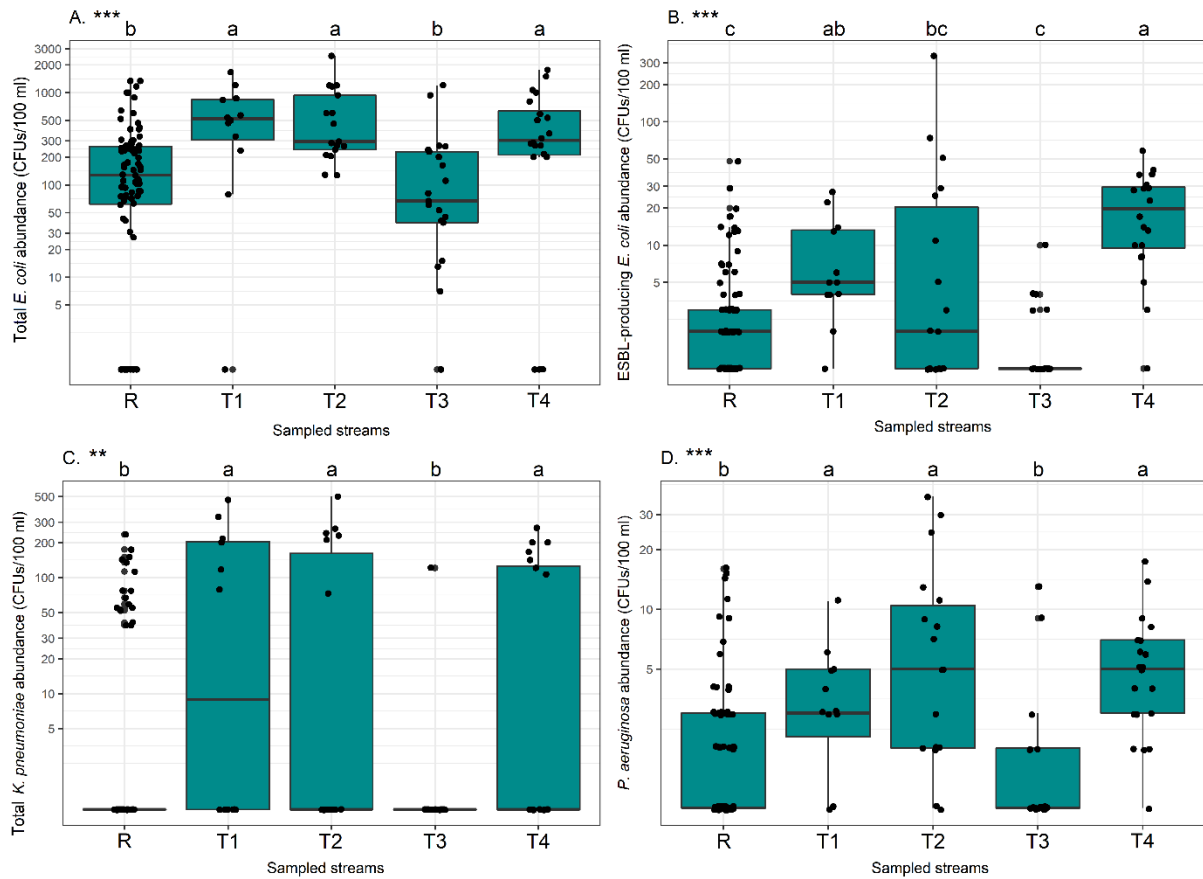


**Table S6. Abundances of human-associated bacteria and ratio between ESBL-producing *E. coli* and total *E. coli* retrieved in the water samples and in the sediment samples of the Ain River floodplain.** We sampled the matrices in July, October, November and December 2019. The means  $\pm$  standard deviation are given.

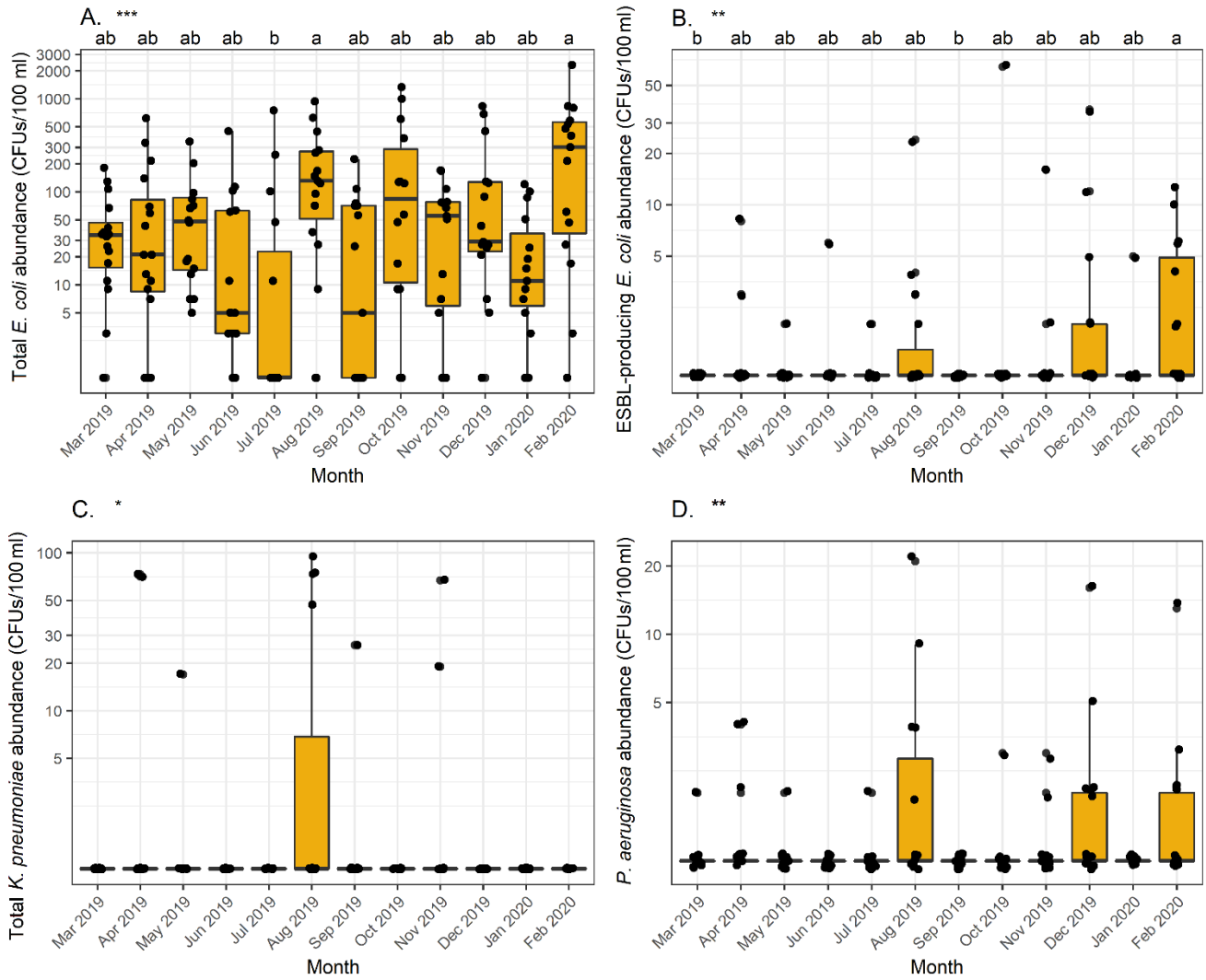
Bacterial abundances (CFUs/100 ml)						
	Number of samples	Total <i>E. coli</i>	ESBL-producing <i>E. coli</i>	Total <i>K. pneumoniae</i>	<i>P. aeruginosa</i>	Ratio ESBL-producing <i>E. coli</i> /total <i>E. coli</i>
<b>Water</b>	52	228.1 $\pm$ 434.0	4.14 $\pm$ 14.2	18.0 $\pm$ 77.4	1.04 $\pm$ 3.02	0.005 $\pm$ 0.015
<b>Sediment</b>	52	79.9 $\pm$ 243.0	1.44 $\pm$ 9.72	7.31 $\pm$ 37.9	0.096 $\pm$ 0.693	0.006 $\pm$ 0.039



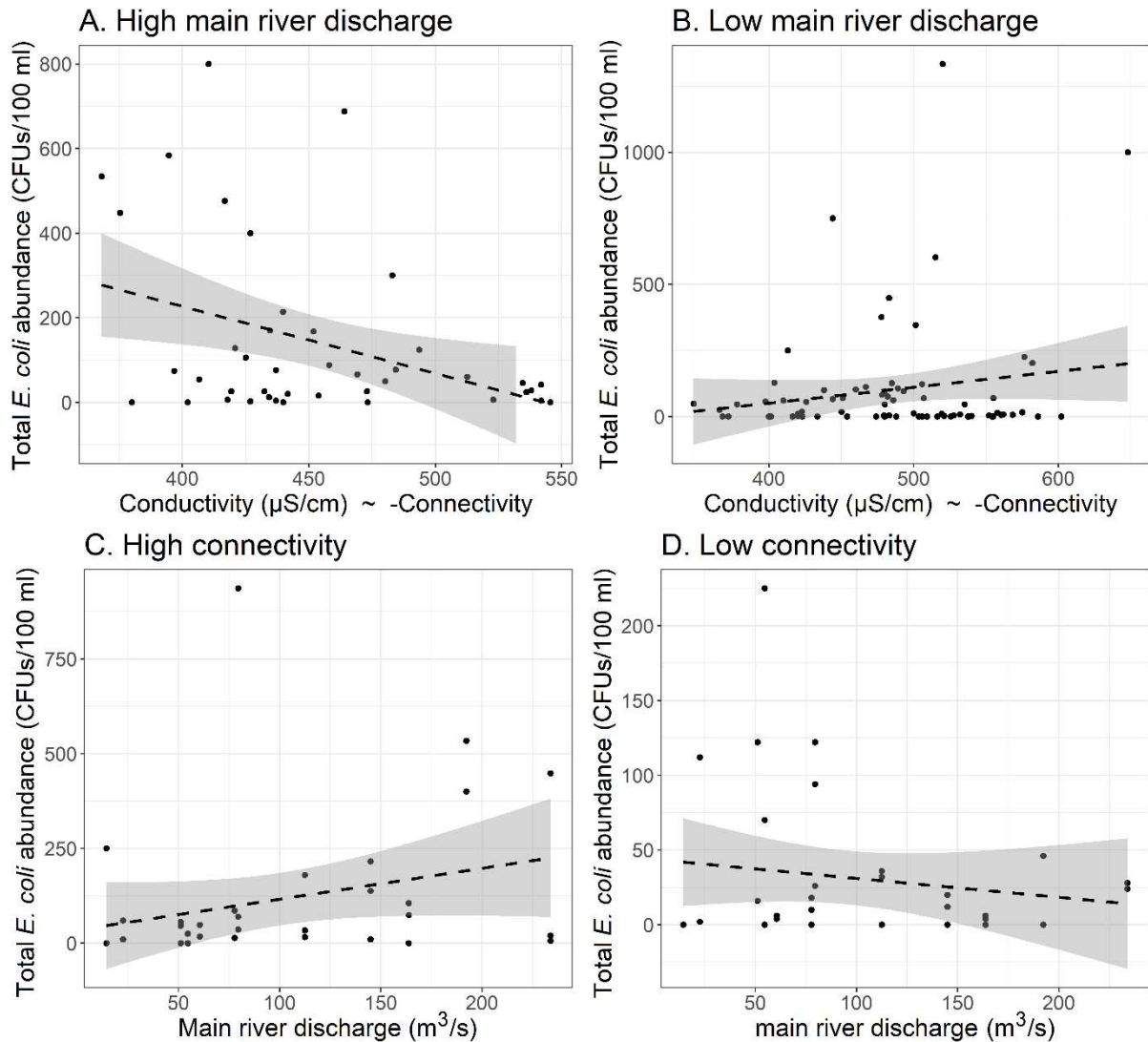
**Fig. S3. Bacterial abundances of total *E. coli* (A), ESBL-producing *E. coli* (B), total *K. pneumoniae* (C) and *P. aeruginosa* (D) retrieved in water sampled in streams of the Ain River floodplain between March 2019 and February 2020, according to the sampling months. Boxplots are presented on a logarithmic scale. Bacterial abundances were determined using a culture-based method. For each panel, asterisks and letters represent the p-value ( $0.05 > * > 0.01 > ** > 0.001 > ***$ ) of the Kruskal-Wallis test, and the groups highlighted by the Dunn's test, performed on bacterial abundances according to the sampling month, respectively.**



**Fig. S4. Bacterial abundances of total *E. coli* (A), ESBL-producing *E. coli* (B), total *K. pneumoniae* (C) and *P. aeruginosa* (D) retrieved in water sampled in streams of the Ain River floodplain between March 2019 and February 2020, according to the five sampling streams. Boxplots are presented on a logarithmic scale. Bacterial abundances were determined using a culture-based method. For each panel, asterisks and letters represent the p-value ( $0.05 > * > 0.01 > ** > 0.001 > ***$ ) of the Kruskal-Wallis test, and the groups highlighted by the Dunn's test, performed on bacterial abundances according to the sampling month, respectively.**



**Fig. S5. Bacterial abundances of total *E. coli* (A), ESBL-producing *E. coli* (B), total *K. pneumoniae* (C) and *P. aeruginosa* (D) retrieved in water sampled in wetlands of the Ain River floodplain between March 2019 and February 2020, according to the sampling months. Boxplots are presented on a logarithmic scale. Bacterial abundances were determined using a culture-based method. For each panel, asterisks and letters represent the p-value ( $0.05 > * > 0.01 > ** > 0.001 > ***$ ) of the Kruskal-Wallis test, and the groups highlighted by the Dunn's test, performed on bacterial abundances according to the sampling month, respectively. No significant differences were highlighted by the Dunn's test for *K. pneumoniae* and *P. aeruginosa* abundances.**



**Fig. S6.** Total *E. coli* abundances in all wetlands during high-flow periods (*i.e.* Ain River discharge  $> 150 \text{ m}^3/\text{s}$ ) according to the conductivity ( $n=44$ ) (A), all wetlands during low-flow periods (*i.e.* Ain River discharge  $< 70 \text{ m}^3/\text{s}$ ) according to the conductivity ( $n=74$ ) (B), highly connected wetlands (*i.e.* W2, W8 and W14) according to the main river discharge ( $n=33$ ) (C), and poorly connected wetlands (*i.e.* W11, W12 and W16) according to the main river discharge ( $n=33$ ) (D). This figure is an illustration of “scenario 2” described in section 2.3.4. *E. coli* abundances were determined using a culture-based method from water sampled in wetlands of the Ain River floodplain between March 2019 and February 2020. The conductivity is used as a proxy of connectivity (section 3.1). For the interpretation of (A) and (B), be careful that conductivity is negatively related to the connectivity.

**Table S7. Results of negative binomial generalized linear models relating the abundances of total *E. coli*, ESBL-producing *E. coli*, total *K. pneumoniae* and *P. aeruginosa*, and the physicochemical parameters of water sampled in the Ain River floodplain between March 2019 and February 2020.** Four scales are considered: the whole floodplain (n=360), the streams (n=153), the wetlands (n=181) and the wetlands corresponding to the scenario 2<sup>a</sup> (n=29). Parameters presented in the table are from the final models (*i.e.* only significant parameters remain). Each parameter is coupled with its regression coefficient, and the corresponding p-value is in brackets. The pseudo-R<sup>2</sup> is also provided for each model. Abundances of human-associated bacteria are related to some physicochemical parameters in floodplain ecosystems.

Scale considered	Total <i>E. coli</i>	ESBL-producing <i>E. coli</i>	Total <i>K. pneumoniae</i>	<i>P. aeruginosa</i>	
Floodplain	DOC	N-ammonium	DOC	N-ammonium	
	0.57 (7.90×10 <sup>-5</sup> )	17.88 (1.80×10 <sup>-5</sup> )	2.21 (1.60×10 <sup>-2</sup> )	2.14 (4.88×10 <sup>-2</sup> )	
	Fluoride	N-nitrate	R <sup>2</sup> = 0.998	DOC	
	-0.77 (8.5×10 <sup>-5</sup> )	6.55 (2.60×10 <sup>-4</sup> )		0.53 (2.20×10 <sup>-3</sup> )	
	‘N-ammonium : N-nitrate’	DOC		Saturated oxygen	
		1.11 (3.10×10 <sup>-7</sup> )		0.03 (1.10×10 <sup>-3</sup> )	
	-0.86 (9.10×10 <sup>-4</sup> )	Fluoride		Conductivity	
	pseudo-R <sup>2</sup> = 0.150		-0.88 (3.45×10 <sup>-2</sup> )		-0.01 (3.98×10 <sup>-2</sup> )
			Dissolved nitrogen		pseudo-R <sup>2</sup> = 0.239
			-5.52 (3.80×10 <sup>-4</sup> )		
		N-nitrite			
	-70.93 (5.90×10 <sup>-4</sup> )				
		pseudo-R <sup>2</sup> = 0.358			
Streams	‘N-ammonium : N-nitrate’	DOC	-	-	
		0.58 (5.47×10 <sup>-3</sup> )			
	6.20 (1.53×10 <sup>-2</sup> )	Conductivity			
	Temperature	0.02 (5.30×10 <sup>-4</sup> )	Temperature		
		-0.10 (1.70×10 <sup>-4</sup> )	-0.20 (3.00×10 <sup>-8</sup> )		
	pseudo-R <sup>2</sup> = 0.176	S-sulphate			
	-0.75 (2.44×10 <sup>-2</sup> )				
		pseudo-R <sup>2</sup> = 0.380			
Wetlands	N-nitrate	N-nitrate	-	-	
	1.88 (3.09 ×10 <sup>-3</sup> )	11.57 (3.30×10 <sup>-4</sup> )			
	DOC	S-sulphate			
	0.90 (2.50×10 <sup>-5</sup> )	2.49 (4.57×10 <sup>-3</sup> )			
	Fluoride	DOC			
	-0.64 (6.03×10 <sup>-3</sup> )	1.52 (8.14×10 <sup>-3</sup> )			
	‘N-ammonium : N-nitrate’	Conductivity			
		-0.04 (5.40×10 <sup>-4</sup> )	Fluoride		
	-0.85 (2.10×10 <sup>-4</sup> )	-2.03 (8.45×10 <sup>-3</sup> )			
	Total nitrogen	Total nitrogen			
-1.85 (2.11×10 <sup>-3</sup> )	-10.62 (7.70×10 <sup>-4</sup> )				
N-nitrite					
-55.53 (2.22×10 <sup>-3</sup> )					
		pseudo-R <sup>2</sup> = 0.451			
pseudo-R <sup>2</sup> = 0.328					

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<b>Scenario 2<sup>a</sup></b>	P-phosphate	b	b	b
	42.48 (2.80×10 <sup>-4</sup> )			
	Dissolved nitrogen			
	9.68 (1.00×10 <sup>-3</sup> )			
	pH			
	1.33 (6.60×10 <sup>-4</sup> )			
	Total nitrogen			
	-11.29 (3.50×10 <sup>-4</sup> )			
	pseudo-R <sup>2</sup> = 0.709			

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<sup>a</sup> *i.e.* scenario 2 highlighted in the results exposed above: we selected total *E. coli* abundances from poorly connected wetlands (*i.e.* W1, W3, W6, W11, W12, W13 and W16) during low-flow periods (*i.e.* May-October 2019).

<sup>b</sup> Not enough non-zero bacterial abundances: the model did not converge

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### **Transition**

Ce travail, centré sur la plaine alluviale de l'Ain, a permis de mieux comprendre les processus conditionnant le devenir des BGN d'origine humaine dans l'environnement. La contamination des écosystèmes alluviaux par les BGN-CP dépend majoritairement de l'hydromorphologie de l'hydrosystème considéré. Bien qu'elle semble minoritaire, la survie des BGN-CP dans certaines zones humides doit être considérée : les écosystèmes peuvent constituer des réservoirs secondaires d'espèces bactériennes pathogènes pour l'Homme. La forte disponibilité en azote et en phosphore favorise la survie des BGN-CP dans les écosystèmes alluviaux. Au-delà des conditions abiotiques étudiées dans ce chapitre, les interactions biotiques peuvent aussi jouer un rôle dans la survie des microorganismes dans l'environnement<sup>58,59</sup>. Le quatrième chapitre de ce travail s'intéressera à cela en étudiant l'interaction *E. coli*-végétation.

## *CHAPITRE 4*

# EFFET DES FACTEURS BIOTIQUES SUR LES BACILLES À GRAM NÉGATIF COMMENSAUX ET PATHOGÈNES : INTERACTION *E. COLI* – VÉGÉTATION



## 6. CHAPITRE 4 : EFFET DES FACTEURS BIOTIQUES SUR LES BACILLES À GRAM NÉGATIF COMMENSAUX ET PATHOGÈNES : INTERACTION *E. COLI* – VÉGÉTATION

**Titre** : Utilisation d'*Escherichia coli* marqué par la GFP pour étudier la persistance des bactéries fécales dans les zones humides végétalisées : une approche expérimentale

**Revue** : Antibiotics, volume 9

**État** : publié, 2020

### Éléments essentiels :


- Grâce à une méthode non-invasive, nous avons suivi l'évolution des abondances d'*E. coli* fluorescents *in vitro* dans des mésocosmes contenant quatre espèces d'angiospermes aquatiques (*Elodea canadensis*, *Mentha aquatica*, *Baldellia ranunculoides*, et *Sparganium emersum*) identifiés pour leur production de métabolites secondaires potentiellement bactéricides ;
- Nous avons inoculé *E. coli* dans l'eau et/ou le sédiment. Dans les conditions lentiques de nos expériences, les sédiments contaminés ne libéraient pas *E. coli* dans la colonne d'eau ;
- Les monocultures d'*E. canadensis*, *M. aquatica* et *S. emersum* ont réduit les concentrations d'*E. coli* dans la colonne d'eau, tandis que les polycultures n'ont pas eu d'effet sur les abondances d'*E. coli* ;
- Les espèces végétales aquatiques pourraient avoir un effet allélopathique sur les BGN-CP qui contaminent les eaux douces de surface et réduire leur survie.

## Résumé

La contamination des eaux de surface par des bactéries pathogènes d'origine humaine est un problème de santé publique important. Les zones humides peuvent être contaminées par des bactéries fécales contenues dans de l'eau provenant de différentes sources, telles que les STEU et l'agriculture. *E. coli* est un commensal de la flore intestinale humaine et le principal indicateur de contamination fécale des eaux de surface. On sait peu de choses sur l'association entre les bactéries fécales et les macrophytes immergés, et sur la façon dont cela peut influencer la qualité de l'eau. Nous nous sommes demandé si les macrophytes pouvaient favoriser ou inhiber la croissance des bactéries pathogènes associées à l'Homme dans les zones humides. Pour répondre à cette question, nous avons cultivé quatre espèces différentes de macrophytes (*Mentha aquatica*, *Baldellia ranunculoides*, *Sparganium emersum* et *Elodea canadensis*, en cultures mono- ou multi-spécifiques) dans des rhizotrons aquatiques, et inoculé les dispositifs avec une souche fluorescente d'*E. coli* (produisant une protéine fluorescente verte) pour simuler la contamination fécale des zones humides. La survie de *E. coli* a été suivie en mesurant la fluorescence pendant 19 jours. Nous avons constaté (i) que les sédiments contaminés ne libéraient pas *E. coli* dans la colonne d'eau dans des conditions lenticques et (ii) que les monocultures d'*E. canadensis*, *M. aquatica* et *S. emersum* réduisaient les concentrations d'*E. coli* dans la colonne d'eau. Cela suggère que ces espèces de plantes aquatiques peuvent être utilisées dans les zones humides artificielles pour réduire l'abondance des bactéries d'origine fécale dans l'eau douce de surface.

Article

# Using GFP-Tagged *Escherichia coli* to Investigate the Persistence of Fecal Bacteria in Vegetated Wetlands: An Experimental Approach

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Received: 20 May 2020; Accepted: 16 June 2020; Published: 18 June 2020



**Abstract:** The contamination of surface water by pathogenic bacteria of human origin is an important public health issue. Wetlands can be contaminated with fecal bacteria by water originating from different sources, such as wastewater treatment plants and agriculture. *Escherichia coli* is a commensal of the human gut flora and the major indication of fecal contamination in surface water. Little is known about the association between fecal bacteria and submerged macrophytes and how this may influence the water quality. We questioned whether macrophytes enhance or inhibit the bacterial growth in wetlands. For this purpose, we grew four different species of macrophytes (*Mentha aquatica*, *Baldellia ranunculoides*, *Sparganium emersum* and *Elodea canadensis*, in mono- or multispecies cultures) in aquatic rhizotrons and inoculated the devices with a fluorescent strain of *Escherichia coli* (producing a green fluorescent protein) to simulate the fecal contamination of wetlands. Bacterial survival was monitored by measuring the fluorescence for 19 days. We found (i) that contaminated sediments did not release *E. coli* in the water column in lentic conditions and (ii) that monocultures of *E. canadensis*, *M. aquatica* and *S. emersum* reduced the *E. coli* concentration in the water column. This suggests that aquatic plant species may be used in constructed wetlands to clear surface freshwater from bacteria of fecal origin.

**Keywords:** fecal bacteria; wetlands; macrophytes; antimicrobial compounds; *Escherichia coli*; GFP

## 1. Introduction

The pathogenic bacterial contamination of surface water is a major health risk for the human population and a threat to future water supplies [1,2]. New contemporary problems, such as antibiotic resistance, could aggravate the existing situation [3].

Fecal bacteria contaminating freshwaters originate from both punctual sources (e.g., wastewater treatment plants (WWTPs) [4], hospitals [5] and agriculture [6]) and nonpunctual ones (e.g., sewage sludge [7], manure [8], rainwater runoff [9] and percolation from fertilized fields [10]). All these sources contain pathogens that contribute to the fecal peril. Many transnational organizations (e.g., WHO and the European Union) set standards and guidelines for assessing the water quality and indicate *Escherichia coli* as a major indicator for fecal contamination [3]. This species is a commensal of human

and animal guts and is released into the environment through feces [11,12]. Once in the environment, the bacteria disperse and reach different ecosystems such as surface water, including wetlands, in which they potentially survive [13,14].

Natural wetlands have beneficial effects on the water quality, while increasing the habitat diversity and maintaining ecological sustainability [15,16]. However, very little is known about the survival of fecal bacteria in these environments. On the one hand, oxygenation of the rhizosphere by the vegetation, crucial for the survival of microorganisms, may promote the survival of fecal bacteria in wetlands [17]. On the other hand, the shade and the allelopathic compounds excreted by the vegetation may negatively affect fecal bacteria in wetlands [18,19]. Several experimental studies have shown that constructed wetlands vegetated with macrophytes efficiently reduce the bacterial load, outlining their potential bioremediation properties [20,21]. The mechanisms of bacteria clearance in these environments may be numerous: natural die-off, UV radiations on the cell wall of bacteria, protozoan predation, antimicrobial substances secreted by macrophyte roots, etc. [22,23].

Here, we aimed at determining experimentally the fate of bacteria of fecal origin in vegetated wetlands and the remediation potential of macrophytes that naturally colonize these wetlands, using *E. coli* as a model. Several studies were carried out on constructed wetlands [22,24,25], whereas, to the best of our knowledge, only one has investigated the potential of natural wetlands [26]. The subject remains poorly studied, and to our knowledge, no study has been aimed at comparing experimentally the efficiency of several macrophyte species colonizing natural wetlands at controlling fecal bacteria survival and multiplication.

To do so, we grew different macrophytes in rhizotrons, which species were selected by the demonstrated or potential production of the secondary compounds and antimicrobial activity. We then inoculated the rhizotrons with GFP-tagged *E. coli* (GFP-*E. coli*) and followed bacterial fluorescence for 19 days. This ex-situ study was carried out implementing nondestructive, cost-effective and simple methods to assess the spatiotemporal dynamics of the bacterial contamination without compromising the plants and rhizotrons settings.

## 2. Results

The bacterial concentration was monitored for 19 days in mixed-species, single-species and unplanted rhizotrons inoculated with a fluorescent GFP-*E. coli* strain. In all inoculated rhizotrons, fluorescence—and so, the concentration of bacteria—increased until reaching a peak in the third day after inoculation. The bacterial concentration started to decrease drastically around day six, until reaching values under the limit of detection (LOD) at day eight.

### 2.1. Influence of the Type of Inoculation in Bacterial Survival

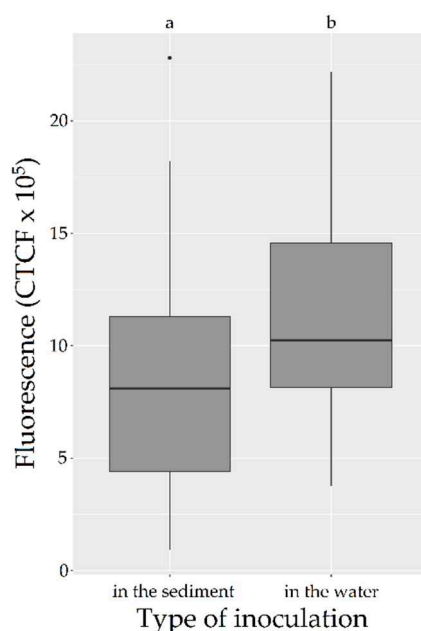
The limit of detection (LOD) was  $4.91 \times 10^5$  CTCF (corrected total cell fluorescence) for the mixed-species rhizotrons and  $4.07 \times 10^5$  CTCF for the unplanted ones. Only fluorescence from day one to day eight was detectable and, thus, considered.

The location of the bacterial inoculation (water or sediment) had an effect on the fluorescence measured in the rhizotron water (ANOVA on the 1-mm,  $p$ -value = 0.0061). The rhizotrons that were inoculated in the sediment ( $n = 6$ ; three with the mixture of four plants and three without plants; Table 1) showed less fluorescence than the ones inoculated in the water ( $n = 6$ ; three with the mixture of four plants and three without plants; Table 1) (Figure 1, Tukey's test on the 1-mm). No interactive effect between the presence/absence of plants and the type of inoculation was observed.

**Table 1.** Experimental design. Planted and unplanted rhizotrons inoculated with GFP-*Escherichia coli* in water or sediment.

Rhizotron Type	Plant Species	Type of Inoculation with GFP- <i>E. coli</i>		
		Water	Sediment	Control <sup>1</sup>
Rhizotrons planted with the four species together ( $n = 7$ )	<i>Baldellia ranunculoides</i> , <i>Elodea canadensis</i> , <i>Mentha aquatica</i> , <i>Sparganium emersum</i> together	$n = 3$	$n = 3$	$n = 1$
Rhizotrons without vegetation ( $n = 7$ )	∅ <sup>2</sup>	$n = 3$	$n = 3$	$n = 1$
Rhizotrons planted with one single plant species ( $n = 16$ )	<i>Baldellia ranunculoides</i>	$n = 3$	∅ <sup>3</sup>	$n = 1$
	<i>Elodea canadensis</i>	$n = 3$	∅ <sup>3</sup>	$n = 1$
	<i>Mentha aquatica</i>	$n = 3$	∅ <sup>3</sup>	$n = 1$
	<i>Sparganium emersum</i>	$n = 3$	∅ <sup>3</sup>	$n = 1$

<sup>1</sup> no bacteria inoculation. <sup>2</sup> no plants in rhizotrons without vegetation. <sup>3</sup> not tested.



**Figure 1.** Effect of the type of inoculation—in water ( $n = 6$ ) or in sediment ( $n = 6$ )—on the fluorescence of GFP-*Escherichia coli* in the rhizotrons. Boxplots of fluorescences measured on days 1, 2, 3, 6 and 8 (fluorescences > limit of detection (LOD)) in the water of rhizotrons inoculated with GFP-*E. coli*. Using an ANOVA with a multiple comparisons Tukey's test on the 1-mm, testing the effects of the type of inoculation on the fluorescence (i.e., bacterial concentration) of the water column. a and b are significantly different ( $p$ -value (ANOVA) < 0.01). CTCF: corrected total cell fluorescence.

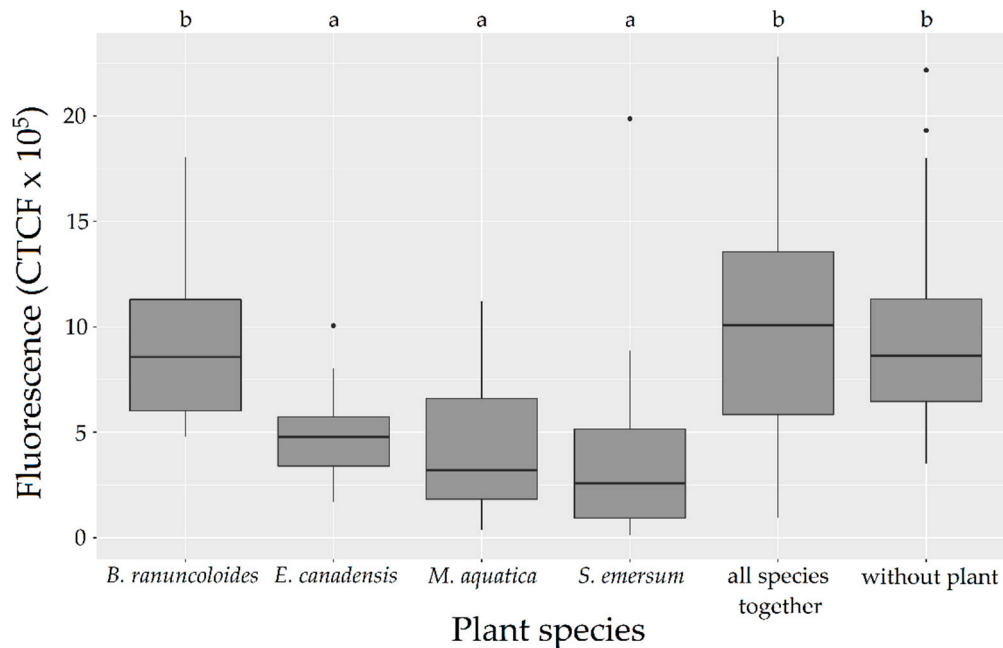
## 2.2. The Role of Vegetation and Plant Species in Bacterial Survival

The LOD was  $1.33 \times 10^5$  CTCF for *Baldellia ranunculoides* rhizotrons,  $1.57 \times 10^5$  CTCF for *Elodea canadensis* rhizotrons,  $1.89 \times 10^5$  CTCF for *Mentha aquatica* rhizotrons and  $1.07 \times 10^5$  CTCF for *Sparganium emersum* rhizotrons. Only fluorescence from the day one to day eight was detectable and, thus, considered.

The fluorescence did not differ between the rhizotrons without plants and those planted with the set of four species (ANOVA on the 1-mm,  $p$ -value = 0.89). For the single-species vegetated rhizotrons ( $n = 12$ ; four plant species in three replicates each; Table 1), the measured fluorescence differed between rhizotrons according to the species planted (ANOVA on the 1-mm,  $p$ -value = 0.000024). The rhizotrons containing *E. canadensis*, *M. aquatica* or *S. emersum* showed less fluorescence than those containing *B. ranunculoides*. In the same way, the measured fluorescence differed between rhizotrons planted with



*B. ranunculoides* ( $n = 3$ ; Table 1), *E. canadensis* ( $n = 3$ ; Table 1), *M. aquatica* ( $n = 3$ ; Table 1), *S. emersum* ( $n = 3$ ; Table 1), the mixture of these four species together ( $n = 3$ ; Table 1) and the ones without plants ( $n = 3$ ; Table 1) (ANOVA on the 1-mm,  $p$ -value =  $3.4 \times 10^{-10}$ ). The rhizotrons containing *E. canadensis*, *M. aquatica* or *S. emersum* were less fluorescent than those containing *B. ranunculoides*, the four tested species together (mixed-species rhizotrons) and those containing no plants (Figure 2; Tukey's test on the 1-mm).



**Figure 2.** Effects of *Baldellia ranunculoides* ( $n = 3$ ), *Elodea canadensis* ( $n = 3$ ), *Mentha aquatica* ( $n = 3$ ), *Sparganium emersum* ( $n = 3$ ), the mixture of these four species together ( $n = 3$ ) and the absence of plants ( $n = 3$ ) on the fluorescence of GFP-*E. coli* in the rhizotrons. Boxplots of fluorescences measured on days 1, 2, 3, 6 and 8 (fluorescences > LOD) in the water of rhizotrons inoculated with GFP-*E. coli*. Using an ANOVA with a multiple comparisons Tukey's test on the 1-mm, testing the effects of plant species on the fluorescence (i.e., bacterial concentration) of the water column. a and b are significantly different ( $p$ -value (ANOVA) < 0.001). The dots are outliers.

No difference of fluorescence was observed between the six mixed-vegetated rhizotrons according to their vegetation densities, as these were comparable (i.e., 50%, 50%, 50%, 75%, 75% and 25% in the six vegetated rhizotrons, measured the third day after inoculation).

We did not observe any fluorescent spot in the rhizosphere (mono- and poly-species rhizotrons) during the experiment.

### 3. Discussion

The aim of this study was to assess experimentally the fate of fecal bacteria in vegetated wetlands and their remediation potential according to the vegetation. To do so, we followed the fluorescence of a GFP-*E. coli* in an experimental model.

In all rhizotrons, we observed an increase of fluorescence in the first days following inoculation, maybe because the bacteria were inoculated with their growth broth. Between days three and eight, even if *E. coli* is mobile, we can assume the sedimentation of the bacteria, since there was no flow and since the measured fluorescence increased in the lower part of the rhizotrons [27,28]. Fluorescence was under the LOD from day eight. We first hypothesized that the bacteria lost the plasmid that borne the GFP-encoding genes, as already reported [29]. To test this possibility, we sampled water aliquots from each rhizotron at day eight and streaked them on a growing medium (Mueller-Hinton agar) further

incubated overnight at 37 °C. Both fluorescent and nonfluorescent *E. coli* grew, but the fluorescent *E. coli* largely dominated (species identification checked with a MALDI-TOF MS). The decay of fluorescence thus rather indicated that most GFP-*E. coli* did not survive in the rhizotrons after day eight. The specific conditions of the rhizotrons (i.e., no flow, no oxygenation of the sediment, no nutrient income and use of tap water) probably accounted for the poor survival of GFP-*E. coli*.

This study aimed to test the capability of natural wetlands to remove bacterial contaminations through vegetation, depending on the source of contamination (water column or sediment). Concerning the source of contamination, the bacterial fluorescence was lower in the water column of rhizotrons inoculated in sediments compared to those inoculated in the water. On the one hand, bacteria found nutrients and protection against predators in the sediment [30]. On the other hand, some studies observed a rapid decay of bacteria in the sediment due to the lack of dissolved oxygen and the absence of water column mixing, as it may be the case in the present experiment [29]. A substantial oxygen concentration in the sediment and water is crucial for a sufficient and visible (in terms of brightness) GFP-gene expression of the transformed plasmid (to allow visual identification within a fine sediments matrix [29]). Thus, the lentic condition of this experiment resulted firstly in a decay of the detected fluorescence and, later, (possibly due to a lack of nutrients) in bacterial population numbers [31]. As the nondestructive method used in this work did not allow to monitor the survival of bacteria in the sediment, it is not possible to conclude on the bacteria survival in this compartment. The present results suggest that the fecal bacterial load, for the most part, was not transferred from the sediment to the water column, either because of habitat preference or poor survival. This suggests that, under lentic conditions, the fecal contamination of the sediment may not significantly contribute to the contamination of the water column in natural wetlands [32]. Moreover, no fluorescent spot was observed near the roots on the inclined side of the planted rhizotrons. This could suggest either that bacteria did not survive in the sediment [33] or that the rhizosphere is not a favorable habitat for fecal bacteria, contrary to what other studies suggested [34].

Concerning the effect of vegetation, we found that monocultures of *E. canadensis*, *M. aquatica* and *S. emersum* reduced the survival of GFP-*E. coli*, whereas monocultures of *B. ranunculoides* and four-species cultures had no effect (Figure 2). *E. canadensis*, *M. aquatica* and *S. emersum* produced secondary compounds [35–37]. In contrast, secondary compounds of *B. ranunculoides* are poorly known [38,39]. The acetone extract from *S. emersum* (made of flavonoids, essential oils, phenylpropanoid glycosides and aromatic alkenes) has a high antimicrobial effect, which may account for the observed decay of bacteria [36]. Previous observations already indicated a negative relationship between *Elodea* and algae or cyanobacteria, and this species has been reported to have a medium allelopathic activity [40]. *Elodea* species contain diglucuronides of the flavones luteolin, apigenin and chrysoeriol and a yet-unidentified phenolic acid similar to caffeic acid [41], and various flavonoids have been demonstrated to inhibit the growth of Gram-negative bacteria [42]. *Mentha aquatica* benefits from a wider knowledge on its role, being largely used in constructed wetlands [43]. While analyzing its essential oils, a total of 34 compounds have been identified. Menthofuran (70.5%) was characterized as the main component, with limonene and pmenthone constituting 9.42% and 7.20% of the oil [44]. The results of the current research revealed that the essential oils exhibited moderate antibacterial effects against the microorganisms—in particular, against Gram-positive bacteria [45,46]. On the other hand, not much is known about *B. ranunculoides*, and the study of its biochemistry diversity is still in its early stages. All *Baldellia* spp. have very strong coriander-like smells whose chemical composition has not yet been studied [47]. It is therefore unclear, any chemical affinities with the aldehydic smell of *Coriandrum sativum* (*Apiaceae*). On the other hand, species of *Alisma* (closely related to *Baldellia* spp.) are reported to produce phenolic glycosides with caffeic acid, chlorogenic acid and quercetin [48]. The content of *B. ranunculoides* extracts are yet to be identified and tested on microbial activity. Even if the observed reduction in the bacterial concentration was rather low compared to conventional remediation processes used in water treatments, for example, the results suggest that *E. canadensis*, *M. aquatica* and *S. emersum* could be used in constructed wetlands for their capacity to

limit bacterial survival and/or multiplication. However, more information is still needed for identifying the identity and quantity of allelopathically active compounds involved and on the factors influencing their production and release by macrophytes. Several studies suggest that the allelopathic effect is also very specific and deeply connected to the macrophyte species and its characteristics [33]. Indeed, plant exudates have the potential to act directly either on resources, competition or chemical interferences between plants and play a direct role in allelopathy mediation [36]. Furthermore, the effects of these secondary compounds on the rhizosphere, hosting bacterial communities essential to the development of vegetation, need to be better clarified.

These results must be nuanced, because multispecies and unvegetated rhizotrons did not show a significant difference in terms of bacterial survival. There is still no clear evidence that, by combining different species with known effects, it would result in a better treatment efficiency for a single contaminant compared to a treatment implying a monoculture [49]. As of now, there is no consistent accordance favoring a monoculture, despite mixed wetlands. Indeed, some studies have indicated that species competition may affect the nutrient removal and vegetation stability in constructed wetlands [50]. Mixed wetlands can be more or less efficient than monocultures, depending on the plant species (growth speed, biomass, competition ability and stubble growth attributes) used in both wetlands and on whether the mixed community can maintain a relatively stable state [51].

It is also possible that the concentration of a given exudate may need to reach a certain value to be effective. This could have implied that the concentrations of the secondary compounds did not reach a breakthrough concentration in the mixed rhizotrons. Monospecies environments may be more efficient, because the allelopathic compound would be much more abundant.

The noninvasive method used in this experiment allowed monitoring the bacterial concentration in the water column of the different rhizotrons for 19 days, without using the classical microbiology technics (more expensive, longer, laborious and potentially invasive). Nevertheless, on the one hand, this method has made it impossible to monitor the bacterial concentration in the sediment. This issue needs to be further explored to conclude on the potential role of the macrophyte rhizosphere as a reservoir of fecal bacteria. On the second hand, the method used in this work did not make it possible to investigate the potential shelter role of plants for bacteria (fixation and formation of biofilm on the surface of submerged parts and presence in vegetal tissues), as presented in several studies. Finally, while this experimental study allowed a better understanding on the fate of *E. coli* in vegetated wetlands, interactions between fecal bacteria and the natural bacterial community present in this habitat should be further investigated for a deeper perspective of fecal bacteria fate in natural wetlands.

It is desirable, in view of their relevance in bioremediation, to focus future studies not only on separate plant species as isolated individuals but, also, on considering the effects of different species as an interconnected whole and the complexity of their allelopathic effects. This knowledge is important for understanding the present existing mechanisms in natural wetlands that can drive the survival or removal of fecal bacterial populations and pollutants' fate and to face the environmental bacterial contamination with effective remediation strategies.

## 4. Materials and Methods

### 4.1. Ecosystem and Conditions of Reference

To ensure that the experimental conditions were as close as possible to the natural ones (sediment composition, plants and temperature), we referred to natural wetlands of the Ain River in the Jura Massif (France) that have already been studied for bacterial contamination [14]. Rhizotrons were filled with artificial sediment prepared according to the sediment composition of six natural wetlands of the Ain River and those referred in the literature [52,53]. Hence, the sediments used were composed by 70% silty sand (supplied by a local quarry from the Ain River floodplain), 15% clay (kaolinite) and 15% organic matter ( $190 \text{ g}\cdot\text{m}^{-3} \text{ N}$ ,  $100 \text{ g}\cdot\text{m}^{-3} \text{ P}_2\text{O}_5$  and  $60 \text{ g}\cdot\text{m}^{-3} \text{ K}_2\text{O}$ ). The mixture was poured in each

rhizotron until covering 10 cm of the box height. Then, 2.5 L of tap water was gently poured, and the rhizotrons were left to settle for 48 h before plant transplant.

Four macrophyte species have been transplanted in the rhizotrons. Three species were chosen based on their abundance in natural wetlands and their referred potential to affect the bacterial community or to product the active secondary metabolites: *E. canadensis* [40], *M. aquatica* [43] and *S. emersum* [37]. The fourth species was *B. ranunculoides* because of its abundance in natural wetlands and to test its unknown effects on fecal bacteria [39,40]. The plants were collected in wetlands of the Ain River (*M. aquatica*, 46°00'36.3" N 5°17'59.3" E; *B. ranunculoides*, 45°58'51.1" N 5°16'57.4" E and *S. emersum* and *E. canadensis*, 45°58'04.7" N 5°17'37.7" E). Plants were stored at 5–8 °C for the time to transport them from the field to the laboratory (5 h), washed and transferred into the rhizotrons. The experiment room was kept at 18 °C to reflect the water temperature of natural wetlands.

## 4.2. Experiment Set up

### 4.2.1. Rhizotrons

Thirty rhizotrons (polystyrene boxes of 27 cm H × 20 cm L × 10 cm W) were filled by 10-cm height of sediment. For comparing the effect of vegetation versus no vegetation on the bacterial success, we planted the four macrophyte species together (one individual per species) in seven rhizotrons: three were inoculated with GFP-*E. coli* in the water, three were inoculated with GFP-*E. coli* in the sediment and one served as a control (mixed-species vegetated but not inoculated with GFP-*E. coli*) (Table 1). We also prepared 7 rhizotrons without vegetation: three were inoculated with GFP-*E. coli* in the water, three were inoculated with GFP-*E. coli* in the sediment and one served as a control (not vegetated and not inoculated) (Table 1). Finally, for studying the effect of a given species on the bacterial success, 16 rhizotrons were planted with four individuals of each of the four different species of macrophytes (four rhizotrons per species—among which, one was not inoculated and served as a control) (Table 1). We only tested the effects of the inoculation (water versus sediment) for mixed-species and unvegetated rhizotrons (Table 1). The rhizotrons were inclined at 30° to observe the possible driving effects of the rhizosphere on the bacterial community [54,55]. The external part of the rhizotrons corresponding to the sediment layer was covered with black plastic film to reproduce natural dark conditions that prevail in natural sediment. We applied a 12-h photoperiod with a growth lamp with blue led light at 30 W (Digyssel, Guangzhou Hua Dun Electronic Technology Co. Ltd., Guangdong, China).

In order to verify that plant size did not differ significantly between replicates, and to assess the possible effect of plant size on bacterial survival, we measured the density of vegetation at the beginning of the experiment and three days after inoculation using 5 classes of density (0%, 25%, 50%, 75% and 100%).

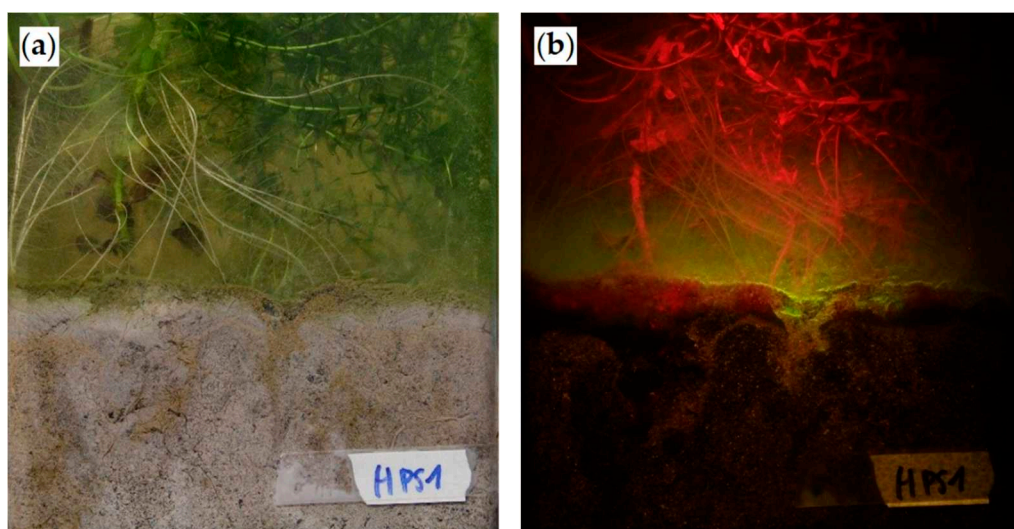
### 4.2.2. Inoculation with GFP-*E. coli*

We used a fluorescent and nonpathogenic strain *E. coli* GFP ATCC® 25922GFP™ (American Type Culture Collection, Manassas, VA, USA). It harbors a multicopy vector encoding the green fluorescent protein GFPmut3. The GFPmut3 fluorophore has an excitation maximum at 501 nm and an emission maximum at 511 nm, compatible with usual fluorescence detection technologies. The plasmid-borne *bla* gene confers the resistance to 100 µg/mL of ampicillin. This *E. coli* strain has been grown in a Luria-Bertrani (LB) broth overnight at 37 °C with gentle shaking to reach the growth plateau (~109 CFU/mL). Then, an aliquot of the culture of 6 mL with a concentration of  $2.4 \times 10^6$  CFU/mL was inoculated in rhizotrons via a sterile pipette for water inoculation and via sterile syringe connected to a tube to reach a 5-cm depth in sediments to simulate contaminations from the surface water and groundwater, respectively. To make the strain visible, it was excited at each measure by lightening the rhizotrons with a FastGene® Blue/Green LED Flashlight (Nippon Genetics Europe GmbH, Dueren, Germany) emitting at 480–530 nm.

### 4.3. Image Analysis

#### 4.3.1. Camera and Settings

Unmodified digital single-lens reflex (DSLR) camera can be a successful tool for detecting fluorescence while being cost-effective, applicable to a wide range of studies and easy to use for the operators [56,57]. A DSLR camera Canon model Eos 7D (Canon Europe N.V., Amstelveen, The Netherlands) on a tripod with an amber filter on the front was used. A black cardboard was placed behind the photographed rhizotron in order to avoid reflection from the background. Each day of measurement, the rhizotrons were photographed in light and total dark conditions while enlightening them with green UV flashlight to spot fluorescence (Figure 3). To ensure the same lighting conditions, the camera and the lamp were kept each time at the same distance from the photographed rhizotron. Camera settings were kept the same throughout the whole duration of the experiment (1/50 s f/4 29 mm ISO 320 for light conditions and 13 s f/18 41 mm and ISO 800 for detecting fluorescence). Photos were taken on days 1, 2, 3, 6, 8, 10, 14 and 19 after inoculation. The limit of detection (LOD) was calculated for each different condition measuring the fluorescence in the control rhizotrons. Fluorescence readings lower than the LOD were removed from the analysis.



**Figure 3.** Example of planted rhizotrons after the inoculation of fluorescent *E. coli* (3rd day after inoculation). (a) Rhizotron under natural light. (b) Rhizotron under fluorescence conditions (enlightened only with a 480–530-nm light and observed with an amber filter). Photo obtained with an exposition of 13 s f/18 and ISO 800 with a Canon EOS 7D.

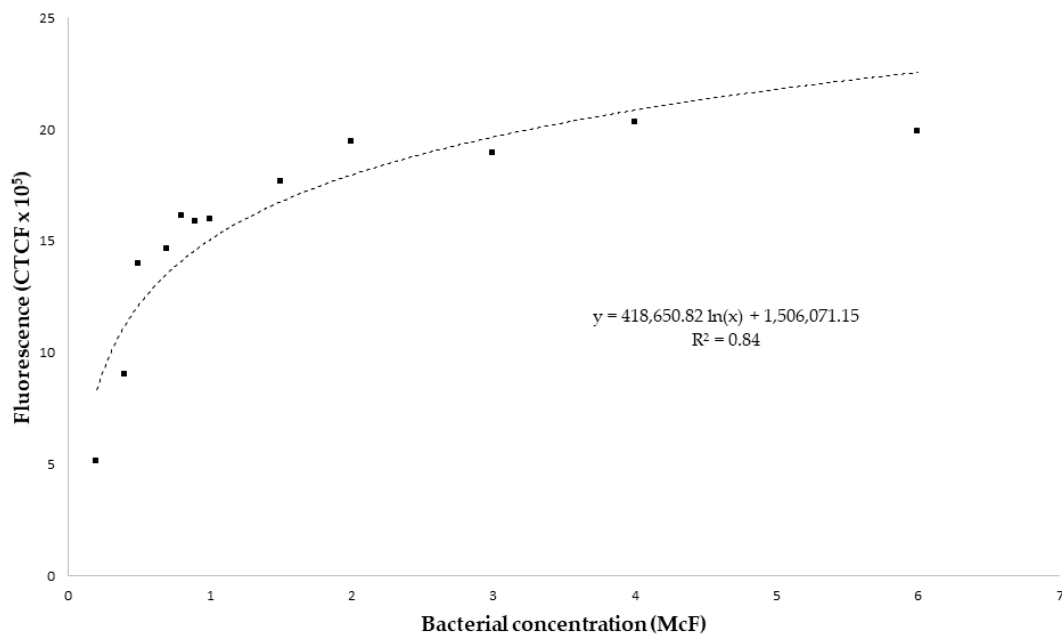
#### 4.3.2. Image Analysis

The images capturing fluorescence were treated with ImageJ2™ (National Institute of Health, Bethesda, MD, USA). This has been shown to be a useful tool for fluorescence intensity analysis [58,59]. The pictures were first converted as an 8-bit grayscale image. The colors channels were split in order to keep only the green fluorescence emitted from bacteria, reducing the noise from the red fluorescence emitted by plants and algae present in the rhizotrons. Noise from burned pixels in the image was also corrected and treated. Then, the CTCF was calculated by selecting three measurement areas (triplicates) with a mean of 20,380.3 square pixels in the water-sediment interface level. We then took three measurements of a mean area of 7318.6 square pixels of the nearby background to correct the fluorescence and calculate the CTCF as follows [60,61]:

$$\text{CTCF} = \text{Integrated density} - (\text{area of selected fluorescence} \times \text{means fluorescence of the backgrounds})$$

### 4.3.3. Calibration Curve

Increasing the concentration of GFP-*E. coli* was prepared in different vials (0.2, 0.4, 0.5, 0.7, 0.8, 0.9, 1, 1.5, 2, 3, 4 and 6 McF) and photographed them three times under the same conditions as the rhizotrons. The CTCF for each vial was calculated as described above, and we deduced a calibration curve between the retrieved CTCF and GFP-*E. coli* concentration (Figure 4). We obtained a curve of 12 points and with a  $R^2$  of 0.84 (Figure 4). The lowest concentration measured was 0.25 McF, which yielded  $5.12 \times 10^5$  CTCF, and the highest value measured was for 4 McF, even though it was not the highest bacterial concentration (6 McF), which yielded at  $2.03 \times 10^6$  CTCF.



**Figure 4.** Fitting curve between the fluorescence measurements (CTCF) and the bacterial concentration (McF). Twelve vials with known concentrations of GFP-*E. coli* (0.2, 0.4, 0.5, 0.7, 0.8, 0.9, 1, 1.5, 2, 3, 4 and 6 McF) were photographed three times under the same conditions as the rhizotrons. The values presented are the means of these three measurements for each vial.

### 4.4. Statistics

The normality and homoscedasticity of the data were tested using the Shapiro-Wilk test and Bartlett's test, respectively. The dataset was square-root (sqrt)-transformed for reaching normality.

Four linear mixed-effects models (lmm) were performed, putting a random effect on the day of sampling to avoid a time-dependent artefact. The first lmm tested the effect of the type of inoculation (water versus sediment) on the fluorescence measured in the rhizotrons. The second lmm tested the effect of the four species together (mixed-species rhizotrons) against the absence of plants on the fluorescence measured in the rhizotrons. The third lmm tested the effect of the four plant species tested individually (single-species rhizotrons) on the fluorescence measured in the rhizotrons. An overall lmm, including the four single-species treatments, the mixed-species treatment and the unplanted rhizotrons were also assessed. An ANOVA was then performed on each lmm, followed by a Tukey's test. The  $\alpha$  value was set to 0.05. All analyses were performed with R 3.6.1 software [62].

## 5. Conclusions

In conclusion, the experiment demonstrated that aquatic plant species may play a role in the survival of bacteria of fecal origin that contaminates surface freshwater. In the lentic conditions of our experiments, we found (i) that contaminated sediments did not release *E. coli* in the water column in

lentic conditions and (ii) that monocultures of *E. canadensis*, *M. aquatica* and *S. emersum* reduced the *E. coli* concentration in the water column.

**Author Contributions:** Conceptualization, E.C., C.P.H. and G.B.; methodology, E.C., C.P.H., D.H., X.B. and G.B.; validation, D.H. and G.B.; formal analysis, C.P.H.; investigation, E.C., C.P.H. and G.B.; data curation, E.C. and C.P.H.; writing—original draft preparation, E.C. and C.P.H.; writing—review and editing, E.C., C.P.H., D.H. and G.B.; supervision, D.H. and G.B.; project administration, G.B. and funding acquisition, G.B. All authors have read and agreed to the published version of the manuscript.

**Funding:** This work was supported by the Mission for Interdisciplinarity of the National Centre for Scientific Research (CNRS; France).

**Conflicts of Interest:** The authors declare no conflict of interest.

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### **Transition**

Ce dernier chapitre nous a permis d'évaluer les interactions *E. coli*-végétation *in vitro*. Certaines espèces de macrophytes semblent capables de sécréter des composés secondaires produisant un effet allélopathique responsable de la réduction de la survie des BGN-CP. Les conditions biotiques des écosystèmes peuvent donc influencer le devenir des BGN d'origine humaine dans l'environnement. Au-delà de la végétation, le microbiome autochtone pourrait également modifier le devenir des BGN-CP dans les écosystèmes, comme supposé dans le chapitre 3 et développé plus tard dans la discussion transversale.



## 7. DISCUSSION TRANSVERSALE

### 7.1. Bacilles à Gram négatif commensaux et pathogènes et *One Health* dans les pays à revenus élevés

#### 7.1.1. Les bacilles à Gram négatif commensaux et pathogènes dans l'environnement

##### Origines

Comme largement démontré jusqu'à présent, nos résultats suggèrent que les deux sources principales de contamination de l'environnement par les BGN-CP sont les effluents des STEU et les activités agricoles<sup>44,20,45,48,19</sup> (chapitres 2 et 3). Ces deux sources contaminent l'aval des cours d'eau et de leur plaine d'inondation. Au-delà des sources de contamination, nous avons recherché l'origine des souches retrouvées dans les écosystèmes alluviaux (chapitres 2 et 3). Premièrement, la spatialité (*i.e.* en aval d'une STEU) et la temporalité (*i.e.* durant les périodes de hautes eaux) militent largement dans le sens d'une contamination des hydrosystèmes par les eaux usées des STEU. De surcroît, la structure de population des BGN-CP retrouvées dans les plaines alluviales confirme que la contamination environnementale est majoritairement d'origine humaine. En effet, dans les approches intra- et inter-hydrosystèmes (chapitres 2 et 3), les phylogroupes A, B1 et B2 prédominaient chez les isolats d'*E. coli* non producteurs de BLSE. Chez les isolats d'*E. coli* producteurs de BLSE, les phylogroupes B2 et A étaient majoritaires. Le phylogroupe A est plutôt commensal, alors que le phylogroupe B2 est associé à des isolats plutôt pathogènes<sup>30,60</sup>. Le typage MLST des isolats B2 et D de l'étude inter-hydrosystèmes (chapitre 2) a confirmé leur origine humaine. En effet, une part importante de ces isolats appartenait à des STs responsables d'infections chez l'Homme (ST10, ST12, ST38, ST69, ST73, ST95, ST131)<sup>31</sup>. Le gène *bla*<sub>CTX-M-15</sub> était le plus retrouvé dans les deux approches (inter- et intra-hydrosystèmes, chapitres 2 et 3). Le gène *bla*<sub>CTX-M-15</sub> est également le plus fréquent chez les *E. coli* producteurs de BLSE responsables d'infection chez l'Homme<sup>61,62</sup>. Finalement, le typage plasmidique des isolats d'*E. coli* totaux et d'*E. coli* producteurs de BLSE collectés dans l'hydrosystème Ain (chapitre 3) a montré que la plupart d'entre eux possédaient un plasmide IncF, prévalant chez les isolats d'*E. coli* d'origine humaine<sup>63</sup>. Tous ces résultats confirment que les écosystèmes aquatiques alluviaux sont contaminés par des bactéries fécales dont l'origine humaine est prédominante.

Une minorité des souches retrouvées dans les plaines alluviales semble avoir une origine animale. Nous avons en effet observé une augmentation des abondances de BGN-CP en aval d'une exploitation agricole (chapitre 3)<sup>64,65</sup>. De plus, certains éléments de la structure des populations des isolats d'*E. coli* retrouvés dans les écosystèmes alluviaux (phylogroupe B1, gène *bla*<sub>CTX-M-1</sub>, Inc11) suggèrent une origine animale<sup>66,67</sup>, minoritaire, de la contamination environnementale par les BGN-CP. Il convient tout de même de noter que l'origine des BGN-CP dépend de la plaine alluviale considérée et des activités anthropiques qui y sont présentes. Les conclusions précédentes résultent des études des plaines alluviales de l'Ain, du Doubs et de la Loue, peu influencées par les contaminations des élevages intensifs, qui sont largement connus pour disperser BGN-CP, BRA et GRA dans leur environnement proche, comme décrit dans le Wisconsin (USA)<sup>68</sup>, entre autres<sup>64,65,69</sup>.

### **Dispersion**

Au sein d'un hydrosystème donné, nous avons observé que la rivière est généralement plus contaminée que les autres masses d'eau car elle reçoit la majorité des effluents de STEU (chapitres 2 et 3)<sup>44,43</sup>. Comme nous l'avons démontré grâce à l'étude de la plaine alluviale de l'Ain (chapitre 3), la dispersion des BGN-CP dans les plaines alluviales dépend de deux facteurs principaux : (i) la variation temporelle du débit de la rivière principale, et (ii) la géomorphologie de la plaine alluviale, régissant la connectivité entre les différents écosystèmes aquatiques (et contrôlant les fréquences et le type de connexion entre les zones humides, le cours d'eau et les nappes). Plus le débit de la rivière principale est élevé, plus la surface inondée est grande, et plus la proportion d'écosystèmes alluviaux soumis aux débordements est importante. De plus, le sous-écoulement des cours d'eau joue probablement un rôle important. Les écosystèmes les plus intensément et/ou fréquemment connectés à la rivière principale sont logiquement les plus exposés à la contamination par les BGN d'origine humaine.

En revanche, nous avons également mis en évidence les processus de dilution et la faible survie des BGN-CP dans les rivières (chapitres 2 et 3), réduisant les concentrations en aval des points de contamination<sup>70</sup>. La plupart du temps, dans la plupart des écosystèmes, les BGN-CP semblent peu survivre dans l'environnement. De manière générale, les processus de dispersion jouent un rôle plus important que les processus de survie dans la contamination des écosystèmes par les BGN-CP.

## Survie

Les zones humides alluviales isolées mais connectées en périodes de hautes eaux peuvent favoriser la survie des BGN-CP (chapitre 3). En effet, leur survie semble favorisée dans ces écosystèmes aquatiques plutôt oligotrophes, isolés et peu profonds en période d'étiage. La modélisation des relations existantes entre les abondances des BGN-CP et certains paramètres abiotiques *in situ* a mis en évidence que l'augmentation de la disponibilité en nutriments, notamment du phosphore et de l'azote, étaient associées positivement à l'abondance des BGN-CP dans ces habitats spécifiques. Bien que nécessitant des conditions particulières, les BGN d'origine humaine semblent donc capables de survivre, voire de se multiplier dans certains écosystèmes. La survie des BGN-CP a également été identifiée dans différents compartiments des écosystèmes dans la littérature, notamment les sédiments<sup>71,72,73</sup> et les sols<sup>46,47,74</sup>, dont la rhizosphère<sup>75</sup>.

Les interactions biotiques entre les BGN-CP et la végétation aquatique ont également été abordées. Nous avons ainsi montré que certaines espèces de macrophytes pourraient avoir un effet allélopathique sur les BGN-CP, conduisant à la réduction plus rapide des abondances d'*E. coli* par rapport aux rhizotrons témoins (chapitre 4). De plus, les BGN-CP semblent défavorisés dans les écosystèmes les plus eutrophes (chapitre 3). Ceci pourrait être dû à des interactions biotiques de type prédation (*e.g.* par des protozoaires) ou compétition (*e.g.* avec des espèces microbiennes à croissance plus lente, peu présentes dans les écosystèmes oligotrophes)<sup>76,77</sup>. Les communautés microbiennes potentiellement moins diversifiées, moins abondantes, ou plus spécialisées des zones humides oligotrophes pourraient ne pas nuire significativement à la survie et à la multiplication des BGN-CP dans ces milieux. Au contraire, dans les zones humides plus eutrophes, riches en nutriments essentiels pour les bactéries, les espèces bactériennes environnementales pourraient entrer en compétition avec les BGN-CP et affecter négativement leur survie.

Bien que les BGN-CP semblent généralement faiblement survivre dans les écosystèmes alluviaux, notre travail a identifié plusieurs paramètres biotiques et abiotiques influençant leur survie dans l'environnement (chapitres 2, 3 et 4). En revanche, la comparaison des STs entre isolats d'*E. coli* environnementaux, cliniques et d'effluents de STEU (chapitre 2), et la comparaison des groupes d'incompatibilité plasmidiques entre les différents écosystèmes alluviaux (chapitre 3) n'a pas permis de ségréguer des populations bactériennes spécifiques. Aucune sous-population d'*E. coli* génétiquement distincte n'a été identifiée dans les plaines alluviales. Ainsi, les processus de dispersion et de survie semblent prédominer par rapport à l'adaptation de sous-populations bactériennes dans les écosystèmes. Afin de s'en assurer, des

analyses plus approfondies de la structure des populations devront être menées, comme développé dans les perspectives de cette discussion transversale.

### **7.1.2. État écologique des écosystèmes et contamination**

Le concept *One Health* suppose que la santé des écosystèmes (*i.e.* état écologique) joue un rôle sur les santés humaine et animale. L'état écologique des écosystèmes conditionne-t-elle en partie la diffusion des BGN-CP ? Cette hypothèse n'a pas explicitement été testée. Néanmoins, nous pouvons ici apporter quelques éléments de réponse. Nos résultats ont montré que la géomorphologie et l'hydrologie jouent donc un rôle prédominant dans la dispersion des BGN-CP dans l'environnement (chapitres 2 et 3). En revanche, nous avons montré que suivant l'espèce bactérienne, les zones humides eutrophes et se réchauffant en été (*K. pneumoniae* ; chapitre 2), et les zones humides oligotrophes isolées présentant une disponibilité en nutriments relativement importante (*E. coli* ; chapitre 3) sont à même de constituer des réservoirs environnementaux pour les BGN-CP. La physico-chimie et l'hydromorphologie des écosystèmes distribués le long des cours d'eau permettent de caractériser l'état écologique des écosystèmes alluviaux<sup>78</sup> ; ces deux paramètres conditionnent également la dispersion et la potentielle survie des BGN-CP dans ces écosystèmes. L'état écologique des systèmes alluviaux et leur contamination par les BGN-CP semblent donc liés.

La concentration en nutriments de l'eau des écosystèmes, et plus particulièrement en azote et en phosphore, est positivement liée à l'abondance des BGN-CP, suggérant que de tels milieux pourraient être favorable à la survie et à la multiplication de ces BGN dans l'environnement<sup>73,79,80</sup>. Au-delà des BGN-CP, les modifications fonctionnelles liées à l'eutrophisation peuvent affecter la dynamique des maladies à transmission vectorielle<sup>81</sup>. Outre l'eutrophisation, la présence d'herbicides et de fongicides peut également favoriser la survie des BGN-CP dans l'environnement en modifiant les paramètres biotiques des écosystèmes, telles que les densités de protozoaires (*i.e.* prédateurs) et de bactéries autochtones (potentiellement compétitrices)<sup>82</sup>.

### **7.1.3. Les bacilles à Gram négatif commensaux et pathogènes : de l'environnement à l'humain ?**

Les principaux résultats de notre travail sur l'origine, la dispersion et la survie des BGN d'origine humaine dans les hydrosystèmes ont été discutés ci-dessus. Comment ces résultats éclairent-ils l'approche *One Health* ?

### **La difficile évaluation du risque**

Le risque est le résultat de l'exposition à un danger. Ici, nous souhaiterions estimer le risque de contamination par une espèce bactérienne pathogène *via* l'environnement. La contamination environnementale par les BGN-CP constitue le danger, alors que l'exposition est la perméabilité entre les compartiments "environnement" et "humain". Or, l'estimation de ce risque dépend de la transmissibilité, de la virulence, de la pathogénicité ou encore de la résistance aux antibiotiques de la souche considérée<sup>83</sup>. Nous n'avons pas abordé dans ce travail le risque associé aux BGN-CP présentes dans l'environnement. Les études ayant évalué ce risque sont quasiment inexistantes. C'est le cas de Leonard *et al.*<sup>84</sup> qui ont estimé que les surfeurs avaient 3 à 4 fois plus de risque d'être porteur d'un *E. coli* résistant aux C3G comparé à des individus non-surfeurs. Ce risque plus élevé est attribué à l'exposition plus grande des surfeurs aux BRA et GRA du compartiment "environnement" (*i.e.* le milieu aquatique marin) que des non-surfeurs. Mughini-Gras *et al.*<sup>85</sup> n'a pas permis d'évaluer un risque à proprement parler, mais a en revanche permis d'estimer les contributions de différentes sources au portage communautaire d'*E. coli* producteur de BLSE, comme discuté dans le premier chapitre.

Au vu des raisons exposées ci-dessus, la "probabilité d'exposition environnementale aux BGN-CP" est préférée au risque à proprement parler.

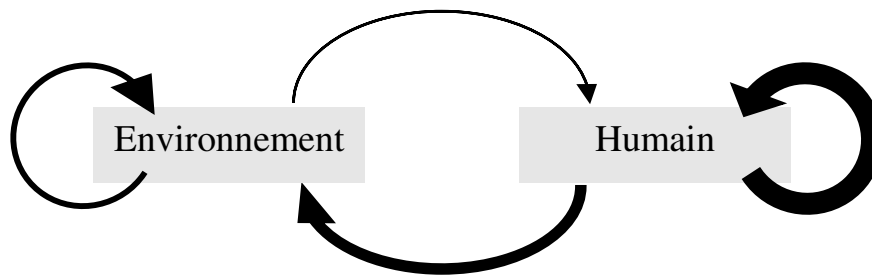
### **La probabilité d'exposition environnementale aux bacilles à Gram négatif commensaux et pathogènes**

Comme décrit précédemment, les BGN d'origine humaine sont présents, parfois en abondance, dans les écosystèmes alluviaux. En effet, la proportion de souches d'*E. coli* appartenant aux phylogroupes D et surtout B2 – plus souvent associé aux infections chez l'Homme – était élevée dans les plaines alluviales étudiées (chapitre 2 et 3), suggérant une probabilité d'exposition environnementale à ces pathogènes pour les populations humaines alentours. La dispersion des BGN-CP dans les hydrosystèmes est restreinte dans l'espace et dans le temps (*i.e.* faible survie générale ; chapitre 3), limitant ainsi la probabilité d'exposition environnementale aux BGN-CP et de retro-contamination des populations humaines. Par exemple, les très faibles concentrations des BGN-CP dans les eaux souterraines limitent la probabilité d'exposition des populations *via* la consommation d'eau provenant des nappes phréatiques. Cette probabilité d'exposition est d'autant plus faible que les eaux destinées à la consommation humaine sont en très grande majorité traitées avant distribution dans le réseau d'eau potable dans les PRE. De plus, la dilution des abondances des BGN-PC dans les écosystèmes alluviaux limitent la probabilité d'exposition environnementale (chapitre 3). Néanmoins, nous avons démontré que certains



écosystèmes peuvent constituer des réservoirs environnementaux pour les BGN-CP (chapitre 3). Les populations humaines peuvent donc être exposées à ces BGN lorsqu'elles entrent en contact avec les BGN-CP contenues dans ces réservoirs secondaires, *via* des activités telles que la pêche (manipulation ou consommation de poissons contaminés)<sup>86</sup> ou la baignade (ingestion d'eau contaminée)<sup>84,87</sup>.

Le typage MLST n'a pas distingué génétiquement les populations de BGN-CP communautaires, cliniques et environnementales (chapitre 2). Ces résultats vont dans le sens d'une forte contamination du compartiment "environnement" par les BGN d'origine humaine. Les compartiments "humain" et "environnement" ont une perméabilité asymétrique dans les PRE : les pathogènes humains sont aisément capables de passer du compartiment "humain" au compartiment "environnement", comme nous l'avons confirmé dans ce travail (chapitres 2 et 3)<sup>44,20,45,48,19</sup> ; l'inverse est en revanche moins probable (Figure 3). En effet, Mughini-Gras *et al.*<sup>85</sup> ont estimé que l'environnement n'était à l'origine que de 2,6 % du portage communautaire d'*E. coli* producteur de BLSE et de pAmpC aux Pays-Bas, loin derrière la transmission interhumaine (67,0 %). Cependant, dans cette même étude, les auteurs ont démontré que l'accroissement de la perméabilité entre les compartiments "humain" et "environnement", *via* les baignades estivales en eaux douces, augmentait l'imputation environnementale des infections communautaires à 6.0 % en été. Cette étude estime que l'exposition environnementale aux BGN-CP est faible, mais néanmoins existante. Même si le rôle du compartiment "environnement" apparaît limité dans la circulation et la transmission des BGN-CP dans les PRE, sa prise en considération est indispensable dans une optique *One Health* (Figure 3). Certes, la réduction de la contamination environnementale par les BGN-CP ne doit pas être la priorité des politiques de santé publique étant donnée la faible probabilité d'exposition environnementale des populations aux BGN-CP. En revanche, des efforts doivent être menés pour réduire la contamination environnementale dans les écosystèmes (*i*) fortement contaminés (danger élevé) et (*ii*) densément peuplés (exposition élevée). De plus, dans une optique de suivi du risque à moyen et long terme dans l'espace et dans le temps, la considération du compartiment "environnement" est indispensable.



**Figure 5. Importance relative de la diffusion des BGN-CP entre les compartiments "environnement" et "humain".** Ce travail a estimé que la probabilité d'exposition environnementale aux BGN-CP est faible dans les PRE, notamment comparée à la transmission interhumaine.

Considérons maintenant brièvement le compartiment "animal" comme source de transmission des BGN-CP à l'Homme. La transmission des BGN-CP à l'Homme *via* la nourriture est limitée dans les PRE (voir "Travaux complémentaires")<sup>88,85,89,90</sup>. La transmission de BGN-CP à l'Homme *via* le contact avec les animaux de compagnie semble également être faible dans les PRE<sup>85</sup>. Ce sont les mesures et normes d'hygiène, notamment alimentaires, qui permettent une faible probabilité d'exposition animale aux pathogènes humains dans les PRE.

## 7.2. Bacilles à Gram négatif commensaux et pathogènes et *One Health* global

La probabilité d'exposition environnementale aux BGN-CP est faible dans les PRE. Or, le concept *One Health* promeut une approche globale, en considérant la diffusion mondiale de clones épidémiques et de la résistance aux antibiotiques, entre autres. Qu'en est-il des échanges de BGN-CP entre les compartiments "humain" et "environnement" au niveau planétaire ?

### 7.2.1. Dans les pays à revenus faibles et intermédiaires

Dans les PRFI, les infrastructures d'hygiène individuelles (toilettes, *etc.*) et collectives (traitement des eaux usées, *etc.*) sont souvent limitées, voire inexistantes. À titre d'exemple, 45 % de la population urbaine en Afrique n'avait pas accès à un système d'assainissement efficace en 2000<sup>91</sup>. La défécation en plein air est parfois commune (*e.g.* Niger : 68 %, Tchad : 67 %, Soudan du Sud : 63 % et Inde : 26 %<sup>92</sup>). L'environnement est donc largement exposé à

la contamination par les BGN-CP. Les infrastructures de traitement de l'eau pour la consommation humaine peuvent être peu efficaces, voire inexistantes, exposant les populations humaines à une contamination *via* leur environnement. Les densités de populations humaines sont parfois extrêmement élevées, et le contact avec les animaux important, favorisant les échanges interhumains et animaux-humains des pathogènes. Les populations des PRFI ont relativement peu accès à des soins adaptés, et sont généralement moins informées sur les mesures d'hygiène que les populations des PRE<sup>9</sup>. C'est également le cas des professionnels de santé et de l'agriculture. L'utilisation d'antibiotiques chez l'Homme et l'animal ne bénéficie pas toujours d'un cadre suffisant, entraînant des utilisations inadaptées et/ou excessives d'antibiotiques. Cela favorise la diffusion et l'émergence de l'antibiorésistance dans ces régions du monde<sup>10,93,94</sup>, d'autant plus que certains de ces pays sont parmi les principaux producteurs mondiaux d'antibiotiques<sup>95</sup>. D'un point de vue organisationnel, les politiques de santé publique sont souvent inadaptées aux enjeux des pays : législations, réglementations, moyens matériels, humains et financiers en sont quelques exemples<sup>9</sup>.

Au-delà du développement, le climat peut également jouer un rôle dans la diffusion des pathogènes humains dans l'environnement dans les PRFI. Les climats chauds et humides (équatorial ou, saisonnièrement, tropical humide) favorisent potentiellement la percolation environnementale des BGN-PC. Dans les climats plus secs (tropical sec, méditerranéen, désertique), la ressource en eau limitée encourage l'utilisation des eaux usées pour irriguer les champs et cultures agricoles<sup>96</sup>, participant également à la diffusion des BGN-CP dans l'environnement. Comme développé plus loin, la température a récemment été associée à des taux de résistance aux antibiotiques plus élevés<sup>97,98</sup> : on peut donc s'attendre à un taux de résistance plus élevé dans les PRFI au climat généralement chaud (*i.e.i* Afrique, Amérique centrale et du sud, Asie du sud-est). De plus, Devarajan *et al.*<sup>99</sup> ont rapporté dans leur étude portant sur *Pseudomonas* spp. que le climat tropical pourrait favoriser les échanges de matériel génétique entre les souches bactériennes.

La probabilité d'exposition environnementale aux BGN-CP est donc très probablement plus élevée dans les PRFI que dans les PRE. Comme dans les PRE, les écosystèmes alluviaux sont plus contaminés par les BGN-CP lors des périodes de hautes eaux<sup>100,101</sup>. En Chine, Xue *et al.*<sup>101</sup> ont montré que les nappes phréatiques étaient contaminées par *E. coli* et les coliformes totaux : 70 % des échantillons d'eau de surface dépassaient la recommandation de 1000 MPN coliformes totaux / 100 ml<sup>102</sup>, et 90% des échantillons d'eau souterraine dépassaient la recommandation de 10 MPN *E. coli* / 100 ml<sup>102</sup>. Les auteurs de cette étude attribuent largement

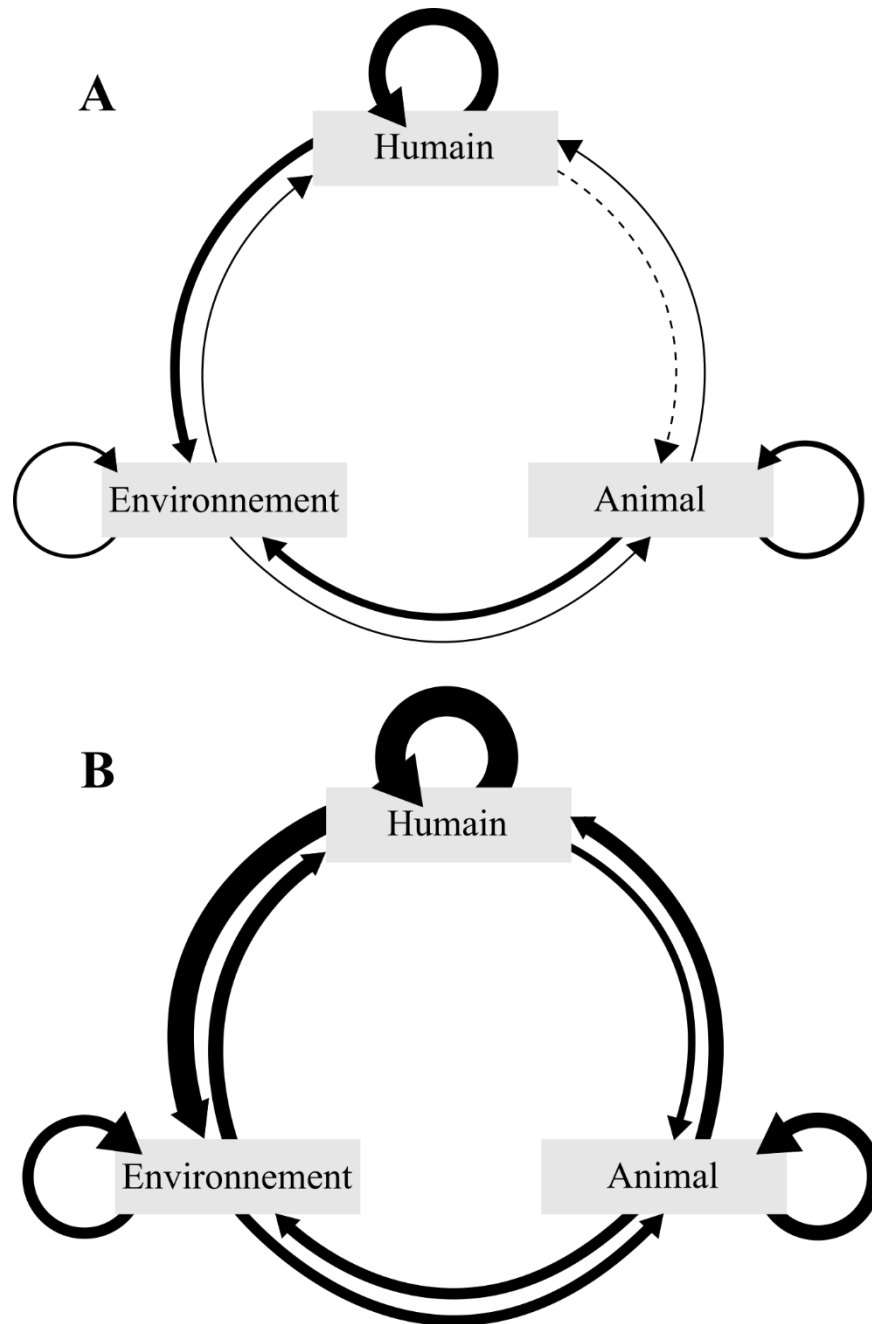
la contamination environnementale au manque de systèmes performants de traitement des eaux usées, notamment dans les zones rurales, généralement plus pauvres que les zones urbaines. Au Bangladesh, une étude récente rapporte que 5, 10 et 38 % des eaux potables d'habitations, de fermes et de marchés étaient contaminées par *E. coli* producteur de BLSE, respectivement<sup>103</sup>.

Même si le compartiment "animal" s'éloigne du cadre de notre travail, la probabilité d'exposition des animaux d'élevage aux BGN-CP semble également élevée dans les PRFI. Au-delà du contact direct avec les animaux d'élevage, il est difficile d'estimer la probabilité d'exposition à de la viande contaminée dans les PRFI par manque de données. Le séquençage de plasmides des *E. coli* producteurs de BLSE provenant de sources humaines et d'animaux d'élevages à Hanoï (Viêt-Nam) a mis en évidence des dissimilarités entre les isolats d'*E. coli* producteurs de BLSE<sup>18</sup>, supposant une faible perméabilité entre les compartiments "humain" et "animal". Malgré cela, des éléments génétiques mobiles porteurs de *bla*<sub>BLSE</sub> identiques partagés entre les deux compartiments ont aussi été relevés, nuanciant les conclusions de l'étude<sup>18</sup>. Les connaissances sur la perméabilité entre les compartiments "animal" et "environnement" sont limitées dans les PRFI.

Notons ici le cas particulier des bidonvilles. Dans ces lieux d'extrême pauvreté aux densités de population très élevées, la situation décrite précédemment est exacerbée. Les contacts interhumains, animaux-humains et environnement-humains sont favorisés. À titre d'exemple, Kayembé *et al.*<sup>104</sup> montrent des taux de contamination des eaux récréatives et des eaux souterraines destinées à la consommation humaine extrêmement élevés dans un bidonville de Kinshasa (République Démocratique du Congo) : durant la saison des pluies, les abondances de *E. coli* ont atteint  $18.6 \times 10^5$  et  $4.9 \times 10^5$  CFU / 100 ml dans la rivière et les puits, respectivement. Ces niveaux de contamination environnementale paraissent invraisemblables comparés aux résultats de ce travail (presque 1000 fois plus élevés en moyenne que ceux observés dans les chapitres 2 et 3). Nadimpalli *et al.*<sup>105</sup> rapportent également une situation très dégradée dans les bidonvilles bangladais, indiens, kenyans et péruviens, et préconisent une considération accrue de la transmission environnementale des pathogènes humains. Les bidonvilles sont donc des hot-spots de contamination et de transmission de BGN-CP. Ils sont également de potentiels lieux d'émergence de nouvelles résistances aux antibiotiques<sup>106</sup>. Une attention particulière ainsi que des efforts inédits doivent être portés à ces zones d'extrême pauvreté afin de limiter (i) la propagation des BGN-CP au sein de ces habitats, mais également (ii) l'émergence de nouveaux pathogènes et de nouvelles résistances ainsi que leur diffusion mondiale. L'amélioration générale des conditions de vie, dont l'accès à l'eau potable, le développement d'infrastructures sanitaires et un contrôle plus strict de l'utilisation des

antibiotiques<sup>94</sup>, est primordiale pour limiter les échanges de pathogènes humains dans et au-delà de ces zones.

Le portage des BGN-CP résistants aux antibiotiques est très élevé dans les PRFI. Par exemple, Rousham *et al.*<sup>103</sup> rapportent un portage digestif alarmant de 67.5 % des *E. coli* producteurs de BLSE chez des individus adultes au Bangladesh. Dans les PRE, ce portage est généralement inférieur à 10 %<sup>107,108,109</sup>. Le portage élevé des BGN-CP résistants aux antibiotiques dans les PRFI est en partie attribuable (i) à la forte transmission interhumaine, (ii) à la mauvaise utilisation/surconsommation d'antibiotiques, et (iii) aux perméabilités accrues entre les trois compartiments *One Health* (Figure 6). En revanche, des études complémentaires doivent être menées pour explorer le lien entre ces différents compartiments, afin de savoir dans quelles mesures chacun des trois compartiments contribue au portage humain des BGN-CP résistants aux antibiotiques. À notre connaissance, aucune étude du type de celle menée par Mughini-Gras *et al.*<sup>85</sup> n'a été conduite dans un PRFI. Même s'il est difficile de connaître précisément la contribution de l'environnement, la circulation des pathogènes humains dans les écosystèmes et la transmission à l'Homme a vraisemblablement un rôle important, voire central, dans la diffusion des BGN-CP dans les PRFI (Figure 6). Le risque environnemental lié aux BGN-CP semble donc largement supérieur dans les PRFI que dans les PRE. La mise en place d'approches *One Health* est toute aussi importante dans les PRFI que dans les PRE, mais les contributions de chacun des trois compartiments semble plus équilibrées dans les PRFI. En effet, le surcroît de l'importance absolue et relative des compartiments "environnement" et "animal" dans les PRFI par rapport aux PRE rend l'application du concept *One Health* plus évidente.



**Figure 6. Estimation de la diffusion des BGN-CP entre les trois compartiments *One Health* : comparaison PRE - PRFI. A.** La diffusion des BGN-CP entre les trois compartiments *One Health* dans les PRE : la perméabilité entre les compartiments est faible, et la diffusion au sein de chaque compartiment est limitée. **B.** La diffusion des BGN-CP entre les trois compartiments *One Health* dans les PRFI: la perméabilité entre les compartiments et la diffusion au sein de chaque compartiment sont élevées.

### 7.2.2. Le concept *One Health* appliqué aux bacilles à Gram négatif commensaux et pathogènes

Certaines études peuvent pousser à écarter l'approche *One Health*, ou en tout cas les compartiments "animal" et/ou "environnement", lorsque l'on s'intéresse à la diffusion des BGN-CP. Les résultats de contamination environnementale présentés dans le présent travail (chapitre 2 et 3), suggèrent une faible probabilité de contamination "environnement" → "humain", ce qui peut poser la question de la légitimité de la prise en compte du compartiment "environnement" dans les PRE. Plusieurs études s'intéressant à la similarité génétique d'isolats provenant des compartiments "humain" et "animal" ont mis en évidence une ségrégation des isolats humains et animaux en comparant leurs génomes<sup>16,17,18,88</sup>, démontrant le faible rôle du compartiment "animal" dans la diffusion des BGN-CP chez l'Homme. *A contrario*, d'autres études décrivent la forte diffusion globale des BGN-CP et soutiennent fortement le concept *One Health*. Par exemple, la diffusion mondiale de clones épidémiques (e.g. *E. coli* ST131<sup>110</sup>) ou de GRA (e.g. *bla*<sub>CTX-M-15</sub><sup>111</sup> ; *bla*<sub>NDM-1</sub><sup>106</sup>), ainsi que leur diffusion dans les compartiments "animal" et "environnement"<sup>112,113</sup>, incitent à une approche globale *One Health*. Notons également que, comme nous l'avons observé dans ce travail (chapitre 2), certaines études montrent des similarités génétiques entre les isolats de BGN-CP provenant des différents compartiments *One Health*<sup>14,15,114</sup>.

La précision des techniques de typage utilisées peut conduire à des résultats et conclusions différentes. Ainsi, comme nous l'avons vu ci-dessus, certaines études – majoritairement basées sur les STs – rapportent une similarité entre les souches des différents compartiments *One Health*, alors que d'autres – majoritairement basées sur le génome complet – montrent une ségrégation des souches suivant ces compartiments. Plus la précision considérée est élevée (espèce < clone < éléments génétiques < génome), plus les isolats des trois compartiments semblent ségrégués, et moins le concept *One Health* peut être un cadre explicatif satisfaisant. Les BGN-CP liés aux compartiments "animal" et "environnement" sont souvent polyclonaux et plus divers que ceux observés dans le compartiment "humain"<sup>115</sup>. Dans une moindre mesure, les souches peuvent évoluer et dériver génétiquement dans l'environnement ou dans leur hôte<sup>115,116</sup>. Bien que les méthodes de génotypage à très forte résolution, tel que le séquençage du génome complet, permettent d'accéder à nombre d'informations utiles et pertinentes (e.g. GRA, plasmides, comparaison de plusieurs génomes grâce au nombre de mutations), elles peuvent aussi conduire à la distinction de souches ayant pourtant une origine commune. Le choix du seuil (nombre de mutations) pour différencier deux génomes est fondamental et

dépend de la dimension spatio-temporelle de la collection considérée. Cet aspect méthodologique doit être pris en compte lors de la comparaison de souches pathogènes des différents compartiments *One Health*.

Pour le cas particulier des BGN-CP dans l'environnement, notre travail suggère que la contribution du compartiment "environnement" est limitée dans les PRE, alors qu'elle semble beaucoup plus importante dans les PRFI. Même dans une aire restreinte, l'importance du compartiment "environnement" va dépendre du type d'écosystème (*i.e.* fonctionnement, climat, pression anthropique, *etc.*), certains écosystèmes étant plus favorables que d'autres en fonction du pathogène considéré.

Le fait que les trois compartiments *One Health* n'aient pas la même importance ne signifie pas que le concept *One Health* n'est pas applicable. C'est justement l'application du concept *One Health* qui nous permet de nous en rendre compte. Le concept *One Health*, holiste, peut se décliner en de multiples approches *One Health*, adaptées aux cas particuliers. D'une part, le concept *One Health* global est applicable dès lors que, à un lieu et temps donné, la contribution de chacun des trois compartiments est pertinente. D'autre part, les approches *One Health* prennent toutes en compte le tryptique humains-animaux-écosystèmes, mais l'importance relative de chacun des trois compartiments peut varier suivant le cas d'intérêt. Ces importances divergentes des compartiments *One Health* dépendent de plusieurs facteurs, dont :

- le pathogène considéré (*e.g.* espèce, clone, type de résistance aux antibiotiques) ;
- le contexte anthropique et écologique (*e.g.* PRE *versus* PRFI, type d'écosystème) ;
- la temporalité (*e.g.* transmissions ponctuelles à l'Homme *versus* flambée épidémique à partir d'un réservoir environnemental dans le cas d'une zoonose).

Par exemple, pour une zoonose telle que le virus Ebola, l'approche *One Health* prend assez logiquement en compte le tryptique humains-animaux-écosystèmes dans un premier temps. Or, la contribution des compartiments "animal" et "environnement" est quasiment limitée à la transmission initiale de l'agent infectieux à l'Homme. Une fois le pathogène passé dans le compartiment "humain", la transmission interhumaine prédomine largement. Bien que les trois compartiments doivent être pris en compte, l'importance de chacun des compartiments, et donc l'approche *One Health*, varie au cours de l'épidémie. Comme nous l'avons vu dans le présent travail pour le cas des BGN-CP, l'approche *One Health* varie bien sûr selon l'espèce bactérienne, voire le clone considéré, mais aussi selon le contexte anthropique et écologique. Même si le concept *One Health* n'est pas toujours évident à appliquer aux BGN-CP, il est nécessaire afin de connaître l'importance de chacun des compartiments et de gérer au mieux la diffusion du pathogène d'intérêt.



### 7.3. Bacilles à Gram négatif commensaux et pathogènes et *One Health* : perspectives de recherche et évolutions futures

#### 7.3.1. Perspectives de recherche

##### **Adaptation des bacilles à Gram négatif commensaux et pathogènes dans l'environnement ?**

Certaines études ont mis en évidence des différences de structure de population des BGN-CP suivant l'hydrologie et/ou l'occupation des sols du bassin versant (en se basant principalement sur les phylogroupes de *E. coli*)<sup>117,118,119</sup>. Au contraire, notre étude de la plaine d'inondation de l'Ain a montré peu de variation génétique entre les populations de BGN-CP dans les écosystèmes alluviaux (en se basant sur les phylogroupes, groupes d'incompatibilité plasmidique et *bla<sub>BLSE</sub>* ; chapitre 3). Notons que l'occupation des sols était relativement homogène dans la plaine alluviale de l'Ain, ce qui nous empêche de tirer des conclusions sur ce point. De plus, la pente de la rivière, et donc les vitesses d'écoulement, étaient élevées pour le tronçon étudié, ce qui pourrait engendrer des transferts rapides de l'amont vers l'aval, et peu d'apports latéraux dans cet hydrosystème. Une caractérisation génétique plus précise (*i.e.* séquençage complet du génome), d'isolats provenant (*i*) d'écosystèmes contrastés dans un même hydrosystème, ou (*ii*) d'écosystèmes fonctionnellement analogues (*e.g.* les zones humides rarement connectées à la rivière principale) à différentes temporalités, pourrait permettre de décrire le comportement des populations de BGN-CP dans l'environnement, et d'évaluer le maintien ou non des déterminants de virulence et de résistance aux antibiotiques.

Dans des conditions défavorables, les BGN-CP peuvent devenir viables mais non cultivables (VBNC). Pommepuy *et al.*<sup>120</sup> ont montré que malgré leur faible activité métabolique, les *E. coli* VBNC produisent des entérotoxines. Néanmoins, très peu d'éléments permettent d'estimer le risque associé à ces bactéries pathogènes VBNC. Sont-elles capables de survivre longtemps et de retrouver leur *fitness* initiale dans un environnement devenant plus favorable ? Dans quelles conditions ? Peuvent-elles échanger du matériel génétique ?

Ce manque d'information est également transposable aux clades cryptiques du genre *Escherichia*. Ces souches, très proches génétiquement de *E. coli* et majoritairement sensibles aux antibiotiques, sont peu retrouvées chez l'Homme<sup>121</sup>. Cependant, très peu de données épidémiologiques sont disponibles. Ces souches ont été identifiées dans les écosystèmes alluviaux les plus oligotrophes dans le présent travail (18.8 et 5.0 % des souches originellement identifiées comme *E. coli*, chapitre 2 et chapitre 3, respectivement). Toutefois, les méthodes

employées (*i.e.* culture sur gélose Drigalski) n'étaient pas parfaitement adaptées à leur détection. Les clades cryptiques du genre *Escherichia* ne semblent pas directement associés à l'Homme, mais leur survie dans les écosystèmes oligotrophes et leur proximité génétique avec *E. coli* questionne sur (*i*) la spécialisation éventuelle des différentes espèces du genre *Escherichia*, et (*ii*) les potentiels échanges de matériel génétique entre ces différentes espèces.

### **Le transfert horizontal de gènes de résistance aux antibiotiques**

Le transfert horizontal de gènes (THG) de GRA entre les BGN-CP et les espèces bactériennes environnementales peut faciliter l'émergence de nouvelles résistances. Ainsi, les gènes *bla*<sub>CTX-M</sub> sont, à l'origine, des gènes chromosomiques conférant une résistance naturelle à des bactéries environnementales appartenant aux espèces *Kluyvera* spp.<sup>122</sup>. Au-delà de l'émergence de nouvelles résistances, le THG de GRA pourrait faciliter la dissémination environnementale de l'antibiorésistance. Cependant, l'amplification de déterminants de résistance aux antibiotiques dans l'environnement par THG est sujet à controverse. Des travaux ont rapporté que le THG de GRA pouvait survenir dans divers environnements : STEU<sup>123</sup>, sols amendés avec des biosolides<sup>124</sup>, environnements aquacoles<sup>125</sup>, rhizosphère<sup>126</sup>. Les biofilms pourraient également faciliter le THG dans l'environnement<sup>127,128</sup>. Toutefois, des études plus globales minimisent le rôle du THG de GRA dans les environnements avec peu ou pas de pression de sélection au profit d'une diffusion passive de la résistance, sans amplification dans l'environnement<sup>129,130</sup>. En effet, une pression de sélection des antibiotiques et/ou d'autres agents antibactériens est essentielle pour que le GRA nouvellement transféré persiste dans l'hôte et se dissémine<sup>129,130,131</sup>. Ainsi, seuls les environnements sous forte pression de sélection (*i.e.* écosystèmes en aval d'effluents d'industries pharmaceutiques) seraient favorables au THG de déterminants de résistance aux antibiotiques<sup>132</sup>. Bien que le THG de GRA semble négligeable et très difficile à démontrer dans l'environnement, les potentiels risques qui pourraient lui être associé motivent les recherches dans ce sens<sup>133,134</sup>.

De récentes études ont rapporté que les biofilms présents sur les microplastiques hébergent un microbiote abondant et très diversifié<sup>135</sup>, facilitant le THG<sup>128</sup>, et favorisant ainsi la dissémination des GRA dans l'environnement. Très peu d'études sont disponibles sur le sujet et ces résultats doivent être confirmés, mais cela semble problématique au vu des quantités importantes de microplastiques que nous rejetons dans l'environnement<sup>136</sup>.

La question du THG se pose également pour les BGN-CP VBNC, qui pourraient potentiellement échanger du matériel génétique avec les espèces bactériennes autochtones des écosystèmes. La proximité génétique entre *E. coli* et les clades cryptiques du genre *Escherichia*

pourrait également permettre le THG entre ces souches. Malheureusement, ces deux derniers points souffrent d'un manque de connaissances qui nous empêche aujourd'hui de mesurer leur importance dans la dispersion environnementale des BGN-CP et de la résistance aux antibiotiques.

### **Interactions biotiques des bacilles à Gram négatif commensaux et pathogènes dans l'environnement**

Comme nous l'avons vu dans ce travail, la végétation peut influencer la survie d'*E. coli* dans les écosystèmes, supposément grâce aux effets allélopathiques des composés secondaires qu'elle produit (chapitre 4). Cette interaction *E. coli*-végétation a été vérifiée *in vitro*, mais reste à vérifier *in situ* dans les écosystèmes pour améliorer la compréhension des phénomènes réels en jeu. Par ailleurs, les plantes constituent un support pour la formation de biofilms<sup>137</sup>, ce qui pourrait, en l'absence de mécanisme allélopathique, soit favoriser la survie des BGN-CP dans l'environnement, soit favoriser les bactéries autochtones au détriment des BGN-CP.

La faune sauvage peut être un vecteur et une source de BGN-CP<sup>86,138,139</sup>. Son rôle semble limité dans la transmission de BGN-CP d'origine humaine dans les PRE<sup>85</sup>. Mais ce rôle pourrait être largement plus important dans des régions du monde où l'interaction humain-faune sauvage est plus élevée. Les espèces sauvages interagissant directement et indirectement avec l'Homme, comme certains rongeurs (rats, raton-laveurs, *etc.*) ou oiseaux (corneilles, goélands, *etc.*), déjà connus comme porteurs de BGN-CP, BRA et GRA<sup>138,139,140</sup>, pourraient jouer un rôle dans la transmission de BGN-CP du compartiment "animal" au compartiment "humain".

Comme supposé mais non démontré dans ce travail, les communautés microbiennes autochtones pourraient limiter la survie des BGN-CP dans les écosystèmes (chapitre 3). Plus la diversité microbienne est élevée dans un écosystème, plus la colonisation de ce dernier par une espèce allochtone est limitée<sup>141,142,143,144</sup>. Cependant, ces résultats reposent sur des analyses *in vitro*, et à notre connaissance, aucune étude de ce type n'a été menée sur les BGN-CP que nous avons examinées ici dans des écosystèmes naturels. Pour pallier à ce manque de compréhension, tous les sites de l'Ain, du Doubs et de la Loue échantillonnés en 2015-2016 (chapitre 2) font actuellement l'objet d'une étude de la structure de leur population microbienne. Brièvement, l'ADN total a été extrait de ces échantillons, et le fragment codant pour les régions v3-v4 de l'ARN ribosomique 16S – propre aux procaryotes – a été amplifié puis séquencé en utilisant la technologie Illumina MiSeq (*next-generation sequencing*). Les résultats sont actuellement en cours d'exploitation. Cette étude a pour but de relier la diversité spécifique et fonctionnelle des communautés microbiennes autochtones des écosystèmes avec la présence/absence et les abondances de BGN-CP (chapitre 2).

### **Recherche et développement de nouveaux antibiotiques**

Parmi les BGN-CP, la dispersion des BRA est particulièrement préoccupante, y compris dans les écosystèmes<sup>38,39,94</sup>. De manière générale, limiter l'émergence de nouvelles résistances et la diffusion communautaire des BRA limite *de facto* leur dispersion dans l'environnement et la probabilité de recontamination des populations humaines. Cela fait presque 30 ans qu'aucun nouvel antibiotique n'a été développé et la résistance aux antibiotiques existants augmente. Des efforts de recherche et de développement de nouveaux antibiotiques doivent être menés afin de limiter l'émergence de BRA et donc leur diffusion entre les trois compartiments *One Health*. Ces efforts doivent impérativement être couplés à une accessibilité globale de ces nouveaux antibiotiques et surtout à leur utilisation encadrée et raisonnée – aussi bien dans les PRE que dans les PRFI – afin d'éviter une « course à l'armement » infinie entre bactéries pathogènes et antibiotiques, comme cela a été le cas jusqu'à aujourd'hui<sup>145</sup>.

### **7.3.2. Evolutions futures et impact sur le concept *One Health***

#### **Les pays à revenus faibles et intermédiaires**

Le recul de la pauvreté dans le monde<sup>146</sup> et le développement global laissent espérer une amélioration générale du niveau de vie dans les PRFI, et de manière subséquente, une diminution de la diffusion des pathogènes humains. Cependant, bien qu'une amélioration des conditions de vie soit attendue, l'accroissement des populations des PRFI ces prochaines années est un défi pour la réduction de la diffusion des BGN-CP<sup>94</sup>. La croissance démographique est concentrée dans les régions du monde où la prévalence des pathogènes humains est la plus élevée, renforçant encore les possibilités de transmission dans chaque et entre les trois compartiments *One Health*. À titre d'exemple, la densité de population des bidonvilles va doubler d'ici 2050<sup>147</sup>, et 90 % de la croissance de la population urbaine est attendue dans les régions les plus touchées par la crise de la résistance aux antimicrobiens<sup>105</sup>. Dans ces régions du monde, le développement des infrastructures individuelles et collectives d'hygiène, l'accès à l'eau potable et à une alimentation sûre, et l'utilisation raisonnée des antibiotiques sont les leviers prioritaires d'action pour la lutte contre la diffusion des BGN-CP résistantes ou non aux antibiotiques. À cet égard, des mesures réglementaires, législatives et de surveillance doivent être mises en place par des politiques locales, soutenues par une (super)vision globale (Organisation des Nations Unies, Organisation Mondiale de la Santé)<sup>145</sup>.

### **Anthropocène et pathogènes dans l'environnement**

Comme nous l'avons brièvement évoqué plus haut dans cette discussion et dans le chapitre 1, certaines études ont noté que l'augmentation des températures induites par les changements climatiques actuels et futurs pourrait favoriser la diffusion environnementale de la résistance aux antibiotiques<sup>97,98,148</sup>. L'échange de matériel génétique entre les BGN pourrait également être favorisé par des températures élevées<sup>99</sup>. De plus, dans les régions climatiques tempérées, la diminution des fréquences des précipitations, mais aussi l'augmentation de leur intensité laissent peser un risque accru de dispersion des pathogènes humains dans l'environnement, *via* les débordements des STEU (à travers les déversoirs d'orages) et les inondations, moins fréquents mais plus intenses. *A contrario*, les sécheresses plus prononcées et plus fréquentes sont susceptibles d'assécher temporairement les milieux aquatiques potentiellement réservoirs de BGN-PC. À l'échelle de la plaine alluviale, la contamination des BGN-CP pourrait atteindre des écosystèmes jusque-là isolés, où elles pourront potentiellement survivre, comme nous l'avons montré (chapitre 3). À l'échelle des cours d'eau, les étiages plus fréquents risquent de provoquer une diminution de la dilution des BGN-CP et l'augmentation subséquente de leurs abondances dans les écosystèmes. La survie des BGN-CP dépendra ensuite de la pérennité des masses d'eau.

L'élévation des températures, l'eutrophisation, la perte générale de biodiversité, ainsi que la destruction et la fragmentation des habitats supposent une modification des communautés microbiennes autochtones dans les écosystèmes<sup>6</sup>. De plus, les polluants (*i.e.* antibiotiques, métaux lourds, biocides) sélecteurs de BRA pourraient également déséquilibrer les communautés microbiennes autochtones<sup>149,150</sup>. Ces modifications pourraient, en diminuant la pression de compétition sur les BGN-CP, leur créer des niches disponibles, et favoriser leur colonisation des écosystèmes<sup>6,141,143</sup>.

Au-delà du risque lié aux BGN-CP, le risque d'émergence de nouvelles zoonoses s'accroît. L'altération des écosystèmes par l'Homme dans les zones riches en biodiversité, et l'augmentation subséquente de la perméabilité entre les compartiments "humain" et "environnement", augmente l'exposition de l'Homme à de nouveaux agents infectieux<sup>5,6</sup>. Nous avons largement constaté ce phénomène depuis le début du XXI<sup>ème</sup> siècle, avec, entre autres, l'émergence et la diffusion du virus Zika, du virus Ebola, de la grippe A (H1N1) ou encore du SARS-CoV-2. Les pandémies mondiales, telle que celle de la Covid-19, provoquent des crises sanitaires, mais aussi sociales, économiques et financières<sup>146</sup>. Les populations les plus pauvres sont les plus exposées à ces pandémies<sup>146</sup>, créant un cercle vicieux qui ralentit l'augmentation de leur niveau de vie, et augmente leur détresse sanitaire.

Les déséquilibres de l'Anthropocène, tels que la perte et la fragmentation des habitats, l'étalement urbain, l'intensification et l'homogénéisation des pratiques agricoles, la perte de biodiversité ou encore les changements climatiques, risquent donc de favoriser considérablement la diffusion des BGN-CP et de la résistance aux antibiotiques entre les trois compartiments *One Health*, ainsi que l'émergence de nouvelles zoonoses. Des études complémentaires doivent être menées pour estimer l'ampleur de l'impact des risques liés à ces changements globaux pour mieux les anticiper et les atténuer. Nous le savons déjà au-delà du cadre de ce travail, mais les gouvernances futures devront notamment veiller à favoriser la préservation de la biosphère et la coopération entre les humains face à l'expansionnisme de nos sociétés.



## CONCLUSIONS

Les bacilles à Gram négatif commensaux et pathogènes d'origine humaine sont largement dispersés dans l'environnement. À l'échelle de la plaine alluviale, cette dispersion dépend majoritairement de la contamination et du débit de la rivière principale, ainsi que de la géomorphologie du lit majeur, qui contrôle la connectivité hydrologique entre les écosystèmes superficiels et souterrains de la plaine alluviale. La survie des BGN-CP est influencée par des facteurs biotiques et abiotiques. Même si leur maintien dans des écosystèmes peu favorables est limité, les BGN-CP survivent, voire prolifèrent, dans certaines conditions spécifiques.

L'environnement joue *a priori* un rôle mineur dans la diffusion des BGN-CP dans les populations humaines des PRE, notamment grâce à la relativement faible contamination générale des écosystèmes, et surtout à la faible perméabilité entre les compartiments "environnement" et "humain". Les transmissions interhumaines sont ainsi les voies de dispersion privilégiées des BGN-CP. En conséquence, la réduction de l'utilisation des antibiotiques et le respect de mesures d'hygiène permettra de réduire la transmission de BRA. La majorité des efforts doivent se concentrer dans les PRFI, dont les populations sont beaucoup plus exposées aux maladies infectieuses et à l'antibiorésistance. Le développement des infrastructures individuelles et collectives d'hygiène, l'accès à l'eau potable et à une alimentation sûre, et l'utilisation raisonnée et encadrée des antibiotiques sont les leviers prioritaires d'action pour la lutte contre la diffusion des BGN-CP dans les PRFI.

Les changements globaux propres à l'Anthropocène présagent une dispersion environnementale accrue des BGN-CP, liée, entre autres, à l'augmentation des températures et à une colonisation des écosystèmes vraisemblablement plus aisée. Les zoonoses font peser un risque sanitaire de plus en plus élevé sur les populations humaines. Même si chacun des trois compartiments *One Health* n'a pas la même importance, la mise en place d'approches *One Health* est une nécessité : tel un écosystème global, tout peut avoir une conséquence directe ou indirecte sur tout à l'échelle mondiale.





## 8. TRAVAUX COMPLÉMENTAIRES

### 8.1. Les bactéries résistantes aux antibiotiques dans les environnements pollués par l'Homme et dans la nourriture

Cette étude s'inscrit dans le projet européen MODERN (*Understanding and modelling reservoirs, vehicles, and transmission of ESBL-producing Enterobacteriaceae in the community and long-term care facilities*). Le projet MODERN a pour objectif d'identifier les réservoirs et de comprendre les dynamiques de transmission des Entérobactéries productrices de BLSE dans cinq villes européennes : Besançon (France), Genève (Suisse), Séville (Espagne), Tübingen (Allemagne) et Utrecht (Pays-Bas).

L'article présenté ici est une partie du projet MODERN. Ma contribution à cet article se limite à des analyses statistiques et relectures, ce qui justifie que ce travail ne soit pas intégré au corps de ma thèse.

**Titre :** Les populations d'*Escherichia coli* et de *Klebsiella pneumoniae* producteurs de  $\beta$ -lactamase à spectre étendu sont différentes dans l'environnement pollué par l'Homme et dans les denrées alimentaires: une étude multicentrique européenne.

**Revue :** Clinical Microbiology and Infection

**État :** publié, 2021

**Éléments essentiels :**

- Les populations d'*E. coli* producteur de BLSE et de *K. pneumoniae* producteur de BLSE des environnements pollués par l'Homme et de la nourriture ont été comparées ;
- Les souches retrouvées dans ces deux compartiments étaient génétiquement distinctes. Des échanges, faibles, sont néanmoins supposés ;
- Le THG semble jouer un rôle limité dans la propagation de *bla*<sub>ESBL</sub> des denrées alimentaires à l'Homme.

**Résumé**

**Objectifs.** Évaluer dans quelle mesure les denrées alimentaires sont une source d'*E. coli* producteur de BLSE et de *K. pneumoniae* producteur de BLSE pour les humains dans cinq villes européennes.

**Méthodes.** Nous avons échantillonné 122 environnements pollués par l'Homme (égouts et rivières polluées, comme indicateur de la contamination humaine) et 714 denrées alimentaires à Besançon (France), Genève (Suisse), Séville (Espagne), Tübingen (Allemagne) et Utrecht (Pays-Bas). 254 isolats d'*E. coli* producteur de BLSE et 39 *K. pneumoniae* producteur de BLSE ont été cultivés. Tous les génomes ont été complètement séquencés afin de comparer leurs séquences type (ST) et leurs *core* génomes, ainsi que la distribution des gènes *bla*<sub>ESBL</sub> et leurs supports génétiques (*i.e.* chromosome ou plasmide).

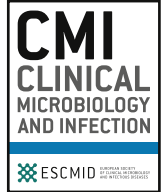
**Résultats.** Les données de séquençage ont révélé que les isolats d'*E. coli* producteur de BLSE et *K. pneumoniae* producteur de BLSE provenant de l'environnement pollué par l'Homme étaient génétiquement différents de ceux contaminant les denrées alimentaires. La souche ST131 d'*E. coli* producteur de BLSE était répandue dans l'environnement pollué par l'Homme (25.5 % des isolats), mais absente des denrées alimentaires testées. *E. coli* producteur de BLSE ST10 était présent dans des proportions similaires dans l'environnement pollué par l'Homme et dans les denrées alimentaires (15 et 10 isolats, respectivement), mais il était surtout porteur du *bla*<sub>ESBL</sub> réservoir-spécifique. *bla*<sub>CTX-M-1</sub> et *bla*<sub>SHV-12</sub> prédominaient dans les isolats d'*E. coli* liés aux denrées alimentaires (32 % et 34 % des isolats, respectivement), tandis que *bla*<sub>CTX-M-15</sub> et *bla*<sub>CTX-M-27</sub> prédominaient dans les isolats de l'environnement pollué par l'Homme (52 % et 15 % des isolats, respectivement).

**Conclusions.** Nous avons trouvé un lien très limité entre les populations d'*E. coli* producteurs de BLSE et de *K. pneumoniae* producteurs de BLSE retrouvées dans les denrées alimentaires et dans l'environnement pollué par l'Homme. Cela suggère que la contamination interhumaine, plutôt que de la chaîne alimentaire, est probablement la voie la plus fréquente de transmission des *E. coli* producteurs de BLSE et des *K. pneumoniae* producteurs de BLSE dans les PRE.



Contents lists available at ScienceDirect

## Clinical Microbiology and Infection

journal homepage: [www.clinicalmicrobiologyandinfection.com](http://www.clinicalmicrobiologyandinfection.com)

## Original article

## Populations of extended-spectrum $\beta$ -lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* are different in human-polluted environment and food items: a multicentre European study

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## ARTICLE INFO

## Article history:

Received 23 March 2021

Received in revised form

15 July 2021

Accepted 18 July 2021

Available online xxx

Editor: E.J. Kuipers

## Keywords:

Extended-spectrum  $\beta$ -lactamase

Environment

*Escherichia coli*

Food

*Klebsiella pneumoniae*

## ABSTRACT

**Objectives:** To assess the extent to which food items are a source of extended-spectrum  $\beta$ -lactamase (ESBL) -producing *Escherichia coli* (ESBL-Ec) and ESBL-producing *Klebsiella pneumoniae* (ESBL-Kp) for humans in five European cities.

**Methods:** We sampled 122 human polluted (hp)-environments (sewers and polluted rivers, as a proxy of human contamination) and 714 food items in Besançon (France), Geneva (Switzerland), Sevilla (Spain), Tübingen (Germany) and Utrecht (The Netherlands). A total of 254 ESBL-Ec and 39 ESBL-Kp isolates were cultured. All genomes were fully sequenced to compare their sequence types (ST) and core genomes, along with the distribution of *bla*<sub>ESBL</sub> genes and their genetic supports (i.e. chromosome or plasmid).

**Results:** Sequence data revealed that ESBL-Ec and ESBL-Kp isolates from hp-environments were genetically different from those contaminating food items. ESBL-Ec ST131 was widespread in the hp-environment (21.5% of the isolates) but absent from the food items tested. ESBL-Ec ST10 was in similar proportions in hp-environments and food items (15 and 10 isolates, respectively) but mostly carried reservoir-specific *bla*<sub>ESBL</sub>, *bla*<sub>CTX-M-1</sub> and *bla*<sub>SHV-12</sub> predominated in food-related *E. coli* isolates (32% and 34% of the isolates, respectively), whereas *bla*<sub>CTX-M-15</sub> and *bla*<sub>CTX-M-27</sub> predominated in isolates from hp-environments (52% and 15% of the isolates, respectively).

**Conclusions:** We found a very limited connection between ESBL-Ec and ESBL-Kp populations retrieved in food items and from hp-environments and *bla*<sub>ESBL</sub>. This suggests that human-to-human contamination,

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<https://doi.org/10.1016/j.cmi.2021.07.022>

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rather than the food chain, is possibly the most frequent route of ESBL-Ec and ESBL-Kp transmission in high-income countries. **Daniel Martak, *Clin Microbiol Infect* 2021;•:1**

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## Introduction

Antimicrobial resistance is a global health issue and extended-spectrum  $\beta$ -lactamase producing *Enterobacterales* (ESBL-PE) are a major cause of antimicrobial resistance dissemination. *Escherichia coli* (ESBL-Ec) and *Klebsiella pneumoniae* (ESBL-Kp) are the two major ESBL-PE species causing infections in humans with third-generation cephalosporin-resistant bacteria, limiting therapeutic options for patients [1]. ESBL-PE carriage by healthy people is frequent, with a prevalence of ~10% in Europe [2]. In addition, some populations, such as those found in long-term care facilities (LTCFs), are at higher risk of colonization and infection [3]. ESBL-PE have also been widely reported in slaughter animals [4], associated with a large proportion of the derived retail meat being contaminated with these antibiotic-resistant pathogens. To a lesser extent, ESBL-PE can also be found in fresh vegetables [5,6]. Sewage systems bring ESBL-PE found in human faeces to the surface water [7]. There are many routes for ESBL-PE dissemination and the One Health concept supports the idea that bacterial populations found in animals, humans and the environment are heavily interconnected [8]. However, the extent of transmission between these compartments remains uncertain, especially in high-income countries where hygiene standards probably limit the contamination from non-human sources [9]. In particular, the role of the food chain is under debate [9–12].

Most studies neglect ESBL-Kp, which also contaminates food items [13] and is responsible for an important burden in humans [14] with a rate of human-to-human transmission higher than that of ESBL-Ec [15].

The exact identification of the route of contamination of an individual is nearly impossible to establish and requires the use of population-level proxies. In European countries, human-polluted (hp-) environments, and especially wastewaters, constitute a good proxy of human contamination [10,16,17].

To estimate the contribution of food-borne ESBL-Ec and ESBL-Kp to human colonization, we used whole genome sequences to assess the genetic relationships, and compared the identity of *bla*<sub>ESBL</sub> between the isolates from retail food and from hp-environments in five European cities.

## Materials and methods

### Study design

We conducted a multicentre study involving five European cities: Besançon (France), Geneva (Switzerland), Sevilla (Spain), Tübingen (Germany) and Utrecht (The Netherlands). We collected samples of food products and from the hp-environment between January 2018 and August 2019, as part of the multicentre MODERN project. Samples from food products and hp-environment were collected within a prospective cohort study of ESBL-Ec and ESBL-Kp carriage in four LTCFs (manuscript in preparation) and during a 4-month follow up of ESBL-Ec and ESBL-Kp carriers after hospital discharge [15].

### Food and environmental samples

The contamination by ESBL-Ec or ESBL-Kp was assessed in 714 food samples collected in LTCF kitchens before any processing (representing collective catering) and in 35 supermarkets located in or nearby each study site representing food bought by ESBL-PE carriers [18] (see Supplementary material, [Appendix S1](#)). The contamination of the hp-environment by ESBL-Ec or ESBL-Kp was assessed by collecting samples ( $n = 122$ ) eight times over a 32-week period in (a) the LTCF discharge sewer, (b) the inflow of the downstream wastewater treatment plant (WWTP), and (c) the river 200 m downstream of the WWTP outflow and >5 m from the riverbank (see Supplementary materials, [Appendix S1](#)).

### Microbiological analysis

For food samples, 25 g of meat or vegetables were incubated overnight at 35°C in 250 mL of tryptic soy broth supplemented with 8 mg/L vancomycin and 0.25 mg/L cefotaxime. Then, 100  $\mu$ L were streaked on ESBL-specific plates (bioMérieux, Marcy-l'Étoile, France) and incubated overnight at 35°C.

Samples from WWTPs were diluted 1:10 in sterile water, 10  $\mu$ L and 100  $\mu$ L were streaked on ESBL-specific plates and incubated overnight at 35°C. One hundred millilitres of river and LTCF sewage samples were filtered on a 0.45- $\mu$ m filter deposited on ESBL-specific plates and incubated overnight at 35°C.

Bacterial colonies suspected to be *E. coli* or *K. pneumoniae* were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Microflex LT, Bruker Daltonik GmbH, Bremen, Germany) with a log value  $\geq 2$  according to the manufacturer's recommendations. We kept a maximum of three *E. coli* or *K. pneumoniae* isolates with different morphotypes per sample. ESBL production was confirmed by double-disc synergy tests (DDST20 and DDST30) as recommended by EUCAST [19]. All isolates were stored in bead-containing cryotubes (Microbank, PRO-LAB Diagnostics, Richmond Hill, ON, Canada) at  $-80^{\circ}\text{C}$  until further analysis.

### Genome sequencing and analysis

Bacterial DNA extraction and sequencing, read assembly, determination of sequence types (STs) with *in silico* multi-locus sequence typing (MLST), *bla*<sub>ESBL</sub> identification and the identification of incompatibility (Inc) groups of plasmids are detailed in the Supplementary material ([Appendix S1](#)).

Core genomes of ESBL-Ec and ESBL-Kp isolates were determined using existing schemes (<https://www.cgmlst.org/ncs>). The home-made pipeline pyMLST analysed the cgMLST (<https://github.com/bvalot/pyMLST>) by aligning genes present in >95% of the isolates and phylogenetic trees were then constructed (see Supplementary materials ([Appendix S1](#))). A network was built with the package IGRAPH on R 3.6.2 software to link genetically related ESBL-Ec, the genomes of which were distant by fewer than ten genes.

### Statistical analysis

The proportions of food samples contaminated by ESBL-Ec and ESBL-Kp were compared with non-parametric tests (Kruskal–Wallis test coupled with a post-hoc Dunn test). We then performed a test of equal proportions to determine in which cases the contamination was higher or lower. The distribution and association of *bla*<sub>ESBL</sub> genes and plasmid incompatibility groups was also assessed. The  $\alpha$  value was set to 0.05. All analyses were performed with R 3.6.2 software (R Core Team Package 2019).

## Results

### ESBL-Ec and ESBL-Kp isolated from food and hp-environment

We obtained 265 food samples from the LTCF kitchens and 449 food samples from 35 supermarkets (Table 1). Overall, ESBL-Ec or ESBL-Kp contaminated 26.7% (93/349) of the meat samples and 1.9% (7/365) of the vegetable samples. Among these samples, chicken (33.2% positive;  $n = 63$ ) and turkey (75.0% positive;  $n = 20$ ) were more frequently contaminated than other types of food products ( $p \leq 2e^{-16}$ ; Table 1). We also collected 122 samples from hp-environment (Table 1). We retrieved 293 isolates: 254 ESBL-Ec and 39 ESBL-Kp. The contamination of food samples by ESBL-Kp was infrequent with only 12 isolates retrieved in the 714 samples analysed (Table 1). Except for two chicken samples, all other food samples positive for ESBL-Kp came from Tübingen. The country-specific distribution of positive samples is shown in the Supplementary material (Table S1).

### Genomic comparison of ESBL-producing *E. coli* cultured from food and hp-environment

The 254 ESBL-Ec isolated from food ( $n = 96$ ) and hp-environment ( $n = 158$ ) were distributed into 77 different STs (Table 2). Twenty-two and 45 STs were exclusively represented by isolates found in food and in hp-environment, respectively. In contrast, 10 STs (99/254 isolates) were found in both food and hp-environment samples (Table 2). ST131 was specifically found in samples of the hp-environment where it accounted for 21.5% of total ESBL-Ec.

We assessed the genetic relatedness of ESBL-Ec isolates retrieved from food and hp-environment by constructing a Bio Neighbour Joining tree from the 254 genomes. It showed that although the genomes of isolates cultured from food and hp-environment were intermixed in the tree, the cgMLST distances were generally high between the two reservoirs (Fig. 1). We identified only five clusters that contained ESBL-Ec isolates from both hp-environment and food, having a cgMLST distance <40 genes. They were represented by ten genomes of ESBL-Ec from the hp-environment and seven genomes of ESBL-Ec from food (see the green sectors in Fig. 1).

To evaluate the interconnections between the ESBL-Ec populations contaminating food and hp-environment, we built a network linking reservoirs with ESBL-Ec isolates whose genomes had a cgMLST distance <10 genes (Fig. 2). To define the cut-off value under which genomes were considered as genetically related, we created epidemiological groups based on the cities of isolation of the ESBL-Ec, considering that links between cities were epidemiologically unlikely. We then built networks that link the different sources: two sources were linked if each of them included at least one genome having less than 10, 15, 20, 30 or 40 differences in core genes. We then counted the number of unlikely links for each cut-off value. The cut-off value of 10, confirming previous findings [20], was chosen because it minimized the number of mistakenly connected compartments (see Supplementary material, Fig. S1).

Most of the links connected sources of the same type (food or hp-environment) and from the same city. Indeed, we found 15 clusters of hp-environment isolates, one cluster of nine isolates, one of five isolates, and 13 clusters of two isolates. Besides, there were 20 clusters of food isolates, one cluster of seven isolates, two of five isolates, four of three isolates and 13 of two isolates). The only link between the two compartments involved genomes of three meat-contaminating isolates and that of an isolate found in the receiving river in Tübingen (Fig. 2).

### *bla*<sub>ESBL</sub> distribution in *E. coli* cultured from food and hp-environment

Thirteen different *bla*<sub>ESBL</sub> were identified. *bla*<sub>SHV-2</sub> was specific to food-related isolates while *bla*<sub>CTX-M-3</sub>, *bla*<sub>CTX-M-8</sub>, *bla*<sub>CTX-M-24</sub> and *bla*<sub>CTX-M-65</sub> were specific to isolates from hp-environments (see Supplementary material, Fig. S2). *bla*<sub>SHV-12</sub> and *bla*<sub>CTX-M-1</sub> were

**Table 1**

Contamination of food and human-polluted environment with ESBL-Ec and ESBL-Kp (Europe, 2018–2019)

	No. of samples	Positives (%)	ESBL-Ec	ESBL-Kp
Food samples				
Beef	61	4 (6.6)	4	0
Pork	51	3 (8.9)	3	0
Chicken	190	63 (33.2)	64	3
Fish	18	2 (11.1)	3	0
Turkey	28	21 (75.0)	16	8
Lamb	1	0	0	0
Overall meat/fish	349	93 (26.7)	90	11
Vegetables	365	7 (1.9)	6	1
Food total	714	100 (14.0)	96	12
Hp-environment				
LTCF sewages	30	22 (73.3)	19	10
WWTP inflows	40	40 (100)	66	8
Rivers downstream WWTPs	52	47 (90.4)	73	9
Hp-environment total	122	109 (89.3)	158	27
Total	836	211	254	39

Abbreviations: ESBL-Ec, extended-spectrum  $\beta$ -lactamase-producing *Escherichia coli*; ESBL-Kp, extended-spectrum  $\beta$ -lactamase-producing *Klebsiella pneumoniae*; hp-, human-polluted; LTCF, long-term care facility; WWTP, wastewater treatment plant.

The table shows the number and nature of food and hp-environment samples collected in the five European cities (Besançon, Geneva, Sevilla, Tübingen and Utrecht), the number and proportion of samples positive for ESBL-Ec or ESBL-Kp and the number of ESBL-Ec and ESBL-Kp isolated from each type of sample. The distribution of the samples according to the city of isolation is detailed in the Supplementary material (Table S1).

**Table 2**  
Distribution of STs among extended-spectrum  $\beta$ -lactamase-producing *Escherichia coli* found in the human-polluted environment and food (Europe, 2018–2019)

All sources		Overall hp-environment		Overall food	
ST	n (%)	ST	n (%)	ST	n (%)
ST131	34 (13.4)	ST131	34 (21.5)	ST10	10 (11.5)
ST10	26 (10.2)	ST10	16 (9.5)	ST155	10 (10.4)
ST155	14 (5.5)	ST38	11 (7)	ST69	9 (9.4)
ST38	14 (5.5)	ST949	10 (6.3)	ST1011	6 (6.3)
ST69	13 (5.1)	ST58	9 (5.7)	ST354	5 (5.2)
ST58	13 (5.1)	ST1193	5 (3.2)	ST58	4 (4.2)
ST949	10 (3.9)	ST155	4 (2.5)	ST533	4 (4.2)
ST1011	6 (2.4)	ST69	4 (2.5)	ST117	4 (4.2)
ST354	5 (2.0)	ST1431	4 (2.5)	ST101	4 (4.2)
ST533	5 (2.0)	ST88	3 (1.9)	ST115	4 (4.2)
ST117	5 (2.0)	ST44	3 (1.9)	ST48	4 (4.2)
ST1193	5 (2.0)	ST3995	2 (1.3)	ST38	3 (3.1)
ST101	4 (1.6)	ST405	2 (1.3)	ST4981	2 (2.0)
ST115	4 (1.6)	ST410	2 (1.3)	ST1249	2 (2.0)
ST48	4 (1.6)	ST227	2 (1.3)	ST3519	2 (2.0)
ST88	4 (1.6)	ST23	2 (1.3)	ST4937	2 (2.0)
ST1431	4 (1.6)	ST752	2 (1.3)	ST602	2 (2.0)
ST4981	3 (1.2)	ST95	2 (1.3)	ST746	2 (2.0)
ST44	3 (1.2)	ST1123	2 (1.3)	ST398	2 (2.0)
ST1249	2 (0.8)	ST2253	2 (1.3)	ST7204	2 (2.0)
ST3519	2 (0.8)	ST34	2 (1.3)	Singletons	12 (12.5)
ST4937	2 (0.8)	ST3541	2 (1.3)	—	—
ST602	2 (0.8)	ST993	2 (1.3)	—	—
ST746	2 (0.8)	Singletons	32 (20.2)	—	—
ST398	2 (0.8)	—	—	—	—
ST7204	2 (0.8)	—	—	—	—
ST362	2 (0.8)	—	—	—	—
ST3995	2 (0.8)	—	—	—	—
ST405	2 (0.8)	—	—	—	—
ST410	2 (0.8)	—	—	—	—
ST227	2 (0.8)	—	—	—	—
ST23	2 (0.8)	—	—	—	—
ST752	2 (0.8)	—	—	—	—
ST95	2 (0.8)	—	—	—	—
ST1123	2 (0.8)	—	—	—	—
ST2253	2 (0.8)	—	—	—	—
ST34	2 (0.8)	—	—	—	—
ST3541	2 (0.8)	—	—	—	—
ST993	2 (0.8)	—	—	—	—
Singletons	38 (15.0)	—	—	—	—
Total	254		158		96

Abbreviations: Hp-environment, human-polluted environment; STs, sequence types.

more often associated with food-related isolates (34% and 32% of the isolates, respectively) compared with isolates from hp-environments (3%,  $p = 1 \times 10^{-11}$  and 12%  $p = 8 \times 10^{-5}$  of the isolates, respectively). *bla*<sub>CTX-M-15</sub> and *bla*<sub>CTX-M-27</sub> dominated in ESBL-Ec from hp-environments (52% and 15% of the isolates, respectively) in contrast with food-related ESBL-Ec (11%,  $p = 1 \times 10^{-13}$  and 6%  $p = 0.043$  of the isolates, respectively). Although we found the ST10 clone in similar proportions in the ESBL-Ec populations in food and in the hp-environment (11.5% and 9.5% of the isolates, respectively;  $p = 0.62$ ), ST10 isolates generally harboured different *bla*<sub>ESBL</sub> genes (11/15 hp-environment samples harboured *bla*<sub>CTX-M-15</sub> whereas 1/10 food-related isolates harboured this gene). Moreover, we found only one hp-environment isolate belonging to ST10 that had <40 different core genes with two food isolates, with these three isolates being collected in distinct cities (Geneva, Besancon and Tübingen).

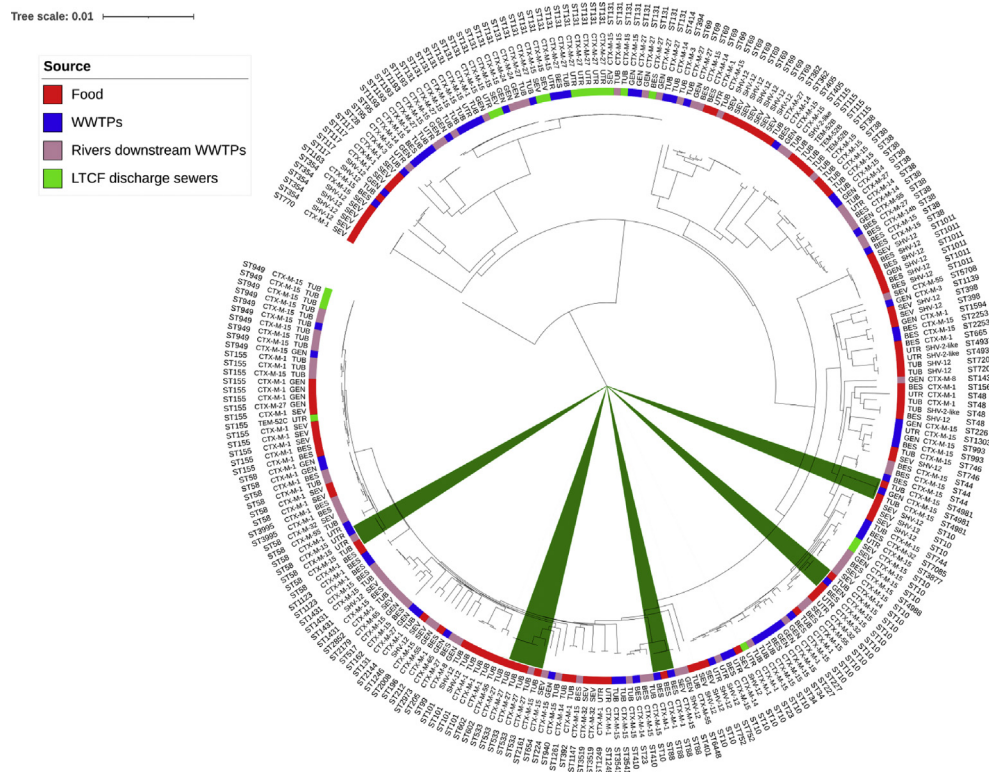
We then analysed the distribution of plasmid incompatibility groups and found that it was homogeneous among the ESBL-Ec isolates of the two reservoirs except for IncB/O/K/Z, which was more often associated with food-related isolates ( $p = 3 \times 10^{-7}$ ), and IncFIA groups, which were more frequently found in isolates from the hp-environment ( $p = 0.019$ ) (see Supplementary material, Table S2).

#### Comparison of genomes and *bla*<sub>ESBL</sub> of ESBL-producing *K. pneumoniae* isolates cultured from food and hp-environment

Among the 39 ESBL-Kp isolates retrieved in this study, 12 were found in food and 27 in the hp-environment. We identified 22 different STs, among which 16 were singletons. Eight STs were specific to the food, 13 were specific to hp-environment, and one ST was shared between the two reservoirs. Indeed, ST219 isolates were found in one river sample of Geneva and Sevilla and in two meat samples (one chicken and one turkey) in Tübingen. We built a maximum-likelihood tree to investigate the relatedness of all ESBL-Kp genomes (Fig. 3). Only the ST219 was found in both food and the hp-environment, but of different cities: two isolates collected from food products in Tübingen had <40 different core genes with one isolate collected in the river in Sevilla. We identified six different ESBL-encoding genes with an overrepresentation of *bla*<sub>CTX-M-15</sub>, carried by 87.2% of the ESBL-Kp isolates (Fig. 3).

#### Discussion

We compared the genomes of ESBL-Ec and ESBL-Kp isolates cultured from food items (96 ESBL-Ec and 12 ESBL-Kp) with those from hp-environment (158 ESBL-Ec and 27 ESBL-Kp) in five



**Fig. 1.** Bio Neighbour Joining tree based on the core genome of the 254 extended-spectrum  $\beta$ -lactamase-producing *Escherichia coli* (ESBL-Ec) isolates cultured from food items and human-polluted (hp-) environment (Europe, 2018–2019). The tree was based on HKY85 distances calculated from the core genome constituted of 2275 genes. Sources: LTCF, long-term care facility; WWTP, wastewater treatment plant; city of isolation: TUB, Tübingen; BES, Besançon; SEV, Sevilla; UTR, Utrecht; GEN, Geneva; sequence types (STs) and *bla*<sub>ESBL</sub> gene are indicated for each isolate. The yellow sectors indicate clusters of isolates retrieved from the same LTCF discharge sewer at different time-points. The green sectors indicate clusters of isolates which genomes had <40 different core genes.

European cities. The main finding of this international study was that the population of these ESBL-Ec and ESBL-Kp cultured from these two reservoirs were genetically different, and that ESBL-Ec generally had *bla*<sub>ESBL</sub> genes of different nature.

Samples were collected in five European cities distributed from north to south in Western Europe to reflect the diversity of climate, food production and veterinary antibiotic consumption that could affect the contamination level of the different reservoirs by ESBL-PE. Our collection of ESBL-Ec found in hp-environment, dominated by ST131 and ST38 mostly carrying *bla*<sub>CTX-M-15</sub> and *bla*<sub>CTX-M-27</sub>, mirrored that of ESBL-Ec found in humans [10,16,17,21–23]. Similarly, we identified pandemic clones of ESBL-Kp (i.e. ST37, ST307) in the hp-environments. Food samples were gathered from LTCF kitchens and supermarkets to make the collection representative for most consumed food, both in collective catering and by ESBL-PE carriers [18] (Table 1, and see Supplementary material, Table S1). The contamination frequency of meat products and vegetables found in this study was consistent with those observed in the literature, with infrequent contamination of vegetables and more frequent contamination of poultry products [5,6,10,24].

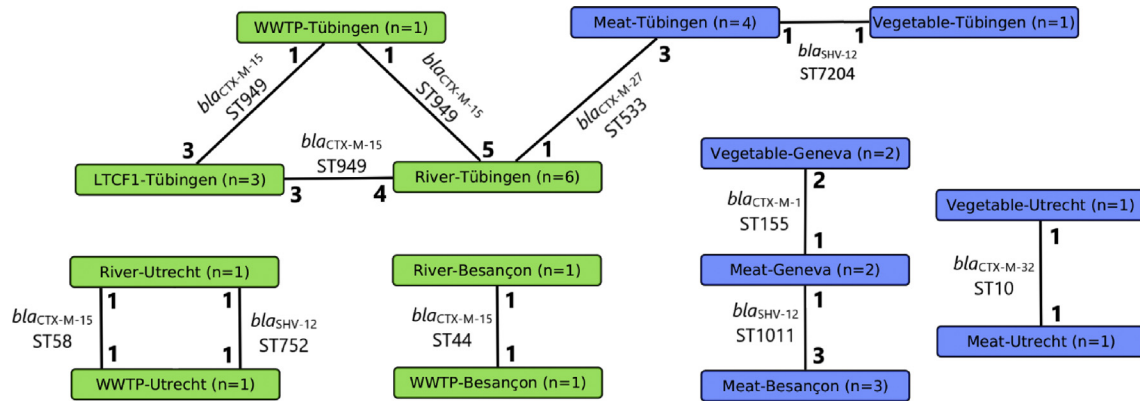
The cgMLST analysis revealed that the populations of ESBL-Ec cultured from food and hp-environment are generally dissociated, with only six isolates out of 254 (four from food products and two from the hp-environment) that could be linked (Figs. 1 and 2). We found a majority of *bla*<sub>CTX-M-15</sub> carriers among ESBL-Kp. The dominance of *bla*<sub>CTX-M-15</sub> in wastewater and retail meat has been previously described [16,25]. The population of ESBL-Kp was mostly represented by distinct clones, with 22 different STs and with no linked isolates (i.e. which genomes had fewer than ten different core genes between the reservoirs). Overall, this suggests

that transmission of ESBL-Ec and ESBL-Kp strains from food items to humans is a rare event. This supports the idea that *E. coli* ST131 and other B2 clonal groups are not food-related and are mostly transmitted from human to human [10,15,24,26]. ST10 has been previously detected in humans, animals and in the environment [27]. Here, ESBL-Ec ST10 was equally distributed between food and the hp-environment but the isolates were genetically distinct and carried reservoir-specific *bla*<sub>ESBL</sub> genes, which limits the chance of a common origin [12].

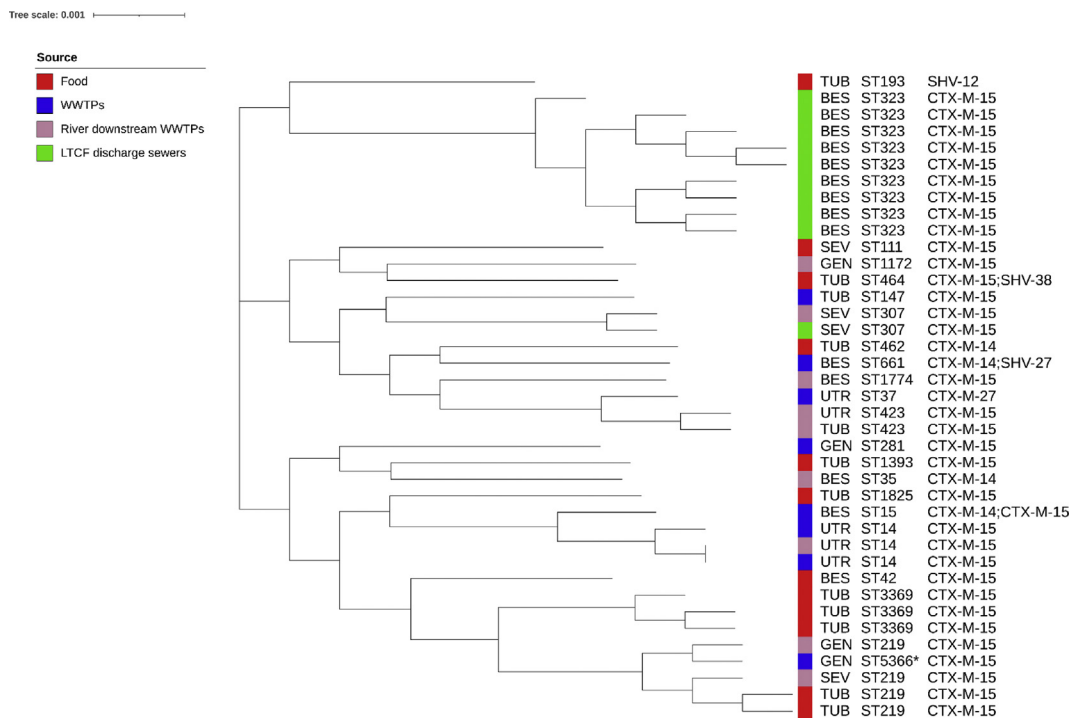
We further compared the distribution of *bla*<sub>ESBL</sub> genes in isolates from food and hp-environment (see Supplementary material, Fig. S2) and found that 5 out of 13 genes were specific to a compartment. Among the other genes, *bla*<sub>SHV-12</sub> and *bla*<sub>CTX-M-1</sub> predominated in food isolates, as reported by others [22,26,28]. Overall, distinct *bla*<sub>ESBL</sub> genes predominated in the two reservoirs. Plasmids carrying *bla*<sub>ESBL</sub> can spread between phylogenetically distinct *E. coli* populations, possibly contributing to the human contamination with ESBL through the food chain [29]. It has been hypothesized that other food-related clones might serve as the source for plasmids or mobile genetic elements with resistance determinants to supply the major clone ST131, which is wide open for plasmid exchange [30]. Even if distinct genes predominated in the two reservoirs, plasmid reconstruction with long-read sequencing could help to identify transfer between the two reservoirs for the other genes.

We found here a limited concordance between the food and hp-environment reservoirs. This is in line with previous studies [10,12] but might appear as contrasting with a monocentric modelling study of the source distribution in a high-income country [9] where food products accounted for 18.9% of human contamination.





**Fig. 2.** Network analysis of the genomes of ESBL-Ec cultured from food and hp-environment (Europe, 2018–2019). The network connects the sources with ESBL-Ec which genomes had <10 core genes of difference. The rectangles represent the sources of the samples with hp-environments in green, food in blue (n = number of isolates of the source linked with isolate(s) of other sources). Only sources linked with  $\geq 1$  other source are shown. Figures indicate the number of ESBL-Ec genomes of the corresponding source linked to the other source. STs and  $bla_{ESBL}$  genes of the isolates linked are indicated.



**Fig. 3.** Maximum likelihood tree based on single nucleotide polymorphisms in the core genes ( $n = 2279$ ) of 39 *Klebsiella pneumoniae* isolates producing extended-spectrum  $\beta$ -lactamase cultured from food samples and human-polluted (hp-) environment (Europe, 2018–2019). Source: LTCF, long-term care facility; WWTP, wastewater treatment plant; city of isolation: TUB, Tübingen; BES, Besançon; SEV, Sevilla; UTR, Utrecht; GEN, Geneva. Sequence types (STs) and  $bla_{ESBL}$  gene(s) are indicated for each isolate. \* only one mutation in allele *phoE* (A314G) differentiates ST5366 from ST219.

However, this contribution could have been overestimated because a model based on identity of *bla* genes was being used, while we also considered their bacterial vehicle. Although we cannot exclude the possibility of human colonization with food-borne ESBL-Ec and ESBL-Kp, our data suggest that, in Western Europe, human-to-human transmission plays a more important role in colonization of humans rather than contamination by food items. However, the situation may be radically different in low- and middle-income countries where interconnections between humans, animals and the environment are stronger [8].

Our study has several limitations. Although we isolated the two most relevant ESBL producers in the community and in hospitals (ESBL-Ec and ESBL-Kp, respectively), we have neglected  $bla_{ESBL}$

borne by other species [22,31]. The ultimate identification of horizontal gene transfer events requires plasmid reconstruction from long-read sequencing data and the comparison of plasmids carrying identical  $bla_{ESBL}$  gene (manuscript in preparation). However, long-read sequencing would not have changed the main conclusions because there is a limited similarity between  $bla_{ESBL}$  genes carried by the isolates of the two reservoirs.

Overall, we found a very limited connection between ESBL-Ec and ESBL-Kp populations and  $bla_{ESBL}$  genes retrieved in retail food and that retrieved from hp-environment. This suggests that the human-to-human contamination, rather than the food chain, is likely the most frequent route of transmission for ESBL-Ec and ESBL-Kp in high-income countries.

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### Transparency declaration

SP reports receiving consulting fees from Illumina and IDbyDNA and honoraria for presentation at Institute of Medical Microbiology, University Cologne, Germany. All other authors have nothing to disclose.

### Role of the funding source

This study was part of a Joint Programming Initiative on Antimicrobial Resistance collaborative research project, under the 2016 Joint Call framework (Transnational Research Projects on the Transmission Dynamics of Antibacterial Resistance). It received funding from the following national research agencies: Instituto de Salud Carlos III (grant no. AC16/00076), Netherlands Organization for Health Research and Development (grant no. 681055, 547001004), Swiss National Science Foundation (grant no. 40AR40-173608), German Federal Ministry of Education and Research (grant no. 01KI1830) and the French Agence Nationale de la Recherche (grant no. ANR-16-JPEC-0007-03).

Elena Salamanca, Mercedes Delgado-Valverde, Alvaro Pascual and Jesús Rodríguez-Baño received support for research from by the Plan Nacional de I+D+i 2013-2016 and Instituto de Salud Carlos III, Subdirección General de Redes y Centros de Investigación Cooperativa, Ministerio de Ciencia, Innovación y Universidades, Spanish Network for Research in Infectious Diseases (REIPI RD16/0016/0001), co-financed by the European Development Regional Fund *A way to achieve Europe*, Operative Program Intelligence Growth 2014–2020.

### Author contributions

All authors contributed to the study design or conduct. DH, DM, AM, TV, SH, ET, JAJWK and JRB wrote the study protocol. DH, JAJWK, SH, JRB, ET and BSC obtained funding. DM, TV, NC, SB, AM, MER, ES and CB collected the samples. DM, AM, AC, SP, ACF, JG, AP, MD-V and DH performed or supervised the microbiological analyses in their local centres. JAJWK, ACF, TV, SP and JG provided a standardized procedure for whole-genome sequencing. JG and SP performed and supervised sequencing. CPH and BV performed statistical analyses. DM, BV and JG performed genetic analyses. DH, JAJWK, SH, JRB and ET supervised the study in their local institution as principal investigators. DM, XB and DH drafted the manuscript and all authors reviewed and contributed to the manuscript. DH coordinated the project.

### Acknowledgements

We would like to thank the staff and residents from the LTCFs of Bellevaux in Besançon, France; Ferrusola and El Recreo in Sevilla, Spain; Luise-Wetzel-Stift and Samariterstift in Tübingen, Germany and Altenahove in Almerker, the Netherlands for their involvement

in the study. We would like to thank Jelle Scharringa and Heike Schmitt from Utrecht, the Netherlands; Marion Broussier from Besançon, France; John Poté and Siva Lingam from Geneva, Switzerland and Michael Eib from Tübingen, Germany for their help on microbiological analyses of the samples, and Elisabeth Stoll and Steffen Ganß from Tübingen, Germany for collecting the samples.

### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.cmi.2021.07.022>.

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## Supplementary data

### Appendix S1: supplementary methods

**Food and hp-environment sampling.** In LTCF kitchens, at least four meat samples and four vegetable samples were collected eight times over a 32-week period from the kitchens of six LTCFs (one LTCF from Besançon, two from Tübingen, two from Sevilla, and one from Utrecht). Meat and vegetable samples were collected from supermarkets between January 2018 and August 2019 including 8 supermarkets in Besançon, 14 in Tübingen, 3 in Sevilla, 6 in Utrecht, and 4 in Geneva.

For hp-environment samples, two hundred and fifty mL of water were collected for WWTP inflow and LTCF discharge sewer samples and 500 mL were collected for river samples. We processed each sample within four hours of collection.

**Whole genome sequencing and bioinformatics.** *E. coli* and *K. pneumoniae* strains showing an ESBL-producing phenotype were restored from deep freezing on Columbia agar supplemented with 5% defibrinated sheep blood and incubated over night at 35°C. To exclude possible contamination, species identity was re-evaluated after the thawing process by MALDI-TOF MS before DNA extraction.

Bacterial DNA was extracted using QIAGEN DNeasy UltraClean Microbial Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Library preparation was performed with Nextera DNA Flex Library Prep Kit (Illumina, San Diego, CA, USA) followed by tagging with Nextera DNA CD Indexes (Illumina). Strains were whole-genome sequenced on the NextSeq platform with Illumina Mid-Output Kit v2, generating 150-bp paired-end reads.

Raw reads were trimmed to a minimum length of 80 base pairs with Trimmomatic v0.35 [1] and *de novo* assembled with SPAdes v3.13.0 [2]. ESBL-encoding genes were identified using ResFinder v3.1 [3]. Sequence types (STs) were determined with *in silico* multi-locus sequence typing (MLST) [4].

We identified the incompatibility (Inc) groups of plasmids present in ESBL-Ec genomes using PlasmidFinder [5].

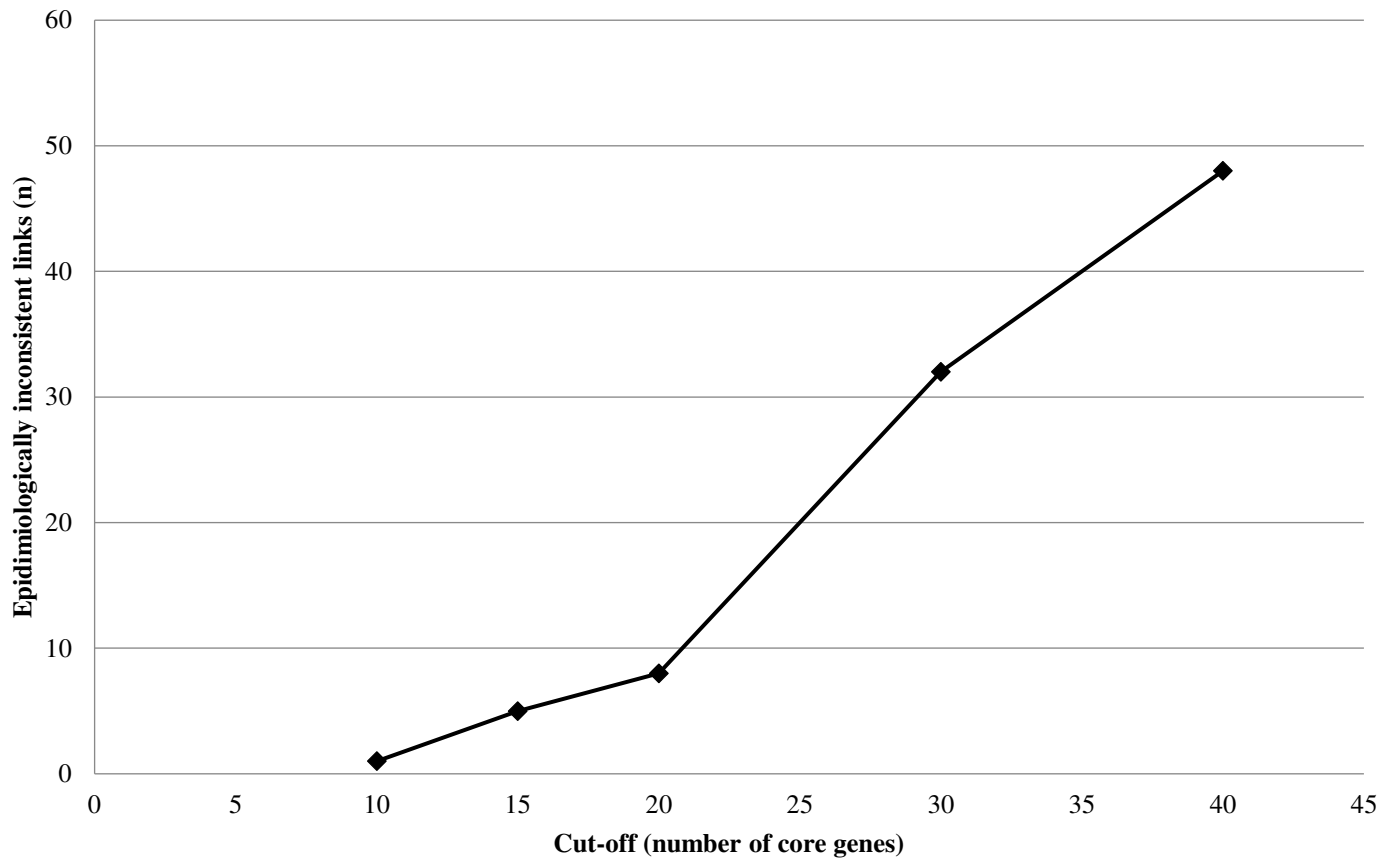
After cgMLST analysis, for ESBL-Ec, a Bio Neighbor Joining (BioNJ) tree based on differences on the core genes was constructed with SplitsTree5 [6] with a HKY85 distance calculation model. For ESBL-Kp, a maximum-likelihood tree was constructed with a GTR model using FastTree 2.1 [7]. We annotated and visualized the trees with iTOL v5 [8].

Phylogroups of *E. coli* were determined *in silico* with the Clermont method [9].

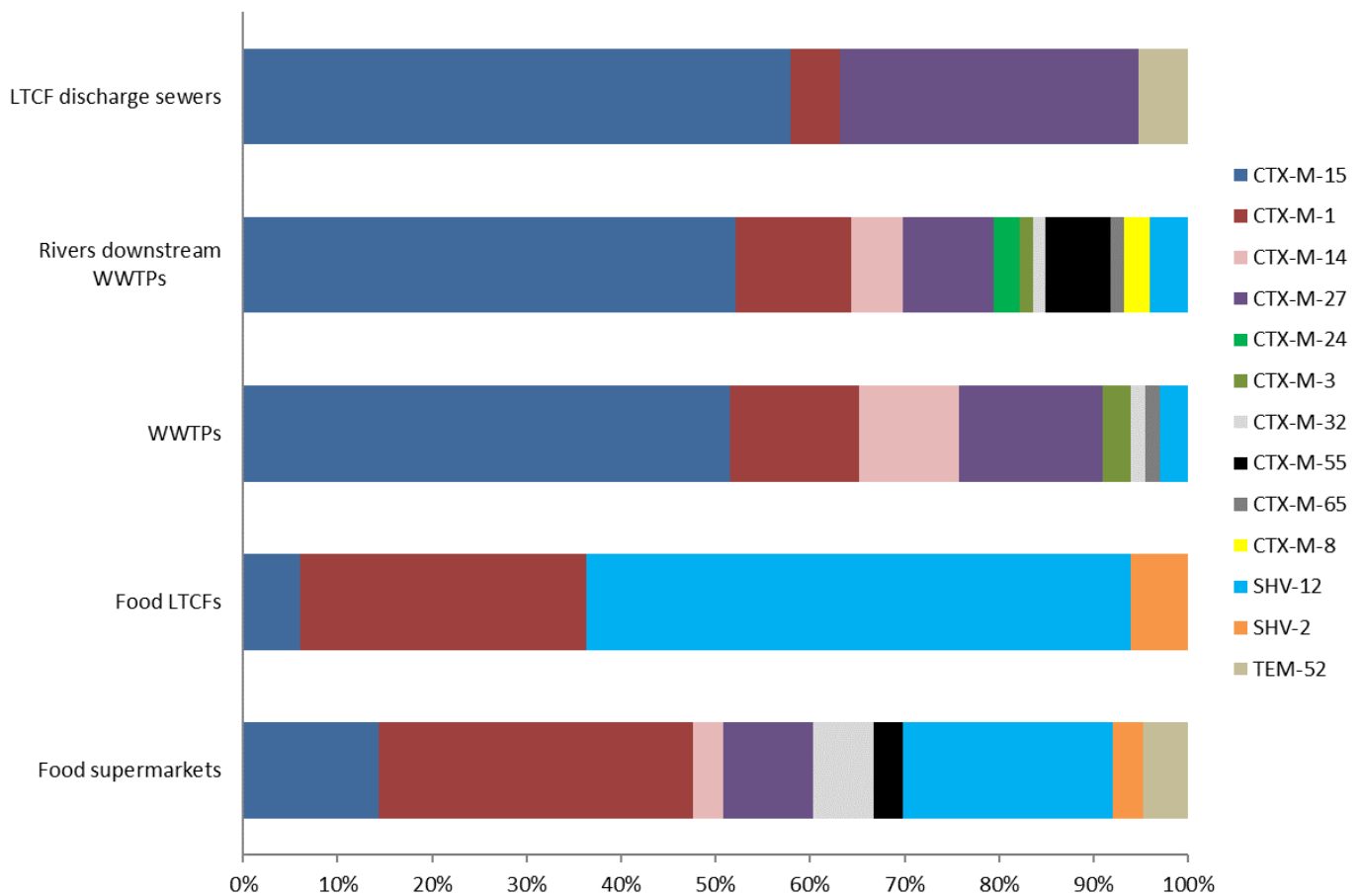
## Supplementary tables and figures

**Table S1. Number and nature of food and hp-environment samples collected in the different European cities and number and proportion of samples positive for extended-spectrum  $\beta$ -lactamase producing *E. coli* (ESBL-Ec) or *K. pneumoniae* (ESBL-Kp). Shaded cells indicate the absence of samples.**

	Besançon (France)		Tübingen (Germany)		Sevilla (Spain)		Geneva (Switzerland)		Utrecht (The Netherlands)		Samples total	Positives total (%)
	n	No. of positive (%)	n	No. of positive (%)	n	No. of positive (%)	n	No. of positive (%)	n	No. of positive (%)		
<b>Food samples</b>												
Beef	10	0 (0)	37	4 (10.8)					14	0 (0)	61	4 (6.6)
Pork	8	1 (12.5)	32	2 (6.3)					11	0 (0)	51	3 (8.9)
Chicken	36	11 (30.6)	32	9 (28.1)	51	33 (64.7)	42	3 (7.1)	29	7 (24.1)	190	63 (33.2)
Fish	4	0 (0)	8	2 (25.0)					6	0 (0)	18	2 (11.1)
Turkey	1	1 (100)	27	20 (74.1)							28	21 (75.0)
Lamb	1	0 (0)									1	0 (0)
<b>Overall meat/fish</b>	<b>60</b>	<b>13 (21.7)</b>	<b>136</b>	<b>37 (27.2)</b>	<b>51</b>	<b>33 (64.7)</b>	<b>48</b>	<b>3 (6.2)</b>	<b>54</b>	<b>7 (13)</b>	<b>349</b>	<b>93 (26.7)</b>
<b>Vegetables</b>	<b>63</b>	<b>1 (1.6)</b>	<b>166</b>	<b>4 (2.4)</b>	<b>32</b>	<b>0 (0)</b>	<b>48</b>	<b>3 (6.2)</b>	<b>56</b>	<b>1 (1.8)</b>	<b>365</b>	<b>9 (2.5)</b>
<b>Food total</b>	<b>123</b>	<b>14 (11.4)</b>	<b>302</b>	<b>41 (13.6)</b>	<b>83</b>	<b>33 (39.8)</b>	<b>96</b>	<b>6 (6.2)</b>	<b>110</b>	<b>8 (7.3)</b>	<b>714</b>	<b>102 (14.3)</b>
<b>Hp-environment samples</b>												
LTCF sewages	7	6 (85.7)	8	4 (50.0)	7	4 (57.1)			8	8 (100)	30	22 (73.3)
WWTP inflows	8	8 (100)	16	16 (100)			8	8 (100)	8	8 (100)	40	40 (100)
Rivers downstream WWTPs	8	7 (87.5)	22	20 (90.9)	6	6 (100)	8	8 (100)	8	6 (75.0)	52	47 (90.4)
<b>Hp-environment total</b>	<b>23</b>	<b>21 (91.3)</b>	<b>46</b>	<b>40 (86.9)</b>	<b>13</b>	<b>10 (76.9)</b>	<b>16</b>	<b>16 (100)</b>	<b>24</b>	<b>22 (91.7)</b>	<b>122</b>	<b>109 (89.3)</b>



**Figure S1. Number of epidemiologically inconsistent links in the network analysis of the genomes of ESBL-Ec cultured from food and hp-environment of five European cities.** The networks created connect the sources with ESBL-Ec which genomes had <10, 15, 20, 30, or 40 core genes of difference (cut-off) out of 2275 core genes. Epidemiologically inconsistent links were defined as the links between sources from different cities and counted depending on the cut-off.



**Figure S2. Distribution of the *bla*<sub>ESBL</sub> genes identified in the 254 extended-spectrum  $\beta$ -lactamase producing *E. coli* isolates cultured from food and hp-environment (Europe, 2018-2019).** The gene types found in the different sources are expressed in proportion over the total number of genes per source. Source (LTCF: long-term care facility; WWTP: wastewater treatment plant) and *bla*<sub>ESBL</sub> gene(s) are indicated for each isolate. For the ease of reading, the letters ‘*bla*’ have been omitted in the color code legend.

**Table S2. Distribution of plasmid replicons (Inc groups) identified in extended-spectrum  $\beta$ -lactamase producing *E. coli* (ESBL-Ec) collected in the different European cities.**

	Col	IncA	IncB/O/K/Z	IncC	IncFIA	IncFIB	IncFIC	IncFII	IncHI1A	IncHI1B	IncHI2	IncHI2A	IncI	IncI1-1	IncI2	IncN	IncN2	IncR	IncX1	IncX2	IncX3	IncX4	IncX5	IncY	p0111
<b>WWTP inflows</b>	83	0	9	1	19	47	2	64	0	0	1	1	4	24	0	0	1	0	5	0	0	5	0	4	4
<b>Rivers downstream WWTP</b>	94	1	5	1	25	58	1	69	2	2	0	0	4	27	2	0	0	0	7	0	3	3	1	7	3
<b>LTCF sewages</b>	41	1	3	0	10	14	0	17	0	0	0	0	0	4	0	3	0	0	0	0	0	0	0	3	0
<b>Hp-environment total</b>	218	2	17	2	54	119	3	150	2	2	1	1	8	55	2	3	1	0	12	0	3	8	1	14	7
<b>Food</b>	177	0	37	3	22	70	3	94	0	3	4	4	4	41	9	2	0	4	14	1	5	5	0	4	29



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**Titre :** Déterminismes environnementaux de la diffusion et de la survie des bacilles à Gram négatif commensaux et pathogènes dans les hydrosystèmes

**Mots clés :** *One Health*, bacilles à Gram négatif, antibiorésistance, *Escherichia coli*, écosystèmes alluviaux, zones humides

**Résumé :** Le concept *One Health* suppose que les santés humaine, animale et environnementale sont interconnectées. Il promeut une approche holistique et transdisciplinaire pour appréhender les problèmes de santé publique. Les risques infectieux et la résistance aux antibiotiques sont des préoccupations majeures du concept *One Health*. L'environnement est souvent relégué au second plan lors de la mise en place d'approches *One Health*. Ce travail de recherche vise à combler le manque de connaissances quant à la diffusion des bacilles à Gram négatif commensaux et pathogènes (BGN-CP) dans l'environnement. *Escherichia coli*, *Klebsiella pneumoniae* et *Pseudomonas aeruginosa* sont trois espèces bactériennes associées à l'Homme qui acquièrent facilement des déterminants de résistance aux antibiotiques. Ces BGN-CP contaminent l'environnement, principalement *via* les eaux usées et les activités agricoles. Dans ce travail, nous avons étudié le devenir des BGN-CP dans les écosystèmes aquatiques des plaines alluviales. *E. coli*, *K. pneumoniae* et *P. aeruginosa* sont largement dispersées dans les hydrosystèmes. Leur survie, influencée par les facteurs abiotiques et biotiques, est généralement faible dans l'environnement. Toutefois, certaines conditions favorisent leur survie, voire leur prolifération dans des écosystèmes spécifiques. Certaines zones humides peuvent constituer des réservoirs environnementaux de BGN-CP. L'environnement joue un rôle mineur dans la contamination par les BGN-CP dans les pays à revenus élevés, où la transmission interhumaine prédomine. En revanche, la situation peut être beaucoup plus complexe dans les pays à revenus faibles et intermédiaires où les compartiments humain et environnement sont plus connectés. Bien que l'environnement est supposé jouer un rôle limité dans la rétro contamination de l'Homme par les BGN-CP dans les pays à revenus élevés, sa prise en compte dans l'approche *One Health* est indispensable car les changements induits par l'Anthropocène pourraient accroître le risque sanitaire pour les populations humaines.

**Title:** Environmental determinants of the dispersal and the survival of commensal and pathogenic Gram-negative bacteria in hydrosystems

**Keywords:** One Health, Gram-negative bacteria, antibiotic-resistance, *Escherichia coli*, floodplain ecosystems, wetlands

**Abstract:** The One Health concept assumes that human, animal and environmental health are interconnected. It promotes a holistic and transdisciplinary approach to public health issues. Infectious risks and antibiotic resistance are key issues of the One Health concept. The environment is often neglected when implementing One Health approaches. This research work aims to fill the lack of knowledge about the diffusion of commensal and pathogenic Gram-negative bacteria (CP-GNB) in the environment. *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* are three bacterial species associated with humans that readily acquire antibiotic resistance determinants. These CP-GNB contaminate the environment, mainly *via* wastewater and agricultural activities. In this work, we studied the fate of CP-GNB in aquatic ecosystems of floodplains. *E. coli*, *K. pneumoniae* and *P. aeruginosa* are widely dispersed in hydrosystems. Their survival, influenced by abiotic and biotic parameters, is generally low in the environment. However, specific factors favour their survival, and even their thrive in specific ecosystems. Some wetlands can be environmental reservoirs of CP-GNB. The environment plays a minor role in the human contamination with CP-GNB in high-income countries, where human-to-human transmission predominates. In contrast, the situation could be much more complex in low- and middle-income countries, where the human and environment reservoirs are more connected. Although the environment is thought to play a limited role in the retro contamination of human by CP-GNB in high-income countries, its consideration is essential in the One Health approach since changes induced by the Anthropocene could increase health risk for human populations.