

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.

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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 β -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).

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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 m³.s⁻¹, the Doubs River has an average monthly discharge of 161.8 m³.s⁻¹ and the Loue River has

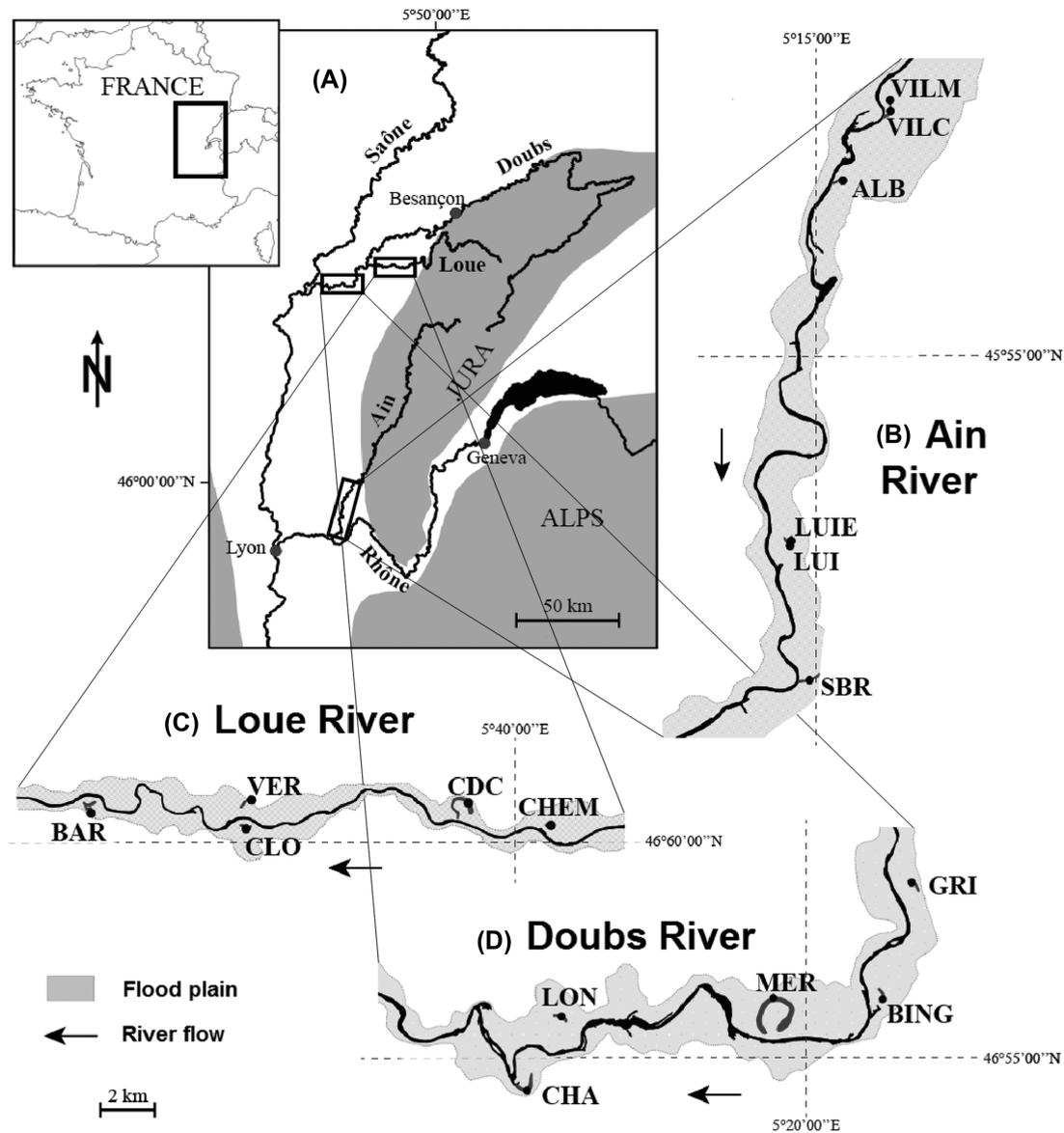


Figure 1. Locations of the 16 studied wetlands. The top left panel represents the locations of the three considered hydrosystems in France. **A.** Location of the river network under study on the map of France. Boxes indicate the locations of the river sections shown in panels b, c and d. The three other 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 Inglas), MER (Mératon), LON (Longepierre) and CHA (Charette).

an average monthly discharge of $49.0 \text{ m}^3 \cdot \text{s}^{-1}$ (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-PO₄³⁻) concentration was measured using the molybdenum blue colouration method after acidification. The N-ammonium (N-NH₄⁺) concentration was measured using the indophenol blue method (Berthelot's reaction, Merck Spectroquant 14 752). The N-nitrate (N-NO₃⁻) 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 ($y = ax + b$) 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, 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 α 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 ²
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

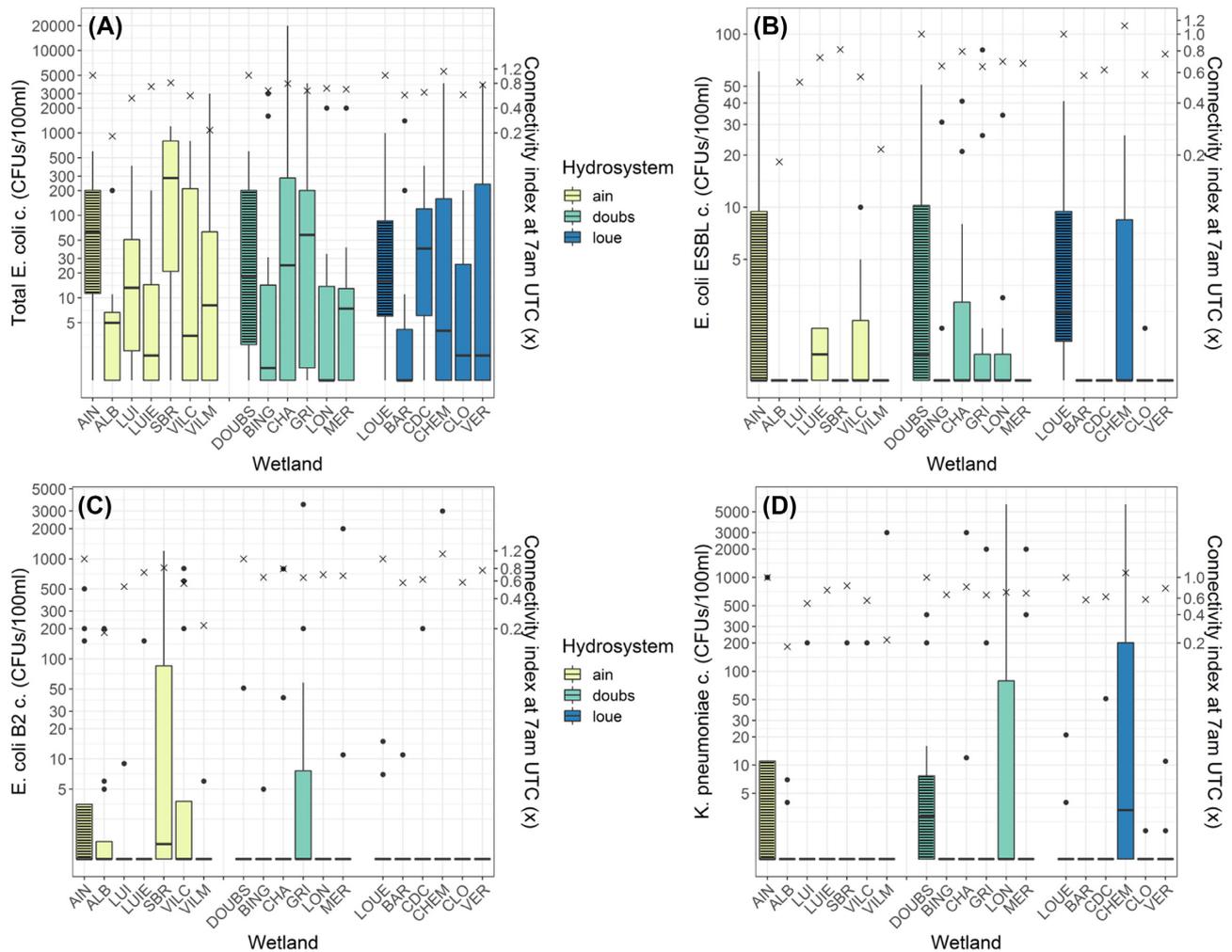


Figure 2. Concentrations (‘c.’) of pathogenic bacterial species in rivers and their wetlands. A. Total *E. coli*, B. ESBL *E. coli*, C. *E. coli* B2, and D. *K. pneumoniae* across space (sites) and according to the connectivity between wetlands and their associated rivers (connectivity index, crossed 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 (↑) or lower (↓) 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
Total <i>E. coli</i>	* river, ↑	x	x	x	x	x	* SBR, ↑	x
ESBL <i>E. coli</i>	*** river, ↑	*** Ain, ↑; Doubs, ↑; Loue, ↑; CHA, ↑; CHEM, ↑; LUIE, ↑	*** CHA, ↑; CHEM, ↑; GRI, ↑; LUIE, ↑; VILC, ↑	x	x	x	x LUIE, ↑; VILC, ↑	* CHEM, ↑
<i>E. coli</i> B2	x	x	x	x	x	x	x	x
<i>K. pneumoniae</i>	** river, ↑	** Ain, ↑; Doubs, ↑; CHEM, ↑; LON, ↑	* CHEM, ↑; LON, ↑	x	x	x	x	** CHEM, ↑
TEMPORAL VARIABILITY – SEASONS								
Total <i>E. coli</i>	-	** summer, ↓	* summer, ↓	*** summer, ↓; winter, ↑	-	x	** summer, ↓	* autumn, ↑
ESBL <i>E. coli</i>	-	*** summer, ↓; winter, ↑	*** summer, ↓; winter, ↑	*** summer, ↓; winter, ↑	-	x	*** winter, ↑	* winter, ↑
<i>E. coli</i> B2	-	x	x	x	-	x	x	x
<i>K. pneumoniae</i>	-	x	x	x	-	* autumn, ↑	x	x
TEMPORAL VARIABILITY – MONTHS								
Total <i>E. coli</i>	-	* September, ↑	* September, ↑	* August, ↓; November, ↓	-	x	x	x
ESBL <i>E. coli</i>	-	*** February, ↑; March, ↑	** February, ↑; March, ↑	* February, ↑	-	x	* December, ↑; January, ↑; February, ↑	x
<i>E. coli</i> B2	-	x	x	* September, ↑	-	x	x	x
<i>K. pneumoniae</i>	-	x	** August, ↑; September, ↑	x	-	** August, ↑; September, ↑	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

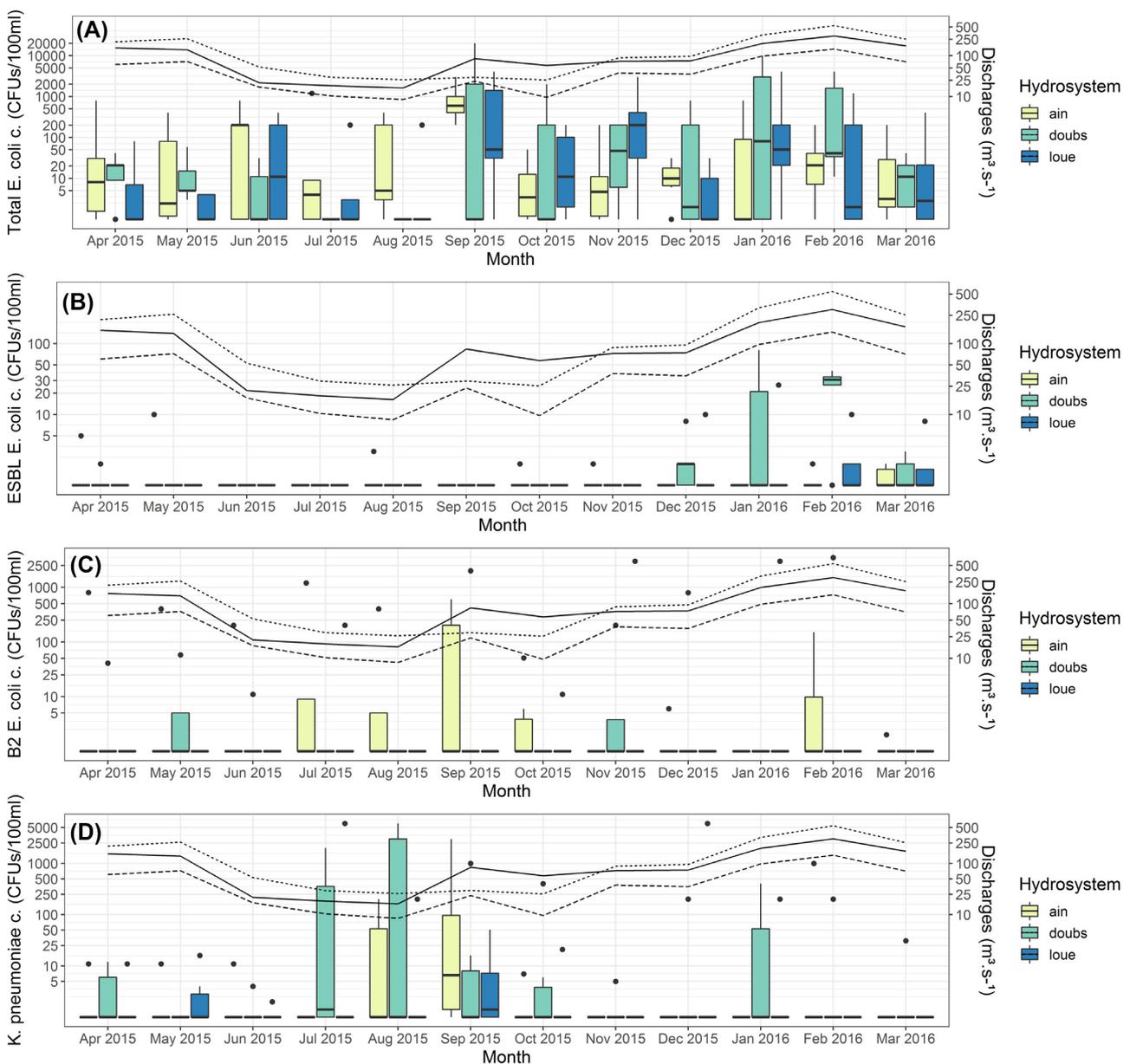


Figure 3. Temporal variation in the concentrations ('c.') of pathogenic bacterial species in wetlands. **A.** Total *E. coli*, **B.** ESBL *E. coli*, **C.** *E. coli* B2, and **D.** *K. pneumoniae* in the 16 wetlands over time (months) and according to river flows. Logarithmic scale. Yellow: Ain wetlands, 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 inhabitants 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	7.91.10⁻⁸	1.88.10⁻¹⁰	0.256	0.00230	-	1.09.10⁻⁸	-	1
Temperature	-0.04	-0.39 **	-0.04	0.51 *	-	-0.44 *	-	1.34 *
pH	4.69 ***	2.51	4.51	7.06 **	-	0.83	-	6.95 *
Saturated O ₂	-0.01	-0.03	-0.01	-0.10 ***	-	-0.003	-	-0.31 *
Conductivity	-0.001	-0.002	0.004	0.02	-	0.006 *	-	-0.01
DOC	0.03	-0.005	0.09	0.0006	-	0.02	-	-0.28
P-PO ₄	14.48 ***	13.01 *	13.0	51.0	-	23.13 **	-	4.68
N-NH ₄	-1.67	-13.78	-23.1	-11.2	-	8.61	-	-0.01
N-NO ₃	0.57 ***	-0.03	0.56	-1.01	-	-0.42	-	4.21 ***
Chlorophyll-a	0.03 *	-0.04	0.04	0.006	-	-0.08	-	-0.16
Water flow	-0.001	0.004	0.00	0.0003	-	0.01 **	-	-0.00001
Connectivity index	-0.45	2.96 *	2.07	4.53	-	9.42 **	-	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.

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