Fate of two strains of extended-spectrum beta-lactamase producing *Escherichia coli* in constructed wetland microcosm sediments: survival and change in antibiotic resistance profiles

Anne-Laure Vivant, Catherine Boutin, Stéphanie Prost-Boucle, Sandrine Papias, Christine Ziebal and Anne-Marie Pourcher

ABSTRACT

Free water surface constructed wetlands (FWS CW) are efficient technologies to limit the transfer of antibiotic resistant bacteria (ARB) originating from urban effluents into the aquatic environment. However, the decrease in ARB from inflow to outflow through the FWS CW may be explained by their transfer from the water body to the sediment. To investigate the behavior of ARB in the sediment of a FWS CW, we inoculated three microcosms with two strains of extended-spectrum beta-lactamase producing *Escherichia coli* (ESBL *E. coli*) belonging to two genotypes. Microcosms were composed of two sediments collected at two locations of an FWS CW from which the strains were isolated. Phragmites were planted in one of the microcosms. The survival curves of the two strains were close regardless of the genotype and the type of sediment. After a rapid decline, both strains were able to survive at low level in the sediments for 50 days. Their fate was not affected by the presence of phragmites. Changes in the *bla* content and antibiotic resistance of the inoculated strains were observed after three weeks of incubation, indicating that FWS CW sediments are favorable environments for spread of antibiotic resistance genes and for the acquisition of new antibiotic resistance.

Key words | antimicrobial resistance, constructed wetland, ESBL *E. coli*, horizontal gene transfer, sediment

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INTRODUCTION

Constructed wetlands (CW) improve the quality of effluents from wastewater treatment plants (WWTP) by removing organic pollutants (Vymazal 2009; Morvannou *et al.* 2015) and by reducing bacterial contamination (Quinonez-Diaz *et al.* 2001; Hench *et al.* 2003; Karathanasis *et al.* 2003; Karim *et al.* 2004; Morató *et al.* 2014). The removal of bacteria from the water in CW is explained by physical (sedimentation, filtration, adsorption), chemical (oxidation, exposure to biocides) and biological processes (predation, competition for nutriments, lytic activity) (Wu *et al.* 2016). It has been reported that antimicrobial resistant genes persist during wastewater treatment (Pruden *et al.* 2013). Furthermore, among resistant bacteria that are highly prevalent in WWTP effluents, extended spectrum beta-lactamase-producing *Escherichia coli* (ESBL *E. coli*) may represent a major threat to public health (Canton *et al.* 2012). In a previous study, we showed that a free water surface constructed wetland (FWS CW), receiving a WWTP effluent, significantly reduced the level of *E. coli* (abatement rate of $1.5 \log_{10}$ to $3 \log_{10}$) and of ESBL *E. coli* (abatement rate $>1.5 \log_{10}$) in the water compartment. Characterization of ESBL *E. coli* isolates, using multilocus sequence typing, demonstrated the circulation of these bacteria from water to the sediments of the FWS CW (Vivant *et al.* 2016), confirming that sedimentation is one of the natural processes involved in the reduction of enteric bacteria in wetlands (Karim *et al.* 2004). Interestingly, strains of ESBL *E. coli* with an identical genotype were isolated from sediments sampled from different areas of the FWS CW, suggesting that a specific population of ESBL *E. coli* persisted in the sediments of the FWS CW. This may pose a public health problem as the survival of antibiotic resistant bacteria

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(ARB) and the release of antibiotic resistance genes (ARG) into the environment is a growing concern.

Even after bacterial death, ARG can persist in sediments when associated with clay particles and organic substances. Indeed, Mao *et al.* (2014) demonstrated high persistence of ARG in river sediments that were more concentrated in ARG than the river water body. Moreover, they reported that available extracellular ARG were assimilated by indigenous bacteria in the river sediments. Similarly, transfer of ARG among bacteria has been reported in river sediment microcosms studies using a plasmid donor bacteria (Bonot & Merlin 2010; Bellanger *et al.* 2014). Horizontal gene transfer is one of the mechanisms that enable bacteria to acquire resistance to antibiotics (Blair *et al.* 2015). Indeed, river sediments exposed to antibiotics showed a high abundance of enzymes involved in horizontal transfer of resistance genes (integrase class 1 and insertion sequence common region transposase of class 2) and of resistance-carrying plasmids (Kristiansson *et al.* 2011b).

On the basis of data on river sediments, we hypothesize that the CW sediments, which contain a large fraction of the microbial biomass of FWS CW (Truu *et al.* 2009), could represent a favorable environment for the survival of ARB and thus induce the formation of ARG reservoirs available for environmental bacteria. To test this hypothesis, we studied the behavior of ESBL *E. coli* in sediments collected in the Marguerittes FWS CW at laboratory scale. The objectives of the study were to (i) compare the level of *E. coli* and ESBL *E. coli* in water and in the sediments of the FWS CW; (ii) investigate the fate of two strains of ESBL *E. coli*, phenotypically and genotypically different, isolated from the FWS CW, in microcosms comprising sediments with or without the presence of young phragmites plants; and (iii) investigate if FWS CW sediments favor the exchange of ARG. To assess ARG exchange in sediment microcosms, we compared the ESBL content and antibiotic profiles of the two strains before inoculation and after an incubation period in the sediment.

MATERIALS AND METHODS

Sample collection

Samples were collected in an FWS CW receiving the effluent of the WWTP of a small town (Marguerittes), located in the South of France. A detailed description of the FWS CW, composed of two successive ponds (P1 and P2) (Supplementary Figure S1, available with the online version of this paper), can be found in Vivant *et al.* (2016). Samples were collected in the two ponds: (i) in the water body and in the sediment at the inlet of pond P1 (point INL) and (ii) in the reed bed of pond P2 (point RB). Samples for enumeration of *E. coli* and ESBL *E. coli* were collected three times over a three month period at points INL and RB. The samples were collected in 1 L sterile flasks, transported in a cooler to the laboratory and analyzed within 24 h.

For the microcosm experiments, three kg of sediments were collected in triplicate at points INL and RB. Each sample was then mixed to obtain a composite sample weighing 9 kg and stored at 4 °C.

Chemical and microbiological characterization of the sediments

Total solids (TS), volatile solids (VS), total Kjeldahl nitrogen (TKN) were determined using standard methods (APHA, 2012). Total phosphorus was measured after mineralization using an automated photometric analyzer (Gallery™ Plus, Thermo Fisher Scientific). Ion and metal analyses were performed at Irstea's Water Chemistry Laboratory (LAMA, France). Ca, K, Mg, Na, Al, Fe, B, Cd, Cr, Cu, Mn, Ni, Pb, and Zn were analyzed using inductively coupled plasma optical emission spectrometry (ICP-OES) according to NF-EN ISO 11885.

Microbial diversity and sediment richness were analyzed by Illumina sequencing through the analysis of the V4–V5 hypervariable 16S rRNA gene regions of each DNA sample. The generated fastq sequence data were processed by quantitative insights into the microbial ecology (QIIME) pipeline (Caporaso *et al.* 2010).

Enumeration of *E. coli* and ESBL *E. coli* in the FWS CW sediments

Samples were serially diluted in sterile peptone water. One mL of each sample (diluted or not) was plated onto Tryptone Bile X-Glucuronide (TBX; Thermo Fisher, France) agar plates for the enumeration of *E. coli*, and on TBX supplemented with 4 mg/L cefotaxime (Sigma Aldrich, France) (TBX-CTX agar) for the enumeration of ESBL *E. coli*. Plates were incubated at 44 °C for 24 h. After incubation, glucuronidase positive *E. coli* (blue colonies) were counted. The results are expressed as colony forming units (CFU) per 100 mL.

When concentrations reached a level of 10 CFU/100 mL, the threshold of the method was increased by using the Colilert®-18test kit (IDEXX laboratories, France). Ten grams of sediment were added to 90 mL of sterile water and Colilert®

reagent was added to each sample according to the manufacturer's instructions. After incubation at 44 °C for 24 h, 10 μL of the enrichment was plated onto TBX–CTX agar and again incubated at 44 °C for 24 h.

Microcosm preparation

Microcosms were prepared in triplicate in 500 mL flasks filled with 200 mL of sediments and capped with a cotton plug. A total of 21 separate sets of microcosms were prepared. Seven consisted of INL sediment (microcosm INL) and seven of RB sediment (microcosm RB). Seven other microcosms consisted of RB sediment planted with *Phragmites australis* (microcosm RBP). Each RBP sediment was planted with phragmites (commercial source of seeds: Aquamoine, France) at a density of two to three rhizomes per flask depending on root development (Supplementary Figure S2, available online). After the addition of the rhizomes, RBP microcosms were left for three days at room temperature to acclimatize of the plants. All microcosms were stored without agitation at 20 °C and maintained under ambient light.

Bacterial strains

Two strains of ESBL *E. coli* (E2 and E6) isolated from the water of the FWS CW were used in this study. Rifampicin resistant mutants were isolated on brain-heart infusion (BHI) agar supplemented with 200 μg/mL rifampicin (Sigma-Aldrich, France) according to Lemunier *et al.* (2005). For each strain, spontaneous Rif^R mutants were selected by comparing growth rates during planktonic growth in BHI at 20 °C without shaking. Mutants with growth characteristics similar to those of the wild-type strains were selected. The stability of the rifampicin resistance trait was analyzed by culturing organisms for 100 generations. Based on these criteria, two Rif^r mutants of the two ESBL *E. coli* strains were selected. The two mutants, named $E2^{rif}$ and $E6^{rif}$, were characterized by determining their *bla* gene content and their antimicrobial resistance and were genotyped using MLST as described by Vivant *et al.* (2016) (Table 1).

The mutants $E2^{rif}$ and $E6^{rif}$ were grown statically at 20 °C for 16 h in 5 mL of BHI. Inocula were prepared by inoculating 25 mL of BHI (1% v/v) and incubating statically at 20 °C to an $O.D._{600\ nm}$ of 1.0. The cultures were then centrifuged at 8,000 *g* for 5 min at room temperature and the pellets were suspended in 0.85% NaCl.

Inoculation and enumeration of *E. coli* in microcosms

Among the 21 sets of microcosms, 18 were inoculated (six per condition) and three (one per condition) served as control. Sediment microcosms were inoculated with the strain $E2^{rif}$ or the strain $E6^{rif}$ at a concentration of *ca.*10^7 CFU/g of sediment (wet weight). Three replicates were performed per strain ($E2^{rif}$ or $E6^{rif}$). Moisture loss during incubation was offset by adding sterile deionized water.

Culturable $E2^{rif}$ and $E6^{rif}$ populations were enumerated in the sediment microcosms inoculated with viable cells by serial plating on TBX agar plates supplemented with 100 μg/L of rifampicin immediately after inoculation (T0) and periodically over 50 days (T3, T7, T14, T21, T50). Enumeration of bacteria recovered on each sampling day was normalized by dividing the mean values by the mean obtained at day 0 (C/C_0). Log transformation was performed.

Table 1 | Initial characteristics of the mutants $E2^{rif}$ and $E6^{rif}$

			***bla* content**							
			$bla_{CTX\text{-}M\ group\ 1}$		$bla_{CTX\text{-}M\ group\ 9}$		bla_{TEM}		bla_{OXA}	
Strain	**ST profile**[a]	**Number of resistances among 15 antibiotics**[b]	**Chr**[e]	**Plas**[f]	**Chr**	**Plas**	**Chr**	**Plas**	**Chr**	**Plas**
$E2^{rif}$	ST162	13[c]	+	−	−	−	+	−	+	−
$E6^{rif}$	unknown ST	3[d]	+	−	−	−	−	−	+	−

[a]ST sequence type determined by MLST analysis using the 7-gene Achtman Scheme.
[b]Number of resistances among the 15 antibiotics tested (cefotaxime, ceftazidime, imipenem, amikacin, gentamicin, kanamycin, netilmicin, streptomycin, tobramycin, ciprofloxacin, ofloxacin, chloramphenicol, doxycycline, cotrimoxazole and colistin).
[c]Resistance to all antibiotics except imipenem and colistin.
[d]Resistance to cefotaxime, ceftazidime and streptomycin.
[e]Genes located on chromosomic DNA.
[f]Genes located on plasmid.

Characterization of the strains after incubation in microcosms

ESBL content and antibiotic profiles of strains E2^{rif} and E6^{rif} were determined before their inoculation in the sediments. The initial profiles of the two strains were compared to those of the 52 isolates developed on TBX-Rif plates after three weeks of incubation in the sediments at ambient temperature. As endogenous Rif^R *E. coli* were never found in the control sediment microcosms and due to the chromosomal location of rifampicin resistance, we considered that all Rif^R *E. coli* present in the microcosms inoculated with E2^{rif} or E6^{rif} strain derived from these two strains.

Identification of ESBL *E. coli*

Genomic and plasmid DNA were extracted from the two strains before inoculation and from the isolates after incubation in microcosms. Genomic DNA was prepared by the boiling method. Briefly, DNA suspension was boiled at 95 °C in a water bath for 10 min, cooled on ice, and then centrifuged at 10,000 g for 5 min. A 100 μL aliquot of the supernatant was transferred to a sterile tube and stored at −20 °C until polymerase chain reaction (PCR) testing. Plasmid DNA was extracted from 5 mL of an overnight culture using the GenElute Plasmid MiniPrep kit (Sigma-Aldrich, France). PCR was performed to detect *bla* genes using primers specific to the genes encoding ESBL from the CTX-M, TEM and OXA families (Supplementary Table S1). PCR amplification was performed in a total volume of 20 μL containing 2.5 μL of DNA template, 2 μL of 10× PCR buffer with $MgCl_2$, 0.5 μL of dNTP mix (10 mM), 0.2 μL of *Taq* polymerase (5 U/μL), and 1 μL of each primer (10 μM). Depending on the primers, different PCR conditions were used (Supplementary Table S2). (Supplementary Tables S1 and S2 are available online.)

Antibiotic resistance

The antibiotic susceptibility of the strains was tested using the disk diffusion method on Mueller-Hinton agar plates. *E. coli* ATCC 25922 was used as the control strain. Fifteen antibiotic disks were used: cephalosporins (cefotaxime 30 μg and ceftazidime 30 μg), carbapenem (imipenem 10 μg), aminoglycosides (amikacin 30 μg, gentamicin 10 μg, kanamycin 30 μg, netilmicin 30 μg, streptomycin 10 μg and tobramycin 30 μg), quinolones (ciprofloxacin 5 μg and ofloxacin 5 μg), chloramphenicol (30 μg), tetracycline (doxycycline 30 μg), cotrimoxazole (trimethoprim-sulfamethoxazole 1.25 μg–23.75 μg) and colistin (10 μg). Overnight cultures were centrifuged at 8,000 g for 5 min at room temperature, and the pellets were suspended in NaCl (0.85%). Bacterial suspensions were then diluted to match 0.5 McFarland turbidity standards. One mL of the diluted bacterial suspension was used to inoculate Mueller-Hinton agar plates (Bio-Rad Laboratories, France). The antibiotic disks were dispensed on the briefly dried Mueller-Hinton agar plates, after which the plates were incubated overnight at 37 °C. Susceptibility test results were interpreted using CLSI (Clinical and Laboratory Standards Institute 2012) breakpoint criteria. Isolates were classified as sensitive, intermediate or resistant. Full resistance includes intermediate plus resistant isolates.

Decay rates and statistical analysis

Two parameters describing the persistence of strains E2^{rif} and E6^{rif} were calculated: (i) the Log_{10} removal at Days 14 and 29 and (ii) the inactivation rate corresponding to the slope of the inactivation curve. Decay rates were calculated using Cerf's model ($C_t = C_0 \times (f \times e^{-k1t} + (1-f) \times e^{-k2t})$), where C_t and C_0 are the concentrations at time t, and t_0, f is the initial proportion of the first fraction, k1 and k2 are the decay constants of the first and second phase, respectively. Decay rate models and their parameters were calculated using XLSTAT 2010 software.

In order to evaluate whether the type of sediment, the genotype of the strains or the presence of vegetation affected the survival of the strains, a non-parametric analysis of variance (Kruskal-Wallis analysis of variance, ANOVA) was performed.

RESULTS

Level of *E. coli* and ESBL *E. coli* in the water and in the sediments of the FWS CW

The average concentrations of *E. coli* and ESBL *E. coli* measured over a three-month period in the water and in the sediments of the two successive ponds (P1 and P2) of the FWS CW are presented in Figure 1. Regardless of their location in the FWS CW, the sediments were more contaminated than the water. Moreover, the concentration of *E. coli* and ESBL *E. coli* in the water was reduced *ca.* 20-fold between the inlet of the pond P1 and the inlet of the reed bed (pond P2), whereas it remained close in the two sediments.

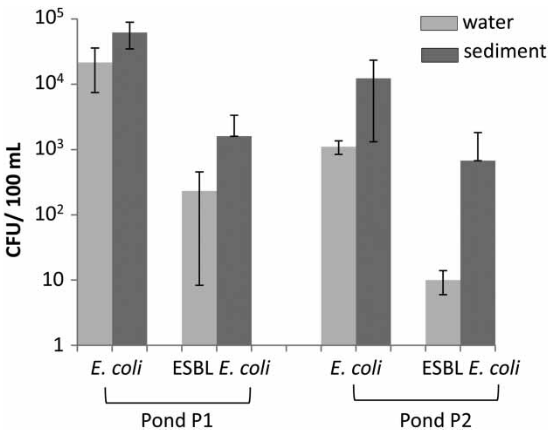

Figure 1 | Average concentration of *E. coli* and ESBL *E. coli*. Samples were collected in water and in sediment located at the inlet of the FSW CW (Pond P1) and in the reed bed (Pond P2).

Persistence of two strains of ESBL *E. coli* in sediment microcosms

In order to evaluate the fate of ESBL *E. coli* after they have been transferred to sediments, two strains of ESBL *E. coli* ($E2^{rif}$ and $E6^{rif}$) were inoculated in microcosms filled with sediments collected at the inlet of the FWS CW (sediment INL) and in the reed bed of pond P2 (sediment RB). As vegetation is fully integrated in the FWS CW, the strains were also inoculated in a constructed microcosm composed of sediment RB planted with *Phragmites australis* (sediment RBP). The main physico-chemical characteristics and the bacterial population composition of the sediments INL and RB are listed in Table 2. Metal and ion concentrations are reported in Supplementary Table S3 (available with the online version of this paper). The two sediments differed in their water content and chemical characteristics. The lowest concentration of total phosphorus, Fe, B, Cr, Cu, Zn, Ni, Pb and Na was observed in sediment RB whereas, the level of Al, Mn, Ca, K and Mg was higher in sediment RB than in sediment INL. The richness and the bacterial composition of the two sediments were close, *Proteobacteria* being the most abundant phylum (38.3% and 36.8% of the OTUs of INL and RB sediments, respectively), followed by *Chloroflexi* (7.9% and 9.4%) and *Bacteroidetes* (7.3% and 8.7%).

The concentrations of both strains declined progressively in the three sediments (Figure 2).

The abatement reached 4.1 to 5.1 and 5.4 to 6.4 Log_{10} after 14 days and 29 days of incubation, respectively (Table 3). However, both strains remained detectable until the end of the experiment (at Day 50). The decay rates of the two strains (0.55 to 1.67 day^{-1}) did not significantly differ (Kruskal-Wallis test, $p > 0.05$), suggesting that the intra species variability of *E. coli* strains did not affect their behavior. Furthermore, there was no significant difference between the decay rates and the log reductions of each strain in the three sediments (Kruskal-Wallis test, $p > 0.05$) indicating that the presence of young *Phragmites australis* had no impact on the persistence of ESBL *E. coli*.

Table 2 | Main physico-chemical characteristics, bacterial richness and diversity of the sediments INL and RB of the FSW CW

	Parameters	Sediment INL	Sediment RB
Physico-chemical	pH	7.5	7.3
characteristics	Total solids (g/kg)	65	566
	Volatile solids (g/kg)	23	34
	Total nitrogen (g/kg)	1.6	1.3
	Total phosphorus (mg/kg)	535	23
Microbiological	Chao1 estimator	24,670	33,088
characteristics	Shannon index	12.2	12.7
	Proteobacteria	38.3%[a]	36.8%
	Chloroflexi	7.9%	9.4%
	Bacteroidetes	7.3%	8.7%
	Planctomycetes	5.5%	3.8%
	Firmicutes	4.8%	6.9%
	Actinobacteria	3.7%	4.4%
	Spirochaetes	3.3%	1.9%
	Acidobacteria	2.9%	3.4%
	Chlorobi	1.9%	2%

[a]Proportion of the most highly represented *phyla* in the two sediments.

Changes in ESBL contents and in the antibiotic resistance profiles

After three weeks of incubation, the remaining cultivable Rif^R *E. coli* still present in the microcosm sediments inoculated with $E2^{rif}$ or $E6^{rif}$ strain were isolated on TBX medium supplemented with rifampicin. The Rif^R *E. coli* isolates were then tested for their ESBL content and their antibiotic resistance. These two characteristics were compared to those of the parental strains $E2^{rif}$ and $E6^{rif}$ before being inoculated in the sediments.

Changes in ESBL contents and antibiotic profiles occurred during the incubation of the sediments. The acquisition of *bla* genes on chromosomic DNA was rare (only

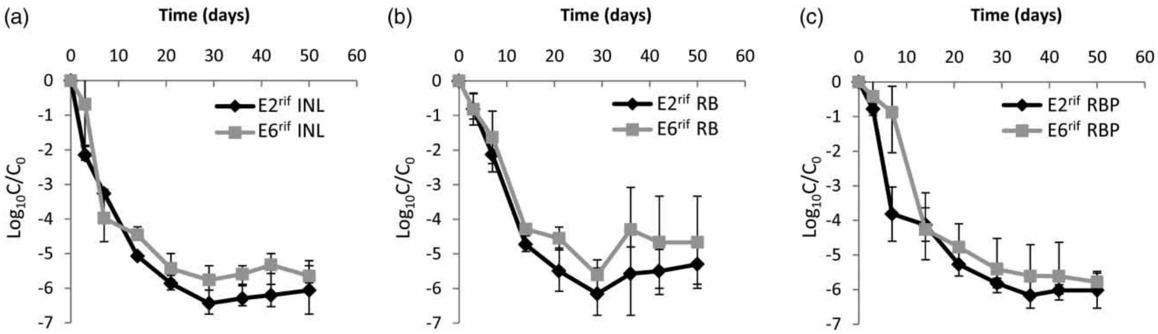

Figure 2 | Fate of the ESBL *E. coli* populations in the sediment microcosms. (a) INL sediment; (b) RB sediment; (c) RB sediment planted with *Phragmites australis*. Bars indicate minimum and maximum values.

observed for $bla_{CTX\text{-}M9}$ gene) whereas most of the isolates of both strains lost $bla_{CTX\text{-}M1}$, bla_{TEM} and bla_{OXA} genes (Table 4). As shown in Figure 3(a), the loss of *bla* genes on chromosomic DNA was the most common event in the three sediments (from 60% to 93%). On plasmid DNA, *bla* genes, not detected initially, were then found in most of the isolates after three weeks of incubation in the microcosms. $bla_{CTX\text{-}M1}$ and bla_{OXA} genes were mainly identified (Table 4). Acquisition of at least one *bla* gene through plasmids was recorded in 89% of the isolates $E2^{rif}$ and in 100% of the isolates $E6^{rif}$ (Figure 3(b)). Moreover, three isolates originating from strain $E2^{rif}$ harbored the four *bla* genes tested (bla_{CTXM1}, $bla_{CTX\text{-}M9}$, bla_{TEM} and bla_{OXA}) on their plasmids, indicating that horizontal acquisitions of *bla* genes can occur in the sediments of the FWS CW within 21 days.

The parental strains $E2^{rif}$ and $E6^{rif}$ presented initially a strong different pattern of resistance as they were resistant to 13 and 3 antibiotics, respectively (Table 1). After incubation in the sediment microcosms, isolates originating from strain $E2^{rif}$ harbored the same antimicrobial resistance as the parental strain (13 of the 15 tested antibiotics), whereas differences in antibiotic resistance were recorded for isolates originating from strain $E6^{rif}$. Indeed, compared to the parental strain, isolates were resistant to 3 to 10 additional antibiotics (Figure 4). It is noteworthy that higher proportions of resistance were acquired in the sediment of the reed bed.

Table 3 | Average decay rates and log removal after 14 and 29 days of the two strains of BLSE *E. coli* inoculated in three sediment microcosms

			Log_{10} removal	
Strain	**Sediment**	**k_1 $(d^{-1})^a \pm$ SD**	**after 14 d.**	**after 29 d.**
$E2^{rif}$	INL	1.67 ± 0.46	5.1 ± 0.06	6.4 ± 0.4
	RB	0.78 ± 0.07	4.7 ± 0.2	6.2 ± 0.7
	RBP	0.84 ± 0.17	4.1 ± 1.0	5.8 ± 0.1
$E6^{rif}$	INL	1.49 ± 0.79^b	4.5 ± 0.2	5.8 ± 0.4
	RB	0.55 ± 0.38	4.3 ± 0.2	5.6 ± 0.6
	RBP	1.14 ± 0.36^c	4.3 ± 0.5	5.4 ± 0.8

[a]Rate constant (day^{-1}) using Cerf's model after the lag period ended.
[b]One of the replicates had a lag phase of 3 days.
[c]Two of the replicates had a lag phase of 7 days.

DISCUSSION

In this study, ESBL *E. coli* represented circa 1% of the *E. coli* population enumerated in the water and in the sediments of the FWS CW. This result is in accordance with the ratio of ESBL *E. coli*/total *E. coli* of 0.56 to 0.75% observed in wastewater samples by Jorgensen *et al.* (2017). The increase of concentration of ESBL *E. coli* in the sediments (10-fold higher than in water) suggests a persistence of these bacteria in the sediments. We previously demonstrated that similar genotypes of ESBL *E. coli* were represented in different compartments of the FWS CW (Vivant *et al.* 2016), confirming the transfer of *E. coli* from the water body to the sediments. This is in agreement with previous studies reporting higher persistence of *E. coli* or of fecal coliforms in sediment than in the water column. Whatever the conditions (microcosms, mesocosms, flow-through chambers, bottles) and the source of inoculation (feces, wastewater effluents, strain inoculum), the authors described a rapid decrease in *E. coli* populations in water and much slower inactivation in sediments (Karim *et al.* 2004; Anderson *et al.* 2005; Kiefer *et al.* 2012; Kim & Wuertz 2015).

Fate of ESBL *E. coli*

As expected, ESBL *E. coli* populations decreased in the sediments over time. This is usually observed for *E. coli* populations in microcosm assays when the matrices are

Table 4 | Lost and acquisition of *bla* genes of E2^{rif} and E6^{rif} isolates after being inoculated 3 weeks in sediment microcosms

		E2^{rif} (n = 18)[a]			E6^{rif} (n = 26)		
			% of isolates[c]			% of isolates	
Gene location	**Gene**	**Parental strain[b]**	**Lost**	**Acquired**	**Parental strain**	**Lost**	**Acquired**
Chromosome	$bla_{\text{CTX-M group 1}}$	+	11.1		+	42.3	
	$bla_{\text{CTX-M group 9}}$	−		11.1	−		11.5
	bla_{TEM}	+	94.4		−		0
	bla_{OXA}	+	61.1		+	76.9	
Plasmid	$bla_{\text{CTX-M group 1}}$	−		88.9	−		100
	$bla_{\text{CTX-M group 9}}$	−		27.8	−		3.8
	bla_{TEM}	−		33.3	−		0
	bla_{OXA}	−		77.8	−		92.3

[a]Number of isolates.
[b]Initial profile of the parental strain (+: presence of the gene, −: absence of the gene).
[c]Percentage of isolates having lost or acquired a gene.

not sterile. Nevertheless, after 50 days of incubation, a few cells were still detectable, indicating that ESBL *E. coli* can survive at low concentrations for a long period at ambient temperature in this environment.

The behavior of the two strains inoculated at an initial concentration of circa 10^7 CFU/g was close to that observed in literature for *E. coli* populations in sediments (Pachepsky & Shelton 2011). No lag period was measured for 15 of the 18 microcosms. Rapid inactivation occurred within the first 21 days, followed by stabilization at a low level until the end of the incubation period. It is noteworthy that at a temperature of incubation of sediment of 20–22 °C, regrowth or a lag phase of *E. coli* is not systematic. Kiefer *et al.* (2012) observed regrowth when the concentrations of *E. coli* in the sediment after the addition of the inoculum (animal feces) was less than 100 MPN/g. Kim & Wuertz (2015) also reported an increase in *E. coli* in sediments in the first two days of incubation after an inoculation at an initial level of *E. coli* of 10^2–10^3 MPN/g. However, no lag period was observed when *E. coli* was added to the sediment at higher concentrations (10^3 MPN/g) (Kiefer *et al.* 2012), suggesting that the initial level of *E. coli* may affect their regrowth.

The decay rates of the two strains in the FWS CW sediment (0.55 to 1.67 d^{-1}) were higher than those reported in studies investigating the persistence of natural populations of *E. coli* from fecal matter in sediment microcosms incubated at 20–22.5 °C. After inoculation of a mix of human, cow and dog feces in the overlying water of a freshwater sediment, the decay rates of *E. coli* ranged between 0.172

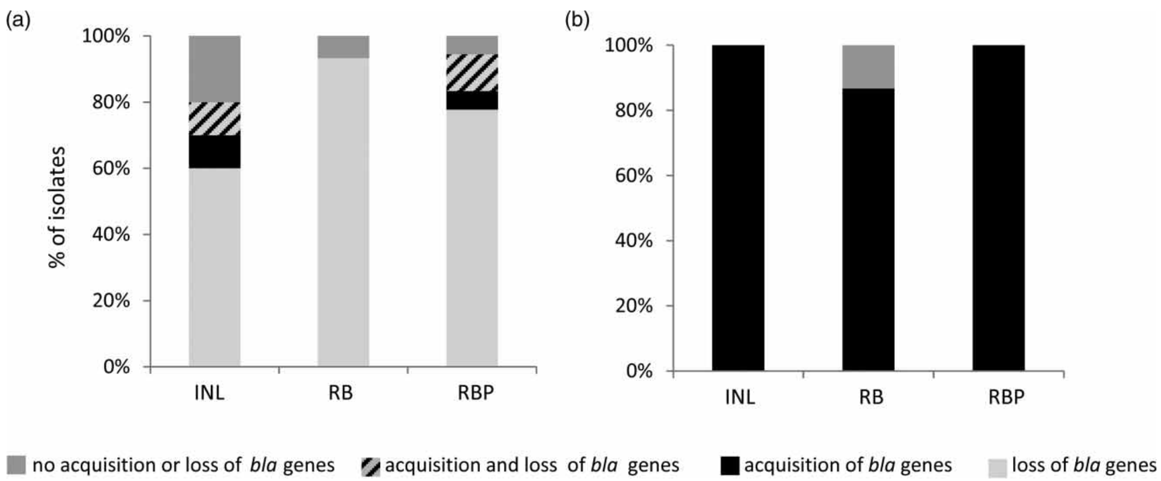

Figure 3 | Modification of the *bla* gene content of ESBL *E. coli* after three weeks of incubation in sediment microcosms (INL, RB and RBP). The figure indicates the proportion of acquired and/or lost *bla* genes on (a) chromosomic DNA and (b) plasmid DNA.

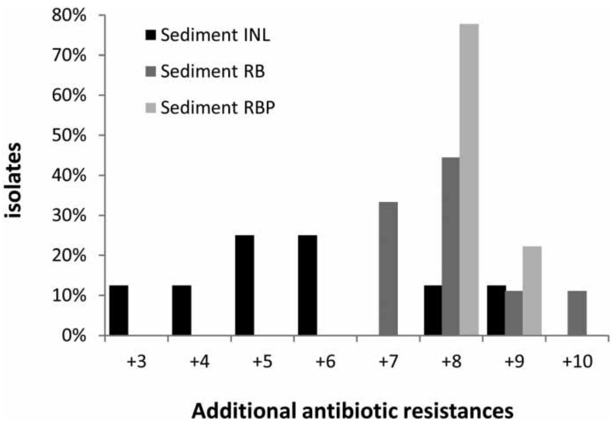

Figure 4 | Number of additional antibiotic resistances acquired by the strain ESBL *E. coli* E6^{rif} in the sediment microcosms (INL, RB and RBP). The strain E6^{rif} initially harbored four antibiotic resistances.

and 0.126 d^{-1} depending on the sampling location (0–2 cm or 4–6 cm from the surface) (Kim & Wuertz 2015). A similar trend (decay rates ranging from 0.172 to 0.04 d^{-1}) was reported by Kiefer *et al.* (2012), who studied the survival of *E. coli* introduced into two streambed sediments through inoculation with animal feces. In a recent study, Abia *et al.* (2016) compared the inactivation over time of a strain of *E. coli* inoculated at a final concentration of circa 10^7 CFU/mL in three riverbed sediments stored at 20 °C. In these controlled conditions, which were close to those of the present study, the decay rates ranged from 0.42 to 0.22 d^{-1}. Moreover, after 28 days, the abatement ranged from 2.5 to 5.5 log, which is also lower than that observed in the sediments of the FWS CW after 29 days (5.4–6.4 log). The higher decline we observed in the FWS CW sediment could be due to the origin of strains and the type of sediment. Nevertheless, Karim *et al.* (2004) reported a lower inactivation rate of fecal coliforms (0.51 d^{-1}) in sediment collected in a constructed surface flow wetland than rates we observed for the strains E2^{rif} and E6^{rif} in the present study.

Although *E. coli* die-off rates depend on several parameters (genotype, structure of the sediment, organic matter and nutrient content) (Pachepsky & Shelton 2011), in our study, the behavior of the two strains was independent of the metal and ion contents of the sediments, the genotype of the strains and the presence or absence of vegetation. It is noteworthy that in our experiment, the height of *P. australis* remained stable in the RBP sediment. The slow growth of the reeds and consequently their low metabolic activity may explain the similar behavior of the strains in RB and RBP sediments. The concentration of total P in the sediment RB, which was circa 10-fold less than in the sediment INL, was probably not limiting, as Toothman *et al.* (2009) reported that the level of total P (which ranged from 6.1 to 671 μg/g) did not affect the concentration of fecal coliform in sediments. According to the literature, the impact of vegetation on bacterial communities is not clear due to the multiple factors involved. Indeed, vegetation modifies sediments physically (the presence of roots), chemically (the production of root exudates) and biologically (modification of the rhizomicrobiome). Moreover, depending on the plant species, the growth stage of the plant (vegetative, bolting and flowering), the quantity and composition of roots and exudates may vary, indicating that the impact of the vegetation can evolve over time. In this context, some studies showed significant effects of vegetation on the abatement of microbial populations in CW while others did not. For example, Headley *et al.* (2013) concluded there was no significant impact of vegetation on *E. coli* removal in their subsurface flow systems, whereas Sidrach-Cardona & Becares (2013) observed a stronger reduction in *E. coli* and coliforms in the planted CW than in the unplanted replicates.

Changes in antimicrobial resistance

Since ARGs have been detected in sediments (Kristiansson *et al.* 2011a; Czekalski *et al.* 2014; Botts *et al.* 2017; Calero-Caceres *et al.* 2017; Chu *et al.* 2018), their horizontal transfer has been suggested. However, horizontal gene transfer has rarely been demonstrated. Bonot & Merlin (2010) reported an early dissemination (within the first 24–48 hours) of a broad-host-range plasmid pB10 in the indigenous population of sediment microcosms inoculated with the donor strain *E. coli* DH5α (pB10). Furthermore, $bla_{\text{CTX-M}}$ ESBL genes may spread across different species or genera (Canton *et al.* 2012). Our study provides further insights into the acquisition and loss of antibiotic resistance in an ESBL *E. coli* population in sediments of a CW. The two strains used in this study (E2^{rif} and E6^{rif}) were multidrug resistant, like 93% of the ESBL *E. coli* isolated in the Marguerittes FWS CW. The presence of multidrug resistant ESBL *E. coli* has also been reported in effluents and surface waters (Ojer-Usoz *et al.* 2014; Jorgensen *et al.* 2017; Gekenidis *et al.* 2018). Understanding the impact of these multidrug resistant bacteria on environmental bacterial communities is thus crucial. We showed that in less than 21 days in sediments, ESBL contents and antibiotic resistance profiles of the ESBL *E. coli* evolved. Therefore, the presence of ARB in sediments, even for a short period and at a low proportion (1% of the total cultivable *E. coli* population), can increase the pool of ARG in the FWS CW. It is well known that within bacterial communities, phages and plasmids provide the required integrase genes to integrate ARG present in the surrounding environment (Casjens 2003; Boyd *et al.* 2009; Wozniak & Waldor 2010). They can be transferred by cell-cell contact (or without), but conjugation and transformation are not the only way to spread ARG widely among bacterial species. Transfer of ARG via phage could also be a major dissemination pathway within communities (Brown-Jaque *et al.* 2015). Interestingly, the characteristic of the sediments seems to affect the antimicrobial resistance profiles of the ESBL *E. coli*. Using a strain of *E. coli* DH10B as a recipient strain for conjugal mating assays, Amos *et al.* (2014) reported that the $bla_{\text{CTX-M-15}}$ gene was easily transferable to other Gram negative bacteria. Although we have not demonstrated the transfer of $bla_{\text{CTX-M}}$ ESBL genes in the CW sediment, we have observed that this environment may favor horizontal gene transfer. Indeed, using a strain of DH5α donor *E. coli* harboring the pB10 plasmid (Bonot & Merlin 2010) inoculated in INL sediment, we observed plasmid transfer to environmental bacteria (in particular to *Pseudomonas plecoglossicida*) within 48 hours (data not shown).

CONCLUSION

This study highlights the persistence of ESBL *E. coli* in sediments of a CW within a period of 50 days in the presence or absence of vegetation, suggesting the ability of ESBL *E. coli* to adapt to FWS CW sediments. Moreover, ESBL *E. coli* of the FWS CW rapidly acquired additional ARG and resistances to antibiotics within the first weeks of incubation in sediments, indicating that FWS CW sediments are favorable environments for the exchange of antibiotic resistance genes. Further research should be conducted to understand the mechanisms of transfer of *bla* genes to autochthonous bacteria in the FWS CW sediments.

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