Water Research 104 (2016) 178–188

Contents lists available at ScienceDirect

Water Research

journal homepage: www.elsevier.com/locate/watres

Free water surface constructed wetlands limit the dissemination of extended-spectrum beta-lactamase producing *Escherichia coli* in the natural environment

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ARTICLE INFO

Article history:
Received 22 June 2016
Received in revised form
3 August 2016
Accepted 5 August 2016
Available online 6 August 2016

Keywords:
Antibiotic resistance
Constructed wetland
Extended-spectrum beta-lactamase
Escherichia coli
Tertiary treatment
Water

ABSTRACT

The fates of *Escherichia coli* and extended-spectrum beta-lactamase-producing *E. coli* (ESBL *E. coli*) were studied over a period of one year in a free water surface constructed wetland (FWS CW) with a succession of open water zones and vegetation ponds (*Typha* or *Phragmites*), that received the effluent from a wastewater treatment plant. ESBL *E. coli* were detected and isolated from all sampling areas of the FWS CW throughout the study period. They represented 1‰ of the total *E. coli* population regardless of the origin of samples. Two main factors affected the log removal of *E. coli* and of ESBL *E. coli*: the season and the presence of vegetation. Between the inlet and the outlet of the FWS CW, the log removal of *E. coli* ranged from 1.5 in the warmer season (summer and fall) to 3.0 in the colder season (winter and spring). The concentrations of *E. coli* decreased significantly in the vegetated areas during the colder season, but increased in the warmer season, suggesting an effect of the plant growth stage on the survival of *E. coli*. Among the 369 ESBL *E. coli* isolates collected during our study, 84% harbored the CTX-M-ESBL type and 55.3% carried *bla* genes on plasmid DNA. Furthermore, 93% of the ESBL *E. coli* isolates were multidrug resistant but the proportion of resistant strains did not change significantly along the FWS CW. ESBL *E. coli* were characterized by MLST analysis using the 7 genes based Achtman Scheme. ESBL *E. coli* isolated from water, sediments, roots and feces of myocastors collected in the FWS CW and in the recipient river were genotypically related, suggesting persistence and circulation of the ESBL producing *E. coli* throughout the FWS CW and in the receiving river. Overall, these observations show that FWS CW could be an efficient treatment for ESBL *E. coli* disinfection of wastewater and could limit their dissemination in the aquatic environment.

1. Introduction

In recent years, concern has been growing regarding the contribution of wastewater effluents to the dissemination of antibiotics, antimicrobial resistant bacteria (ARB) and antibiotic resistance genes (ARGs) in the natural environment. Indeed, ARB or ARGs have been detected in effluents from wastewater treatment plants (WWTP) (Brechet et al., 2014; Diallo et al., 2013; Dolejska et al., 2011; Ferreira da Silva et al., 2007), in surface water (Akbulut et al., 2014; Amos et al., 2014; Bajaj et al., 2015; Zhang et al., 2014), in ground water (Li et al., 2014) and in sediments

Abbreviations: ANOVA, analysis of variance; ARB, antibiotic resistant bacteria; ARG, antibiotic resistant gene; CFU, colony forming unit; CW, constructed wetland; ESBL, extended spectrum beta-lactamase; FWS CW, free water surface constructed wetland; MLST, multilocus sequence typing; ST, sequence type; TBX, Tryptone Bile X-Glucuronide; UPGMA, unweighted pair group method with arithmetic mean; WWTP, wastewater treatment plant.

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http://dx.doi.org/10.1016/j.watres.2016.08.015
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(Kristiansson et al., 2011). Agricultural practices such as land spreading of sewage sludge or irrigation with WWTP effluents can contribute to the spread of ARB and ARGs in the environment (Biswal et al., 2014; Jechalke et al., 2015). Several studies have shown that WWTP effluents can be a major route by which ARB and ARGs are introduced into ecosystems, and especially aquatic systems. Whatever the country (Czech republic, Ireland, Portugal, Poland, Netherlands, France), the treatment plant capacity (from 72, 000 to 400,000 equivalent inhabitants), the type of wastewater (city, suburban, hospital, healthcare institution), WWTPs reduce the burden of ARB but do not completely eliminate them (Biswal et al., 2014; Blaak et al., 2015; Brechet et al., 2014; Dolejska et al., 2011; Ferreira da Silva et al., 2007, Galvin et al., 2010; Korzeniewska et al., 2013). This situation results in the release of a large amount of ARB and to an increase in the number of ARGs in the environment.

To tackle this environmental concern and improve wastewater effluents quality, additional treatments can be employed. Although tertiary treatments cannot be expected to completely eliminate ARB and ARGs, they are more efficient for the removal of pollutants than secondary treatments. Given their low cost and their ease of maintenance, constructed wetlands (CWs) may be a useful water polishing process for the removal of ARB. Indeed, depending on their characteristic, CWs have been shown to be able to efficiently remove organic pollutants from WWTP effluents (Morvannou et al., 2015; Vymazal, 2009). However, not enough data are currently available on the efficiency of CWs in removing ARB or ARGs (Berglund et al., 2014; Chen et al., 2015; Nolvak et al., 2013). Most of the studies performed to date were pilot experiments, or were performed in experimental CWs with a small surface area. In Sweden, Berglund et al. (2014) studied the impact of experimental surface-flow wetlands supplied with groundwater spiked with 12 antibiotics, on the removal of eight ARGs. These authors reported no proliferation of ARGs whose concentration was not affected by the CW treatment. Chen et al. (2015) reported that concentrations of 11 ARGs decreased by 1–3 $\log_{10}$ from the influent to the effluent of a CW designed to treat rural wastewater produced by a Chinese village. In a study using pilot-scale CWs with different configurations at a Spanish WWTP, Sidrach-Cardona and Becares (2013) reported that all CWs (planted or not with *Phragmites* or *Typha*) removed ARB better than WWTPs. Finally, in a recent study, Ibekwe et al. (2016) analyzed the antimicrobial susceptibility of strains of *Escherichia coli* isolated from an experimental CW feed with wastewater from a piggery. Both the influent and effluent contained resistant strains to tetracycline, erythromycin, ampicillin, streptomycin and sulfisoxazole and the level of *E. coli* in influent were 2 $\log_{10}$ higher than that in final effluent, suggesting that CWs can significantly reduce ARB.

Among resistant bacterial isolates, extended spectrum beta-lactamase-producing *E. coli* (ESBL *E. coli*) are a major threat to public health (Canton et al., 2013). Resistance to last-resort antibiotics (carbapenem and colistin) has been identified in ESBL *E. coli* (Poirel et al., 2016; Yao et al., 2016) and its prevalence in WWTP effluents is high (Brechet et al., 2014; Reinthaler et al., 2010). The aim of this study was therefore to investigate the fates of *E. coli* and ESBL *E. coli* in a free water surface CW (FWS CW) receiving secondary effluent from a small WWTP. The specific objectives were to: (i) monitor the concentrations of *E. coli* and ESBL *E. coli* in the effluent of the WWTP over a period of one year in both the FWS CW and the river receiving the treated water; (ii) determine the antibiotic susceptibility patterns of ESBL *E. coli* isolates; (iii) characterize the *bla* genes involved in ESBL production among *E. coli* isolates; and (iv), investigate the clonal diversity of the ESBL *E. coli* isolated in the different FWS CW compartments (water, sediment, roots, wild animal feces) by multilocus sequence typing (MLST).

2. Materials and methods

2.1. Study site

The FWS CW is located in the municipality of Marguerittes (43° 51′ N and 04° 26′ E), in southern France. It was designed as a tertiary treatment system to treat domestic wastewater from a municipal WWTP (15,000 equivalent inhabitants). The FWS CW is composed of a 1 ha horizontal flow system of artificial ponds receiving between 2250 m^3 to 3050 m^3 of treated wastewater per day.

The FWS CW consists in two major ponds (Fig. 1). WWTP secondary effluent is entirely discharged into the first pond with microphytes (P1), this being the biggest pond in volume (3575 m^3) and surface area (3575 m^2). The P1 pond is lined with *Typha latifolia*. Water is released into a second pond (P2) composed of a succession of five basins: a reed bed, open waters, a sea grass bed, a delta zone and a macrophyte basin planted with both *Typha latifolia* and *Phragmites australis*. Pond P2 has a volume of 3820 m^3 and a surface area of 6370 m^2 (30% of the surface is vegetated and 70% is free water). The reed bed, planted with *Phragmites australis,* is the most vegetated part of the FWS CW (1400 m^2 out of the 3200 m^2 of vegetated surface). The residence time in the FWS CW is three days and the nominal theoretical residence time is 3.3 days (ranging from 2.4 to 5.9 days). The treated water is discharged into a river named Canabou. Air temperature data were collected from a *Météo-France* weather station located close to Marguerittes.

2.2. Field sampling procedure

Nine samples of water were collected once or twice a month (a total of 16 times between January 2015 and December 2015) (Fig. 1). Sample W1 refers to the WWTP effluent. Samples W2 and W3 were collected at the inlet and the outlet of pond P1, respectively; sample W4 was collected in the reed bed; sample W6 in the open waters; sample W9 in the sea grass bed and sample W12 at the outlet of the FWS CW in the macrophyte basin. Two more samples (W13 and W14) were collected in the River Canabou, upstream and downstream of the outfall of the FWS CW.

Additional samples of sediment (S1, S3, S4, S5, S6, S8, S9) and roots of *Phragmites australis* (RR1, RR2) and *Typha latifolia* (RT1, RT2) were sampled twice over the year in the FWS CW and in the river. Their locations are shown in Fig. 1. Myocastor feces were collected eight times over the year in the FWS CW.

The samples were collected in sterile flasks and transported in a cooler to the laboratory. All samples were analyzed within 24 h.

2.3. Enumeration and isolation of E. coli and ESBL E. coli

Water samples were filtered on a hydrophilic mixed cellulose ester membrane (0.45 μM) (PALL Life Sciences, VWR, France). The filters were placed on Tryptone Bile X-Glucuronide (TBX; Thermo Fisher, France) agar plates for detection of *E. coli* and on TBX supplemented with 4 mg/L cefotaxime (TBX-CTX) (Sigma Aldrich, France) for detection of ESBL *E. coli*. The plates were incubated at 44 °C for 24 h. After incubation, blue colonies (glucuronidase positives) were counted. In the absence of colony growth, *E. coli* and ESBL *E. coli* were enriched by dissolving one pack of Colilert®-18 test kit (IDEXX, France) in 100 mL of water sample. Following incubation at 44 °C for 24 h, 10 μL of the enrichment was plated onto TBX supplemented or not with cefotaxime (4 mg/L) and the plates were then incubated at 44 °C for 24 h. The results are expressed as colony forming units (CFU) per 100 mL of sample.

The presence of ESBL *E. coli* was examined in the sediments, on the roots and in the myocastor feces. The sediments were directly

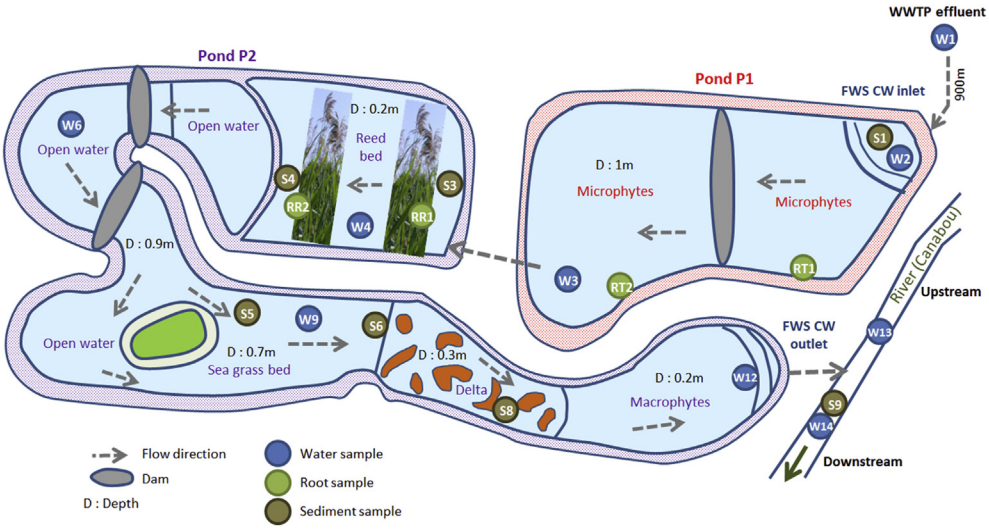

Fig. 1. Schematic representation of the free water surface constructed wetland.

plated onto TBX-CTX whereas the roots and the myocastor feces were homogenized by vortex in NaCl (0.85%) before plating. Plates were incubated at 44 °C for 24 h. In the absence of ESBL *E. coli*, 10 g of samples were added to 90 mL of sterile water and one pack of Colilert®-18 test kit and treated as described above.

For each sample (enriched or not), up to five ESBL isolates were randomly picked from the TBX-CTX plates when possible. Isolates were purified on TBX-CTX and then grown separately at 44 °C in brain heart infusion (BHI) medium (Thermo Fisher, France) supplemented with 4 mg/L cefotaxime (BHI-CTX). Isolates were stored at − 80 °C in BHI medium containing 25% glycerol. In total, 369 ESBL-producing isolates were collected and stored in the lab culture collection for subsequent analysis.

2.4. Antibiotic resistance

The antibiotic susceptibility of isolates was tested using the disk diffusion method in agar medium. *E. coli* ATCC 25922 was used as the control strain. Sixteen antibiotic disks were used, including penicillin (6 μg), 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 8000 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 suspensions was used to inoculate Mueller-Hinton agar plates (Bio-Rad Laboratories, France). Antibiotic disks were finally 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. Multi-resistance defines resistance to at least two different families of antimicrobial agents.

2.5. ESBL identification

Genomic and plasmid DNA were extracted from the 369 ESBL-positive *E. coli* isolates. 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 PCR testing. Plasmid DNA was extracted from 5 mL of overnight culture (BHI-CTX, 37 °C) 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 (Table 1). PCR amplification was carried out in a final volume of 20 μL containing 2.5 μL of DNA template, 2 μL of 10X PCR buffer with $MgCl_2$, 0.5 μL of dNTP mix (10 mM), 0.2 μL of Taq polymerase (5U/μL), and 1 μL of each primer (10 μM). Different PCR conditions, listed in the Supplementary Table S1, were used depending on the primers concerned.

2.6. Genotyping

Seventy-eight ESBL *E. coli* strains were genotyped by MLST. Internal fragments from seven housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA* and *recA*) were amplified and sequenced as described online (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli/documents/primersColi_html). The alleles, the sequence types (ST), and the clonal complexes of each strain were assigned in accordance with the *E. coli* MLST database (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli/). A UPGMA (unweighted pair-group method with arithmetic mean) tree was generated from the allelic profiles of the strains with the software START2 (Jolley et al., 2001).

2.7. Calculations and statistical analysis

The efficiency of *E. coli* removal by the FWS CW, represented by the log removal of the *E. coli* population, was calculated as follows:

$$Efficiency = Log10\left|\frac{CFU_{W12}}{CFU_{W2}}\right|$$

where, CFU_{W2} and CFU_{W12} are the concentrations of *E. coli* (expressed in CFU/100 mL) at the inlet and the outlet of the FWS CW, respectively. The efficiency was calculated for each measurement (16 times over the year) and geometric means were performed to gather values per month.

Spearman's rank correlation was used to test the dependence

Table 1
Specific primers used in this study to target the *bla* genes encoding ESBL from CTX-M, TEM and OXA families.

Primers	Target	Sequence 5′ → 3′	References
CTX-M1 F469	bla_{CTX-M} group 1	CAGCTGGGAGACGAAACGTT	(Hartmann et al., 2012)
CTX-M1 R532		CCGGAATGGCGGTGTTTA	
CTX-M9 F446	bla_{CTX-M} group 9	GAGGCGTGACGGCTTTTG	(Hartmann et al., 2012)
CTX-M9 R513		CGTAGGTTCAGTGCGATCCA	
Mab/F	bla_{TEM}	ATAAAATTCTTGAAGAC	(Korzeniewska et al., 2013)
Mab/R		TTACCAATGCTTAATCA	
OXA F	bla_{OXA}	ACACAATACATATCAACTTCGC	(Korzeniewska et al., 2013)
OXA R		AGTGTGTTTAGAATGGTGATC	

between the efficiency of *E. coli* removal and seasonal temperatures.

To determine which zone of the FWS CW had a significant impact on the bacterial contamination, a non-parametric analysis of variance (Kruskal-Wallis ANOVA) with pairwise comparisons using Mann-Whitney's test was used. Model assumptions of normality and homogeneity of variance were respectively checked using the Shapiro-Wilcoxon test and the Bartlett's test.

A Venn diagram was constructed to represent the distribution of *bla* genes on the plasmid DNA of the ESBL *E. coli*.

Generalized linear models were fitted using the family = "binomial" argument that uses a logit transformation to determine whether or not a specific antibiotic resistant pattern appeared in a specific zone of the FWS CW. The analysis was performed with the *Glm* function in the *lme4* package (Bates et al., 2015) for R version 3.2.3 (R Core Team, 2015). Finally, the Shannon index (H′) was used to evaluate the diversity of ESBL profiles among the FWS CW. The index H′ was calculated as follows:

$$H' = -\sum_{i=1}^{R} p_i \ln p_i$$

where i is an ESBL profile and p_i the proportion of ESBL *E. coli* belonging to the ESBL profile,

$$p_i = n_{i/N}$$

where n_i is the number of ESBL *E. coli* belonging to an ESBL profile and N is the total number of ESBL *E. coli*.

3. Results

3.1. Removal of E. coli in the water compartment of the FWS CW

The average concentrations of *E. coli* in the six areas of the FWS CW evaluated over a period of one year are listed in Table 2. The levels of *E. coli* gradually decreased along the FWS CW from $4.8.10^4$ CFU/100 mL at the inlet to $2.5.10^2$ CFU/100 mL in the outlet. However, as shown by the minimum and maximum values in Table 2, concentrations of *E. coli* varied considerably within each sampling location. This could be due to variations in environmental factors. For example, air temperatures varied between −1 °C and 36 °C during the sampling period (Supplementary Fig. S1).

To investigate whether the efficiency of bacterial removal of the FWS CW was impacted by seasonal variations in temperature, the *E. coli* $\log_{10}$ removal by the FWS CW observed for each month was compared to average monthly air temperatures at the study site (Fig. 2). The average monthly temperatures ranged from 6.8 °C (in February) to 27.4 °C (in July). A significant negative correlation was found between *E. coli* removal and air temperature using Spearman's rank correlation test (rSpearman = −0.56, P = 0.041), showing a decrease in efficiency of the bacterial disinfection process by the FWS CW under high temperature conditions. Indeed, the removal of *E. coli* by the FWS CW fluctuated from 1.5 $\log_{10}$ at the highest temperatures to 3 $\log_{10}$ at the lowest temperature.

To account for variations in temperature over the sampling period, the levels of *E. coli* at each sampling point were grouped according to the season (Fig. 3). During the coldest months (January to April and December) (Fig. 3A), the concentrations of *E. coli* (ranging from $2.4.10^4$ to $3.5.10^5$ CFU/100 mL) were similar in the WWTP secondary effluent (W1) and in the inlet of the FWS CW (W2) (Mann-Whitney's test, P = 0.399). Then, along the FWS CW (from W2 to W12), a progressive decline in the concentrations of *E. coli* was observed, with a 2.8 $\log_{10}$ decrease between the inlet (W2) and the outlet (W12) of the FWS CW, leading to a final concentration of *E. coli* of 1.10^2 CFU/100 mL. Two areas of the FWS CW (the pond P1 and the reed bed of the pond P2) significantly influenced the levels of *E. coli* (Mann-Whitney's test, P = 0.037 and 0.007, respectively). The first decrease in the concentration of *E. coli* (0.5 $\log_{10}$) occurred between the inlet (W2) and the outlet (W3) of

Table 2
E. coli concentration in the free water surface constructed wetland.

Sample	CFU/100 mL		
	Mean[a]	Min	Max
W2	$4.8.10^4$	1.10^3	$2.4.10^5$
W3	$7.0.10^3$	20	$6.9.10^4$
W4	$1.2.10^3$	38	$4.2.10^3$
W6	$3.8.10^2$	40	$2.1.10^3$
W9	$1.6.10^2$	1	$1.2.10^3$
W12	$2.5.10^2$	<1	$1.6.10^3$

[a] Arithmetic mean.

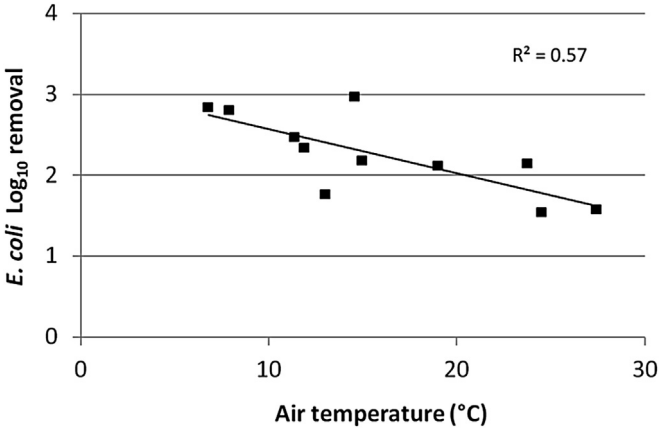

Fig. 2. Effect of seasonal variations in temperature on the efficiency of *E. coli* removal by the free water surface constructed wetland. R^2 represents the coefficient of determination of the linear model between seasonal temperatures and *E. coli* removal.

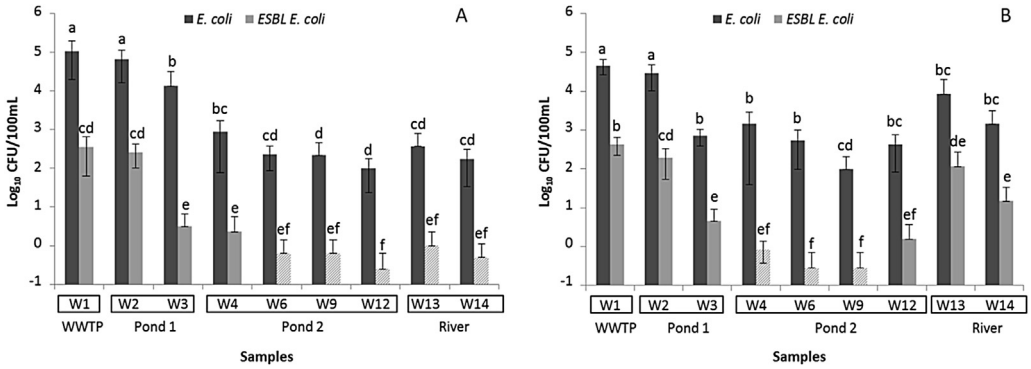

Fig. 3. Concentration of *E. coli* and ESBL *E. coli* in waters of the wastewater treatment plant (W1), throughout the free water surface constructed wetland (W2-W12) and from the river (W13 and W14) from January to April 2015 and December 2015 (A) and from May to November 2015 (B). The errors bars represent the standard deviation of all the samples. Letters indicate significant differences in concentration according to Mann-Whitney's test ($P < 0.05$). Hatched bars indicate that ESBL *E. coli* concentrations were close to the limit of detection.

the P1 pond. The second significant decrease (2 $\log_{10}$) occurred when the water passed from inlet (W3) to outlet (W6) of the reed bed located in the P2 pond. Interestingly, in the river, the water upstream of the FWS CW discharge (W13) contained a higher concentration of *E. coli* than downstream (W14).

During the warmest months (May to November), the levels of *E. coli* in the secondary WWTP effluent and in the inlet of the FWS CW remained stable (Fig. 3B) and variations were in the same order of magnitude as those observed during the coldest season. A significant decrease in the concentration of *E. coli*, reaching nearly 1.5 Log_{10}, was observed in the P1 pond (Mann-Whitney's test, $P = 0.002$). Then, unlike in the coldest months, the concentration of *E. coli* fluctuated but did not decrease along the FWS CW. Even if the differences were not statistically significant, an increase in concentration of *E. coli* was observed in the two vegetated areas (Mann-Whitney's test, reed bed W3-W4: $P = 1.000$ and macrophyte basin W9-W12: $P = 0.055$). The water discharged into the river was significantly more contaminated during summer and fall than in winter and spring (Mann-Whitney's test, $P = 0.028$). The river was also more contaminated upstream of the FWS CW discharge than downstream. The *E. coli* concentration measured in the river was 1.5 $\log_{10}$ higher during summer and fall than during winter and spring.

3.2. Removal of ESBL E. coli in the water compartment of the FWS CW

ESBL *E. coli* were detected in all samples with concentrations ranging from 1 CFU to 1.10^3 CFU/100 mL, 1000 fold lower than that of *E. coli*, irrespective of the sampling point (Fig. 3). As observed for *E. coli*, there was a significant reduction in the concentration of ESBL *E. coli* in the P1 pond, regardless of the season (Mann-Whitney's test, $P = 0.01$ and 0.02 according to the season) (Fig. 3A and B). Then, as described before, during winter and spring, the ESBL *E. coli* concentrations decreased gradually in the P2 pond and were significantly lower at the outlet of the pond (Mann-Whitney's test, $P = 0.021$) while during summer and fall, concentrations fluctuated. The concentrations firstly decreased and this diminution was statistically significant (Mann-Whitney's test, $P = 0.031$) but at the outlet of the P2 pond the contamination increased so that the inlet and outlet of the P2 pond presented a same level of contamination (Mann-Whitney's test, $P = 0.136$). In the river, the water upstream of the FWS CW discharge had higher concentrations of ESBL *E. coli* than downstream, especially in summer and fall.

3.3. bla gene typing and antibiotic susceptibility of the ESBL E. coli isolates

Over the sampling period, 369 ESBL *E. coli* were collected in the different water compartments. Seventy nine, 215 and 75 isolates were collected from the secondary effluent of the WWTP, the FWS CW and the river, respectively. *bla* gene type and antibiotic susceptibility were determined to characterize the ESBL-producing isolates.

As shown in Fig. 4, $bla_{\text{CTX-M}}$ type genes were found to be dominant in the ESBL-producing isolates. Eighty-four percent of the isolates harbored such genes, with a higher proportion of CTX-M1 type (66%) than CTX-M9 type (54%). Both $bla_{\text{CTX-M1}}$ and $bla_{\text{CTX-M9}}$ genes were detected simultaneously in 36% of the isolates. Genes coding for bla_{TEM} and bla_{OXA} were present in 30% and in 18% of the isolates, respectively. A small proportion (9%) of the isolates did not carry any of these genes. The genes $bla_{\text{CTX-M}}$ ($bla_{\text{CTX-M1}}$ and $bla_{\text{CTX-M9}}$), bla_{TEM} and bla_{OXA} were carried on plasmids in 55.3% of the ESBL-producing isolates (204 isolates). Of these 204 isolates, 119 carried at least one $bla_{\text{CTX-M1}}$ gene, 118 carried a $bla_{\text{CTX-M9}}$ gene, 45 a bla_{TEM} gene, and 20 a bla_{OXA} gene. As illustrated by the Venn diagram in Fig. 5, 61% of the isolates with plasmid-encoded-*bla* genes carried only one of the genes tested ($bla_{\text{CTX-M9}}$ and $bla_{\text{CTX-M1}}$ accounting for 25% and 24%, respectively). Among the remaining isolates, 32% and 6.5% harbored two and three *bla* genes,

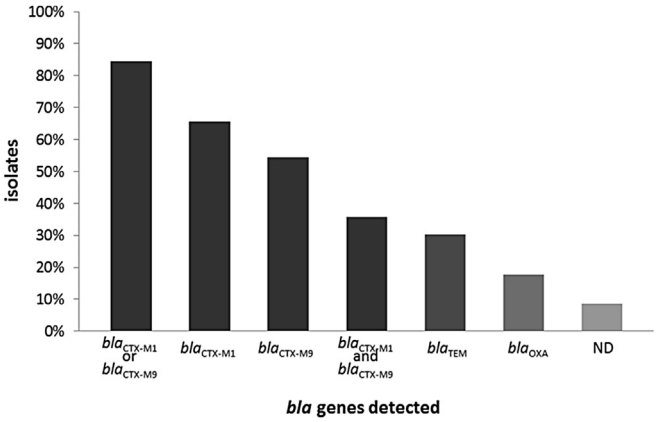

Fig. 4. Type of ESBL detected in the ESBL *E. coli* isolated from waters of the wastewater treatment plant, free water surface constructed wetland and river (n = 369). ND indicates that none of the *bla* genes in this study were carried by the strains.

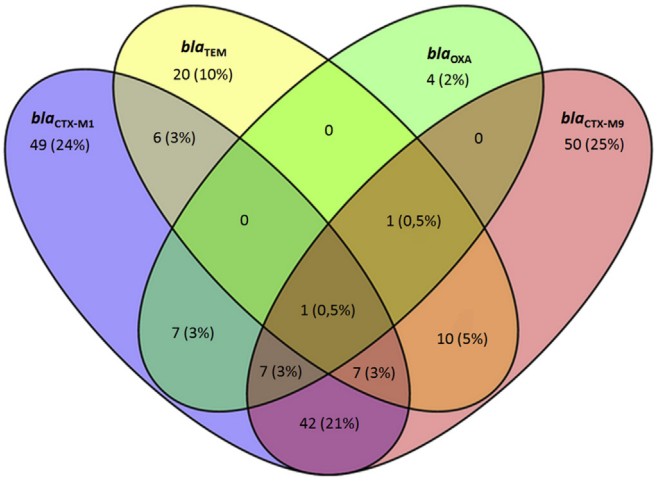

Fig. 5. Venn diagram comparing the distribution of *bla* genes on plasmid DNA of the ESBL *E. coli* isolated from waters from the wastewater treatment plant, free water surface constructed wetland and the river (n = 204).

respectively. One isolate harbored the four *bla* genes tested (bla_{CTX-M1}, bla_{CTX-M9}, bla_{TEM} and bla_{OXA}).

Antimicrobial susceptibility of the 369 ESBL-producing isolates was tested on 16 antibiotics. Seven percent of the strains only showed resistance to Beta-lactamins (cefotaxime and penicillin) and were consequently classified as non-multi-drug resistant. The resistance spectrum of the other isolates ranged from three to 14 antibiotics (Fig. 6). On average, the strains were found to be resistant to six antibiotics but 38 isolates (10.3%) presented a higher level of resistance, being resistant to 11 to 14 of the 16 antibiotics. Twenty-nine percent of the isolates were found to be resistant to 3 to 5 antibiotics and 45% to 7 to 10 antibiotics. All the isolates were at least resistant to the beta-lactam antibiotics cefotaxime and penicillin, and all were susceptible to the last-resort antibiotics imipenem (carbapenem family) and colistin. Most isolates were resistant to streptomycin (80%), followed by doxycycline (68%), cotrimoxazole (59%), ciprofloxacin (50%), ofloxacin (49%), kanamycin (46%), chloramphenicol (25%), tobramycin (21%), gentamicin (19%), amikacin (11%) and netilmicin (8%).

3.4. Distribution of the antibiotic resistant patterns and bla gene content of the ESBL-producing isolates in the FWS CW water

The results of association analysis using the *Glm* (generalized linear model) function indicated that the proportion of resistant strains did not significantly change along the FWS CW (Chi Square Test, P = 0.710). However, a slight increase in the percentage of resistance was observed after the reed bed in the open water of pond 2 (W6) (Fig. 7).

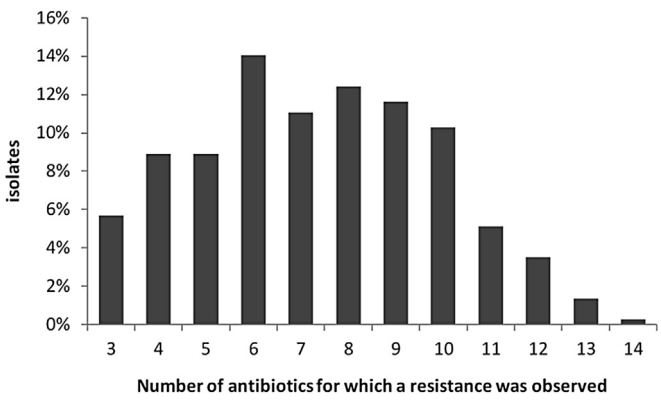

Fig. 6. Resistance spectrum of the ESBL *E. coli* isolated from water (n = 369).

bla gene profiles of the isolates were determined by the absence or presence of the different *bla* genes (bla_{CTX-M1}, bla_{CTX-M9}, bla_{TEM} and bla_{OXA}) in each isolate. Fig. 8 shows the *bla* gene profiles according to their sampling location. Their proportion among the ESBL-producing isolates ranged from 0% to 28% depending on the sampling location. In contrast to the resistance profiles that remained stable along the FWS CW, the *bla* gene profiles differed depending on the sampling site. Their number decreased from the inlet (with 13 ESBL profiles in W2) to the outlet of the FWS CW (W12) where eight ESBL profiles were identified. As indicated by the Shannon index (H′), the diversity of the ESBL profiles decreased, especially between W2 and W3 and between W6 and W9. Two of the *bla* gene profiles were isolated in a specific area. The *bla* gene profiles "aapp" (bla_{CTX-M1} and bla_{CTX-M9} absent, bla_{TEM} and bla_{OXA} present) and "pppp" (all four *bla* genes present) were only found in the outlet of pond P1 (W3) and in the outlet of the FWS CW (W12), respectively. Conversely, three *bla* gene profiles ("apaa", bla_{CTX-M9} present; "paap", bla_{CTX-M1} and bla_{OXA} present and "ppap", bla_{CTX-M1}, bla_{CTX-M9} and bla_{OXA} present) were detected at all the sampling points of the FWS CW. Interestingly, the proportion of isolates with the "paap" profile increased along the FWS CW (from 3%, in the inlet to 25% in the outlet). The same phenomenon was also observed, although to a lesser extent, for the "ppap" profile (from 4% in the inlet to 10% in the outlet).

3.5. Diversity and distribution of the ST profiles of ESBL producing E. coli in different compartments of the FWS CW including the river

To identify the distribution and transfer of ESBL *E. coli* in and between the different compartments of the FWS CW, including the river, ESBL *E. coli* strains were also isolated from other FWS CW compartments, e.g. the sediments, myocastor feces, and roots of *Typha* and *Phragmites* (data not shown). The results of the analyses of all these supplementary compartments were positive for the presence of ESBL *E. coli* (data not shown). MLST analysis (7-genes Achtman scheme) was performed on a representative set of 78 ESBL-producing isolates. Forty-four originated from the water compartment and belonged to the previously analyzed 369 ESBL *E. coli*. The isolates were selected for the MLST analysis to represent the different zones of the FWS CW. The supplementary isolates originated from sediments (16 isolates), roots (8 isolates) and feces (10 isolates). The genotypic characteristics of these 78 isolates i.e. sequence type (ST) profile and clonal complex (CC) are listed in Supplementary Table S2. Eight isolates could not be assigned to any known ST profile according to the *E. coli* MLST database due to the lack of correspondence with the *purA* reference sequence (Supplementary Fig. S2). The predominant profile was ST1722 (11 isolates) followed by ST744 and ST131 (10 isolates each) and the unassigned ST (8 isolates), while 10 STs were represented by a single isolate (Table 3). Due to their similarity, ST10, ST34, ST167 and the unassigned ST were grouped in the clonal complex CC10, and ST88 and ST90 were grouped in the complex CC23. ST profiles were uniformly distributed in the water and root compartment but not in the sediment and feces one, as 50% of the isolates had the same assignation (Table 3).

The UPGMA tree (Fig. 9) generated from the allelic profiles (ST) evidenced transfer of ESBL *E. coli* in the different compartments of the FWS CW, as seven of the 12 clusters contained isolates of two different origins (water and sediment, water and myocastor feces or sediment and roots) or three different origins (water, roots and sediment or water, myocastor feces and sediment). Half the isolates

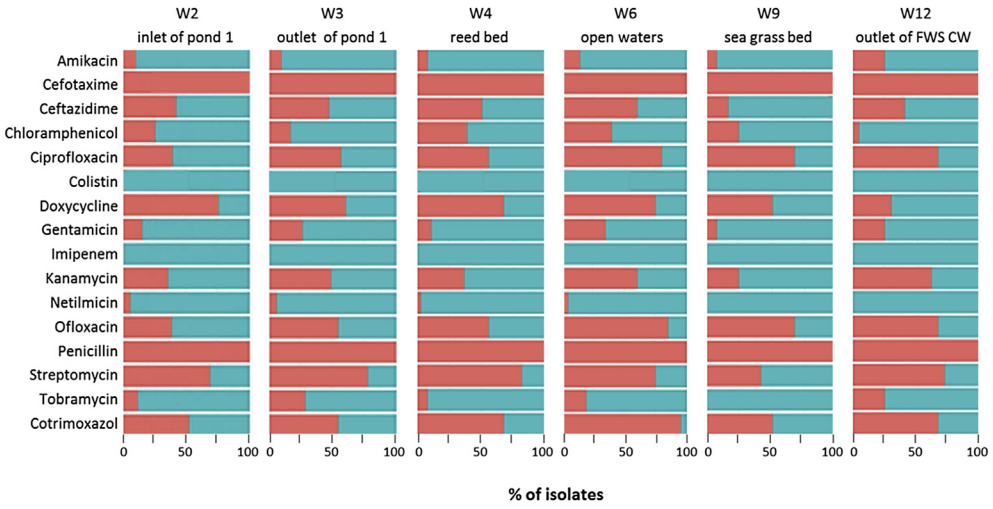

Fig. 7. Distribution of antibiotic resistance of the ESBL *E. coli* along the free water surface constructed wetland.

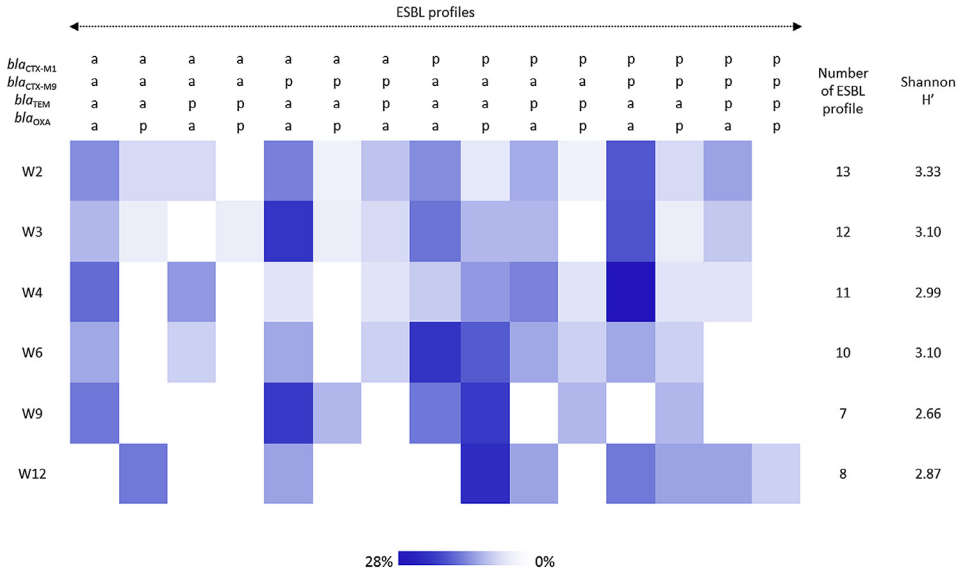

Fig. 8. Distribution of the ESBL types of the ESBL *E. coli* along the free water surface constructed wetland. Absence (a) or presence (p) of, respectively, bla_{CTX-M1}, bla_{CTX-M9}, bla_{TEM} and bla_{OXA}.

originating from the sediments clustered together (ST744) even though the sediments were sampled at different sites in the FWS CW (points S1, S3, S4, S6 and S8). Similarly, one (unassigned) cluster contained isolates collected in the water at different sampling sites (W2, W6, W12 and W14), evidence the strains circulated through the FWS CW into the river. It is noteworthy that the two isolates taken from the river upstream of the discharge of the FWS CW belonged to STs represented by single isolate.

4. Discussion

We confirm that FWS CW is an effective way to reduce *E. coli* contamination of WWTP effluent as previously reported (Headley et al., 2013; Mulling et al., 2013; Vacca et al., 2005; Wood et al., 2015). In our study, a log decrease (from 1.8 to 3.8 $\log_{10}$) in *E. coli* contamination was observed. In comparison to the data presented in the review of Wu et al. (2016), the efficiency of the FWS CW we studied was high considering that the hydraulic retention time (HRT) was only three days. Indeed, in most FWS CW of equivalent size and HRT, the microbial reduction did not exceed 1 $\log_{10}$ (Kadlec et al., 2010; Karpiscak et al., 2001). The highest removal rate was observed in several studies in which the CWs were smaller and the HRT higher. For example, in the 5.64 m^2 pilot scale CW studied by Headley et al. (2013), a 1.3 to 1.5 $\log_{10}$ microbial reduction was measured for a HRT of around 5.5 days. Hench et al. (2003) reported a similar reduction of 2.3 $\log_{10}$ in 400 L wetland mesocosms with a HRT ranging from 6 to 8 days.

FWS CWs are complex systems that use the natural functions of vegetation, soil, and microorganisms to treat water. Consequently, multiple parameters can affect the efficiency of the FWS CW. Seasonal temperatures are one of these parameters. Our data showed that an increase in temperature reduced the efficiency of microbial removal in the FWS CW. During the colder seasons, a regular decrease in *E. coli* was recorded in all the water compartments (vegetated or not), whereas during the warmer seasons, the concentration of *E. coli* decreased in areas with no vegetation but

Table 3
Allelic profiles frequencies of the ESBL *E. coli* in the different compartments.

ST profile	ST complex	Frequency % (n)				
		Total	Water	Sediment	Root	Feces
1722	none	14.1 (11)	13.6 (6)	–	–	50 (5)
744	none	12.8 (10)	–	50 (8)	25 (2)	–
131	131	12.8 (10)	18.2 (8)	6.3 (1)	–	10 (1)
UA[a]	10	10.2 (8)	18.2 (8)	–	–	–
88	23	6.4 (5)	6.8 (3)	6.3 (1)	12.5 (1)	–
3045	none	6.4 (5)	11.4 (5)	–	–	–
683	none	5.1 (4)	–	6.3 (1)	37.5 (3)	–
354	354	5.1 (4)	2.3 (1)	–	–	30 (3)
38	38	3.8 (3)	6.8 (3)	–	–	–
117	none	3.8 (3)	4.5 (2)	6.3 (1)	–	–
90	23	3.8 (3)	4.5 (2)	–	12.5 (1)	–
167	10	2.5 (2)	–	12.5 (2)	–	–
5493	none	1.3 (1)	2.3 (1)	–	–	–
58	155	1.3 (1)	2.3 (1)	–	–	–
847	none	1.3 (1)	2.3 (1)	–	–	–
46	46	1.3 (1)	–	6.3 (1)	–	–
34	10	1.3 (1)	2.3 (1)	–	–	–
10	10	1.3 (1)	2.3 (1)	–	–	–
69	69	1.3 (1)	–	–	12.5 (1)	–
2914	none	1.3 (1)	2.3 (1)	–	–	–
120	none	1.3 (1)	–	–	–	10 (1)
362	none	1.3 (1)	–	6.3 (1)	–	–

[a] UA, unassigned.

increased in the vegetated areas, resulting in more *E. coli* being discharged into the river during the warmer seasons. The higher removal efficiency of the FWS CW during colder seasons contradicts previously published data. Several authors reported that, in their systems, bacterial removal, including *E. coli* and fecal bacteria, was higher during summer when temperatures were high (Arora and Kazmi, 2015; Karathanasis et al., 2003; Morató et al., 2014), but the temperatures and type of wetland systems in these studies differed from our experimental conditions. For example, Arora and Kazmi (2015) showed bacterial removal was maximal when the temperature reached 38–40 °C whereas in our study, monthly average temperatures did not exceed 27 °C. In the same way, the study by Karathanasis et al. (2003), involved a single separated lined constructed wetland (vegetated or not) while the FWS CW in our study is a succession of vegetated and vegetation free basins. Finally, in two other studies, the authors found no effect of temperature on the efficiency of microbial removal (Headley et al., 2013; Vacca et al., 2005).

Seasonal temperatures explained 60% ($R^2 = 0.57$) of the variation in *E. coli* removal in our system. This suggests that other factors may influence the efficiency of the FWS CW in bacterial disinfection. Seasonal fluctuation is also known to be related to the intensity of UV, the status of the plants, and to wildlife activity. During warmer seasons, the number of days with sunshine and the intensity of UV irradiation increase. Solar disinfection is known to be an effective treatment against bacterial populations. The P1 pond in our study is free of vegetation and is thus well exposed to solar UV. The notable reduction in *E. coli* contamination (about 2 $\log_{10}$) measured during the warmer seasons could be a consequence of UV disinfection. In a recent study, Schmidtlein et al. (2015) demonstrated that solar exposure of a secondary effluent for 180 min decreased *E. coli* contamination more than 10 fold. Moreover, in our study, floating plants (*Lemna* sp.) colonized the P1 pond from mid-June to mid-August (warm season) and were removed by hand in August. These plants provide attachment sites for bacteria (mixed biofilm). We can thus assume that the water was less contaminated at this time because of the transfer of the bacteria from the water to floating plants.

In contrast to areas of open water, the vegetated zones of the FWS CW (W4 and W12) appear to promote the growth of *E. coli* during the warmer seasons. This may be due to the combined effect of environmental factors that are specific to the vegetated zones in the warmer months. Indeed, the water flowing through the reed bed and the macrophyte basin is not exposed to UV irradiation. Although Sidrach-Cardona and Becares (2013) observed more efficient reduction of *E. coli* and of coliforms in the planted CW than in their unplanted replicates, Quinonez-Diaz et al. (2001) reported that the vegetation may protect microorganisms by creating a natural barrier against UV, thereby reducing the removal of bacteria. In addition, the rhizosphere environment could be more favorable for the establishment of the bacterial population during the warmer period. The composition of root exudates (concentration of amino acids, phenolic compounds, sugar and sugar alcohols) differs with the plant development stage (vegetative, bolting and flowering), resulting in a change of the rhizo-microbiome (Huang et al., 2014). Sugars and amino acids, which are chemoattractants for microbes, become more abundant in root exudates at later plant growth stages (Aulakh et al., 2001; Badri and Vivanco, 2009). Furthermore, as reported by Díaz et al. (2012), *E. coli* could find anaerobic conditions in the reed bed that could improve their persistence by decreasing competing aerobic populations. Finally, in our study, the presence of wild animals, such as moorhens and myocastors, which may increase the concentration of *E. coli*, especially during the warm season when vegetated areas provide a favorable habitat for nesting and food supplies. It thus appears that the configuration of the studied FWS CW, ending in a vegetated area, favors the survival of the bacteria in the warmer season. In such a context, it would be more efficient to end the water treatment process with an open water zone to avoid an increase in bacterial contamination before the water is released into the river.

It is noteworthy that during the one-year sampling period, there was no negative impact of the FWS CW on the level of fecal contamination of the river which flowed through a rural area receiving effluents from on-site sanitation systems. On the contrary, regardless of the season, the discharge of the FWS CW decreased both the level of *E. coli* and ESBL producing *E. coli* present in the river (dilution effect by the water discharged into the river).

ESBL *E. coli* were detected in the different zones of the FWS CW and in the river at a 1000 fold lower concentration than *E. coli*, which is in agreement with the data reported by Brechet et al. (2014). These authors reported that ESBL *E. coli* accounted for 0.1% of all *E. coli* in urban effluents. Contamination by ESBL *E. coli* was significantly reduced through the FWS CW as reported for *E. coli*. Interestingly, ESBL producing *E. coli* showed a similar behavioral pattern to *E. coli*, i.e. removal was lower in summer than in winter, suggesting that resistance to beta-lactamines did not influence the fitness of ESBL producing *E. coli*.

The ESBL producing *E. coli* isolated in our study showed a strong multidrug resistance phenotype. Ninety-three percent of the ESBL *E. coli* isolated in the FWS CW showed resistance to at least three of the 16 antibiotics tested. This result is in accordance with the high proportion of multidrug resistant ESBL *E. coli* strains (97.3%) isolated from effluents sampled in Spanish wastewater treatment plants (Ojer-Usoz et al., 2014). Interestingly, the profile of multidrug resistance did not change in the different areas of the FWS CW suggesting that no new resistance appeared in the water circulating through the FWS CW. The emergence of resistance may depend on the type of CW. Sidrach-Cardona and Becares (2013) compared the percentage resistance of *E. coli* to three antibiotics between the inlet and the outlet of seven independent pilot scale CWs. They observed high variability of the percentage of resistance depending on the configuration of the CWs and of the antibiotic tested. In our study, most of the ESBL isolates from the FWS CW produced CTX-M-type ESBL with a predominance of CTX-M group 1. This is consistent

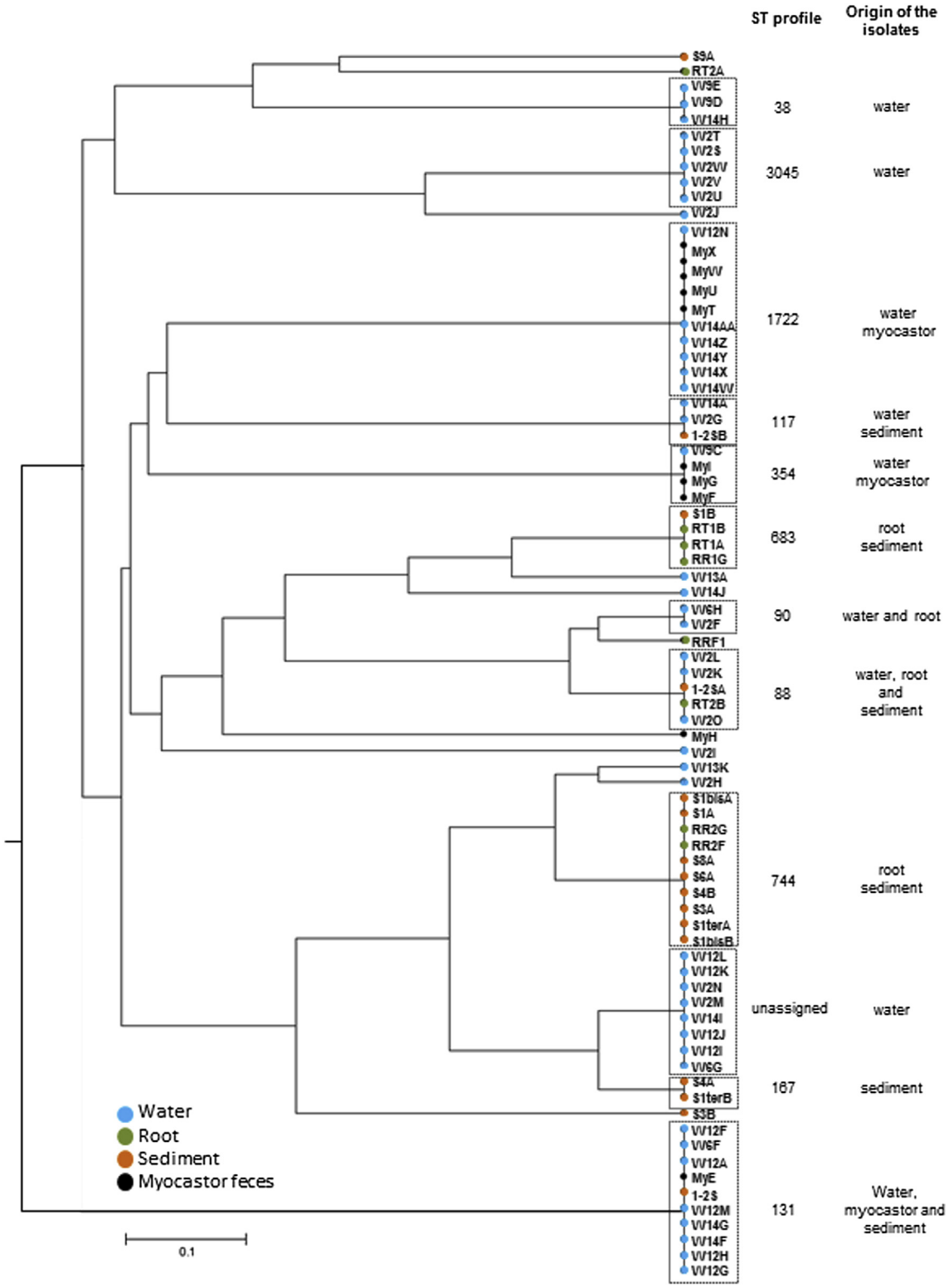

Fig. 9. UPGMA (unweighted pair group method with arithmetic mean) tree generated from allelic profiles of the ESBL *E. coli*. ID, ST and allelic profile of the ESBL *E. coli* are shown. The two major phylogroups are denoted A and B. The most predominant STs (ST1722, ST744, ST131 and ST617) are boxed in the figure.

with previous studies reporting that among CTX-M enzymes, the CTX-M-15 (CXT-M group 1) and CTX-M-14 (CTX-M group 9) are the most common ESBL enzyme in human, animal and environmental compartments all over the world (Cantón et al., 2012; Dolejska et al., 2011). Similarly to our results, Brechet et al. (2014) and Galvin et al. (2010) reported that a large part of ESBL *E. coli* isolated in effluents from urban and hospital WWTP harbored $bla_{\text{CTX-M}}$ (88 and 95.7% respectively). They also reported that CTX-M group 1 was the most frequent group of ESBL enzymes. Interestingly, we observed that more than half the ESBL strains harbored *bla* genes

on plasmid DNA. This is the first report of a high prevalence of plasmid carrying *bla* genes in environmental strains, suggesting that WWTP effluent could be a substantial environmental source of *bla* genes. However, the decrease in the concentration of ESBL producing *E. coli* and the decrease in their diversity along the course of the FWS CW suggests that this tertiary treatment limits the dissemination of *bla* genes into the natural environment (i.e. surface water).

The fate of the ESBL *E. coli* in the FWS CW is complex. A combination of natural die-off, predation and competition for nutrients, as well as transfer of the bacteria from one compartment to another (sedimentation, adsorption on the surface of the roots) may explain the reduction in the population through the FWS CW. In FWS CWs, free surface water, sediment and vegetation constitute an intricate environment in which bacteria circulate. We found that ESBL producing *E. coli* were present in the sediments and on the roots of *Phragmites* and *Typha* collected in the FWS CW, suggesting that ESBL *E. coli* were transferred from water to sediments and vegetation. The MLST analysis provided evidence that clonal isolates circulated in the FWS CW and in the surrounding environment. ESBL producing *E. coli* harboring identical STs were found in different areas and compartments of the study site, including wild animals, suggesting clonal populations circulate at our study site. Of these, four STs were predominant, including ST131, which has been identified as a pandemic clonal ST in humans (Ewers et al., 2012). Although none of the 78 ESBL *E. coli* collected in the FWS CW harbored the *eae* gene encoding the intimin adherence protein (data not shown), according to the MLST database, various *E. coli* submitted: ST10, ST34, ST38, ST46, ST69, ST88, ST90, ST117, ST120, ST131, ST167, ST354, ST362 and ST2914, also identified in this study, are pathogenic and virulent for humans (EPEC, EAEC, EHEC).

5. Conclusion

Although *E. coli* circulated in the different compartments of the FWS CW, this study demonstrates that FWS CWs could be efficient systems to limit the dissemination of fecal contamination, including ARB, to the aquatic environment. It is also important to note that vegetated areas of the FWS CW increased the survival of *E. coli* during the warmer months and hence reduced the efficiency of the FWS CW.

Acknowledgments

The authors are grateful to the French National Agency for Water and Aquatic Environments (ONEMA) for their interest in this study and financial support. We are most grateful to the genomics platform of Nantes (Biogenouest Genomics) core facility for technical support. MLST data are publicly available at http://www2.warwick.ac.uk/mlst, which is currently supported by a grant from the Biotechnology and Biological Sciences Research Council (BBSRC).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2016.08.015.

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