Diversity and antibiotic resistance of *Aeromonas* spp. in drinking and waste water treatment plants

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A B S T R A C T

The taxonomic diversity and antibiotic resistance phenotypes of aeromonads were examined in samples from drinking and waste water treatment plants (surface, ground and disinfected water in a drinking water treatment plant, and raw and treated waste water) and tap water. Bacteria identification and intra-species variation were determined based on the analysis of the 16S rRNA, *gyr*B and *cpn*60 gene sequences. Resistance phenotypes were determined using the disc diffusion method.

Aeromonas veronii prevailed in raw surface water, *Aeromonas hydrophyla* in ozonated water, and *Aeromonas media* and *Aeromonas puntacta* in waste water. No aeromonads were detected in ground water, after the chlorination tank or in tap water. Resistance to ceftazidime or meropenem was detected in isolates from the drinking water treatment plant and waste water isolates were intrinsically resistant to nalidixic acid. Most of the times, quinolone resistance was associated with the *gyr*A mutation in serine 83. The gene *qnr*S, but not the genes *qnr*A, B, C, D or *qep*A, was detected in both surface and waste water isolates. The gene *aac(6')-ib-cr* was detected in different waste water strains isolated in the presence of ciprofloxacin. Both quinolone resistance genes were detected only in the species *A. media*. This is the first study tracking antimicrobial resistance in aeromonads in drinking, tap and waste water and the importance of these bacteria as vectors of resistance in aquatic environments is discussed.

Keywords:
Aeromonas
Quinolone resistance
Surface water
Waste water

1. Introduction

The genus *Aeromonas* comprises ubiquitous bacteria, considered indigenous to aquatic environments (Janda and Abbott, 2010). Members of this genus are able to inhabit surface water (rivers, lakes), sewage, drinking water (tap and bottled mineral), thermal waters and sea water (Biscardi et al., 2002; Maalej et al., 2003; Pablos et al., 2009). Some species, mainly the psychrophilic *Aeromonas salmonicida* and the mesophilic *Aeromonas hydrophila* and *Aeromonas veronii* are recognized causative agents of fish disease (Janda and Abbott, 2010). *Aeromonas* spp. are also important human opportunistic pathogens with ability to cause various types of diseases, which include intestinal, blood, skin and soft tissue and trauma-related infections (Aminov, 2009; Lamy et al., 2009; Janda and Abbott, 2010). Among the leading pathogenic species are *A. hydrophila*, *Aeromonas caviae* (later synonym of *Aeromonas punctata*) and *A. veronii* (Lamy et al., 2009). The environmental ubiquity associated with the potential pathogenicity of these bacteria has been illustrated also in recent

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natural disasters (Dixon, 2008). Evidences for the water-human transmission of *Aeromonas* spp. are also available (Khajanchi et al., 2010).

Over the last years, a greater public awareness and scientific understanding of antimicrobial resistance contributed to consider environmental reservoirs and paths of dissemination as critical points for antimicrobial resistance control. Among such reservoirs and paths of dissemination, water environments in which enter antibiotic resistant organisms from human and animal sources play a pivotal role (Baquero et al., 2008; Kümmerer, 2009; Taylor et al., 2011). The importance of municipal waste water treatment plants as sources of antimicrobial resistance and the risks of contamination of surface waters has been demonstrated in numerous publications (e.g. Gõni-Urriza et al., 2000; Ferreira da Silva et al., 2007; Servais and Passerat, 2009; Novo and Manaia, 2010). As a consequence of surface and ground water contamination, emerges the hypothesis that antimicrobial resistance can reach drinking water, serving as a vehicle of resistance transfer to the water consumers. Indeed, antimicrobial resistance has been detected in drinking water (Schwartz et al., 2003; Faria et al., 2009; Xi et al., 2009; Vaz-Moreira et al., 2011). Considering this urban water cycle, ubiquitous bacteria, which can colonize different types of water, are of special interest to assess potential forms of antimicrobial resistance dissemination. Given their ubiquity and patterns of acquired antimicrobial resistance, members of the genus *Aeromonas* are good example of such bacteria.

In a recent comprehensive review on the genus *Aeromonas*, Janda and Abbott (2010) alerted for the little attention given to the general low susceptibility of aeromonads to various classes and combinations of antimicrobial agents. Nevertheless, the potential of aeromonads to develop and disseminate antibiotic resistance either in clinical settings or in the environment has been demonstrated in numerous publications (Walsh et al., 1997; Gõni-Urriza et al., 2000; Huddleston et al., 2006; Blasco et al., 2008; Cattoir et al., 2008; Gordon et al., 2008; Lamy et al., 2009; Arias et al., 2010a,b). Moreover, recent and emerging antibiotic resistance seems to be common in different species of *Aeromonas*. For instance, different variants of the plasmid-mediated quinolone resistance *qnr* gene were detected in environmental isolates of the species *A. punctata*, *Aeromonas media* or *Aeromonas allosaccharophila* (Cattoir et al., 2008; Picão et al., 2008; Xia et al. 2010). In spite the ubiquity of aeromonads in aquatic environments and the likelihood to develop antimicrobial resistance, the ecology and patterns of resistance of members of this genus present in drinking and waste water treatment plants has not been addressed in scientific literature. The current work aimed at filling this gap and was based on the hypothesis that *Aeromonas* spp. could serve as a vehicle for antibiotic resistance dissemination within the urban water cycle. According to this hypothesis, this work was designed to track aeromonads and their antibiotic resistance profiles in different parts of the urban water cycle. Our main goal was the identification of major sources of antibiotic resistant aeromonads and of critical points for their elimination. Specifically, it was intended to i) identify the different aquatic environments within the urban water cycle where aeromonads are more prevalent, and possible factors contributing for their reduction; ii) determine the predominant species in each of those environments; iii) infer about the possible relationship *Aeromonas* species-antibiotic resistance pattern.

2. Materials and methods

2.1. *Water sampling*

Water samples were collected from different aquatic environments within an urban water cycle in the region of Northern Portugal (Fig. 1). These sites included: i) a drinking water treatment plant (WTP) and the respective distribution system, which supplies water to a population of about 1.5 million of inhabitants; ii) household tap water, served by the WTP and iii) a municipal waste water treatment plant (WWTP), serving 85 000 inhabitant equivalents, in the same geographical area (Ferreira da Silva et al., 2006; Vaz-Moreira et al., 2011). In the WTP, samples were collected from raw surface water (A), ground water (alluvial wells) (B), after sand filtration and ozonation (C) and after chlorination (D), the drinking water final treatment step. Four samples were collected downstream the WTP, in the drinking water distribution system respectively, after the first or the second re-chlorination stations (E-H). Tap water samples were collected in eleven houses (I-S), from taps used 1–4 times a month, served by the WTP referred to above and situated within an area of 25 km (Vaz-Moreira et al., 2011). Waste water samples corresponded to raw (T) and treated waste water (U). Sites A to H (drinking water treatment plant and distribution system) were sampled in November 2007 and in September 2009, sites I to S (taps) were sampled in April, July and October 2009 and sites T and U (waste water treatment plant) were sampled nine times between November 2004 and November 2009.

2.2. *Isolation, enumeration and preliminary identification*

This work was integrated in a wider study designed to assess the diversity and antibiotic resistance of culturable bacteria, belonging to different groups, present in selected niches within the urban water cycle. For the microbiological characterization of the water samples it was used the membrane filtration method, as described before for waste and surface water (Ferreira da Silva et al., 2006; Vaz-Moreira et al., 2011). Volumes of 10–500 ml (WTP, drinking water distribution system, taps) or of 1–10 ml (WWTP) of water samples or decimal dilutions thereof were filtered through cellulose nitrate membranes (0.45 μm pore size, 47 mm diameter, Albet), which were placed onto different culture media and incubated up to 7 days. No selective culture medium for aeromonads was used. *Aeromonas* spp. analysed in this study were isolated among the culturable heterotrophs recovered on different culture media - Plate Count Agar (PCA, Pronadisa), on m-endo-agar-LES (Difco), on mFC agar (Difco), on Tergitol-7 agar (TTC, Oxoid), on Pseudomonas Isolation Agar (PIA, Difco), on R2A agar (Difco) or on Bile Esculin Agar (BEA, Pronadisa). The culture media PCA, PIA, BEA and R2A were incubated at 30 °C and mFC agar, m-endo-agar-LES and TTC were incubated at 37 °C. Given this work was designed to

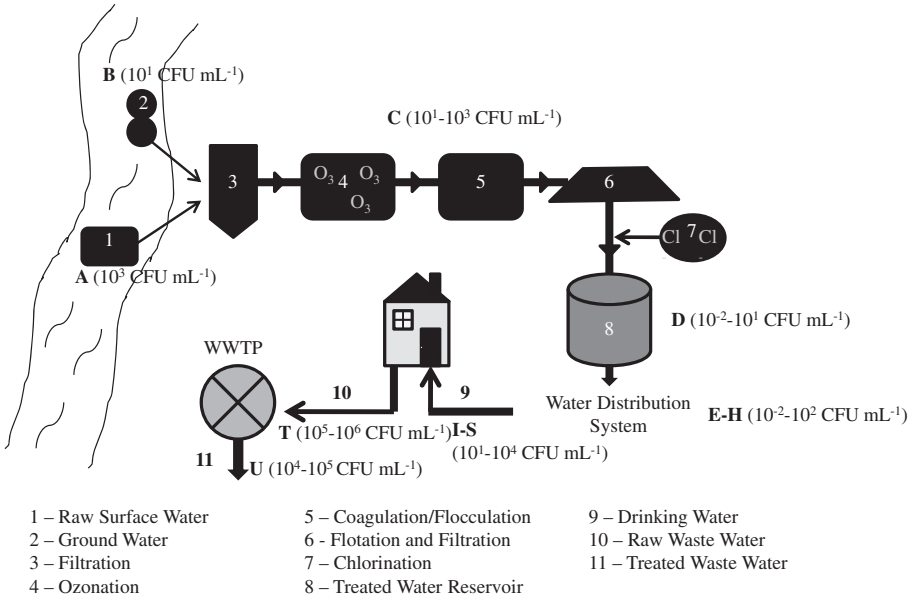


Fig. 1 – Schematic representation of the drinking and waste water treatment plants, indicating the treatment stages and sampled sites (A–U). Ground water disinfection involves only stages 6–7. CFU mL^{-1} corresponds to culturable heterotrophic counts in the plates from which the aeromonads were isolated.

recover culturable bacteria from different bacterial groups, representatives of all colony types were selected for further culture isolation and purification, according to the following criterion: all colonies of morphotypes represented by less than five colonies, half of the colonies of morphotypes represented by five to 10 colonies, and about one third of colonies with morphotypes represented by more than 10 colonies. Cytochrome *c* oxidase positive isolates with the morphology of Gram-negative rods and forming yellow colonies on GSP agar (Merck) at 30 °C were presumptively identified as aeromonads (Corry et al., 2003; Abulhamd, 2009; Kivanc et al., 2011). This set of aeromonads included a total of 121 isolates, from which 72 and 8 were isolated, respectively, from the first and second sampling campaigns in the WTP; 1, 3, 2, 6, 1, 1, 5 and 13 were isolated, respectively, from each sampling date in the WWTP. A group of 9 WWTP isolates recovered on PCA or mFC agar supplemented with 4 mg L^{-1} ciprofloxacin (AppliChem) (Novo and Manaia, 2010) were also examined in this study.

2.3. *Identification at the species level and determination of intra-species variation*

Identification at the species level was based on the analysis of the 16S rRNA gene sequence and intra-species variation was assessed on basis of the comparison of two additional housekeeping genes, *gyr*B and *cpn*60 (Yáñez et al., 2003; Miñana-Galbis et al., 2009). PCR amplifications of fragments of the genes 16S rRNA, *gyr*B and *cpn*60 were conducted using the primers and the conditions described before (Table 1). PCR products were purified with GFX PCR DNA purification kit (GE Healthcare) and the nucleotide sequences were determined.

Partial nucleotide sequences of the genes 16S rRNA, *gyr*B and *cpn*60 were aligned using Clustal W from MEGA 4.0 software (Tamura et al., 2007) and compared with the homologous sequences of the type strains of the different *Aeromonas* species, available in the GenBank database. For the gene *cpn*60, the nucleotide sequences of the type strains of the species *Aeromonas sanarellii* and *Aeromonas taiwanensis* were not available in the GenBank database, and were determined in this study using the type strains provided by BCCM/LMG culture collection with the numbers LMG 24682^{T} and LMG 24683^{T}, respectively. These nucleotide sequences were deposited in the GenBank database with the accession numbers JF920655 and JF920656 for *A. sanarellii* and *A. taiwanensis*, respectively.

Nucleotide sequence relatedness was estimated based on the model of Jukes and Cantor (1969) and dendrograms were created using the neighbour-joining method. The maximum-likelihood method was also applied to assess tree stability. In the analysis were used 1283, 779 and 555 nucleotide positions of the 16S rRNA, *gyr*B and *cpn*60 gene sequences, respectively. Non-repetitive nucleotide sequences were deposited in the GenBank database with the accession numbers JF920473-JF920563, JF938599-JF938689, and JF920564-JF920654 for 16S rRNA, *gyr*B and *cpn*60 sequences, respectively.

In an attempt to discriminate strains of the same species, each pair of isolates was compared based on the nucleotide sequence of each of the three genes. Strains differing at least in a nucleotide position were classified as representing distinct sequence types (ST). This comparison was represented in a dendrogram constructed based on 2617 nucleotide positions of the concatenated sequences of 16S rRNA, *cpn*60 and *gyr*B genes (Fig. 2).

2.4. *Determination of antibiotic resistance phenotypes*

The susceptibility to 12 antibiotics was determined using the agar diffusion method (CLSI, 2007). The antibiotics tested were nalidixic acid (NA, 30 μg); ciprofloxacin (CIP, 5 μg); amoxicillin

Table 1 – Primers and PCR conditions used.

Gene	Primers	Sequence	Fragment length (bp)	Annealing temp. (°C)	Reference
16S rRNA	27F	GAGTTTGATCCTGGCTCAG	1465	55 °C	Lane, 1991
	1492R	TAC CTT GTT ACG ACT T			
*gyr*B	*gyr*B-3F	TCCGGCGGTCTGCACGGCGT	1130	58 °C	Yáñez et al., 2003
	*gyr*B-14R	TTGTCCGGGTTGTACTCGTC			
*cpn*60	C175	GAAATYGAACTGGAAGACAA	763	52 °C	Miñana-Galbis et al., 2009
	C938	GTYGCTTTTTCCAGCTCCA			
*gyr*A	AsalgyrAF	TCCTATCTTGATTACGCCATG	481	50 °C	Goñi-Urriza et al., 2002
	AsalgyrAR	CATGCCATACCTACCGCGAT			
*par*C	AcparCF	GTTCAGCGCCGCATCATCTAC	245	54 °C	Goñi-Urriza et al., 2002
	EcparCR	TTCGGTGTAACGCATTGCCGC			
*qnr*A	qnr Am F	AGAGGATTTCTCACGCCAGG	580	54 °C	Cattoir et al., 2007
	qnrAm R	TGCCAGGCACAGATCTTGAC			
*qnr*B	qnrBm F	GGMATHGAAATTCGCCACTG	264	54 °C	Cattoir et al., 2007
	qnrBm R	TTTGCYGYYCGCCAGTCGAA			
*qnr*S	qnrSm F	GCAAGTTCATTGAACAGGGT	428	54 °C	Cattoir et al., 2007
	qnrSm R	TCTAAACCGTCGAGTTCGGCG			
*qnr*C	qnrC-F	GGGTTGTACATTTATTGAATC	447	50 °C	Wang et al., 2009
	qnrC-R	TCCACTTTACGAGGTTCT			
qnrD	qnrD-F	CGAGATCAATTTACGGGGAATA	582	55 °C	Cavaco et al., 2009
	qnrD-R	AACAAGCTGAAGCGCCTG			
aac(6')-Ib	aac(6)-F	TTGCGATGCTCTATGAGTGGCTA	482	55 °C	Park et al., 2006
	aac(6)-R	CTCGAATGCCTGGCGTGTTT			
*qep*A	qepA-F	TGGTCTACGCCATGGACCTCA	1137	56 °C	Périchon et al., 2007
	qepA-R	TGAATTCGGACACCGTCTCCG			
*cph*A	cphA-F	TCTATTTCGGGGCCAAGGG	230	55 °C	Balsalobre et al., 2009
	cphA-R	TCTCGGCCCAGTCGCTCTTCA			
bla_{TEM}	blaTEM fw	ATAAAATTCTTGAAGACGAAA	939	55 °C	DiPersio et al., 2005
	blaTEM rv	GACAGTTACCAATGCTTAATCA			

(AML, 25 μg); ticarcillin (TIC, 75 μg); cephalothin (CP, 30 μg); ceftazidime (CEF, 30 μg); streptomycin (STR, 10 μg); sulphamethoxazole/trimethoprim (SXT, 25 μg); tetracycline (TET, 30 μg); gentamicin (GEN, 10 μg); colistin sulphate (CT, 50 μg) and meropenem (MER, 10 μg). For the antibiotics AML and CT, which are not included in the CLSI list, were used the following criteria: $S \geq 21/R < 14$ and $S \geq 10/R < 10$, respectively. Whenever diameters larger than R but smaller than S were observed, were referred to as intermediary, and were excluded from the resistance percentage calculations. The strains *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* DSM 1117 (=ATCC 27853) were included as quality controls.

2.5. *Screening of resistance genetic determinants*

Mutations in the chromosomal genes *gyr*A and *par*C and the presence of resistance genes *qnr*A, *qnr*B, *qnr*S, *qnr*C, *qnr*D, *aac(6')-Ib* and *qep*A were screened in quinolone resistant isolates. The presence of genes *cph*A and bla_{TEM} associated with beta-lactam resistance were screened in all isolates. The primers and PCR conditions used were described before (Table 1).

Point mutations in the genes *gyr*A and *par*C were identified after comparison with homologous nucleotide sequences of quinolone susceptible strains available in the GenBank - *A. punctata* CIP 7616^T (AY027899 and AF435418), *A. hydrophila* subsp. *hydrophila* CIP 7614^T (AY027901 and AF435419) and *Aeromonas sobria* CIP 7433^T (AY027900 and AF435420) as described before (Goñi-Urriza et al., 2002). Strains *E. coli* L0 (*qnr*A1 +), *Klebsiella pneumoniae* B1 (*qnr*B1 +) and *Enterobacter cloacae* S1 (*qnr*B4+ and *qnr*S1+) were used as positive controls for the presence of the determinants *qnr*A, *qnr*B and *qnr*S. The strains *E. coli* DH10B transformant pHS11 and *E. coli* DH10B transformant p2007057 were used as positive controls for *qnr*C and *qnr*D, respectively. *Salmonella enterica* serovar *typhimurium* GSS-HN-2007-003 was used as positive control (Xia et al., 2009) for the presence of gene *aac(6')-Ib*. Strain *E. coli* TOP10 + pAT851 was used as positive control for gene *qep*A. PCR products were purified and the nucleotide sequences were determined and compared. A representative of each distinct nucleotide sequence was deposited in the GenBank (JF938596-JF938598).

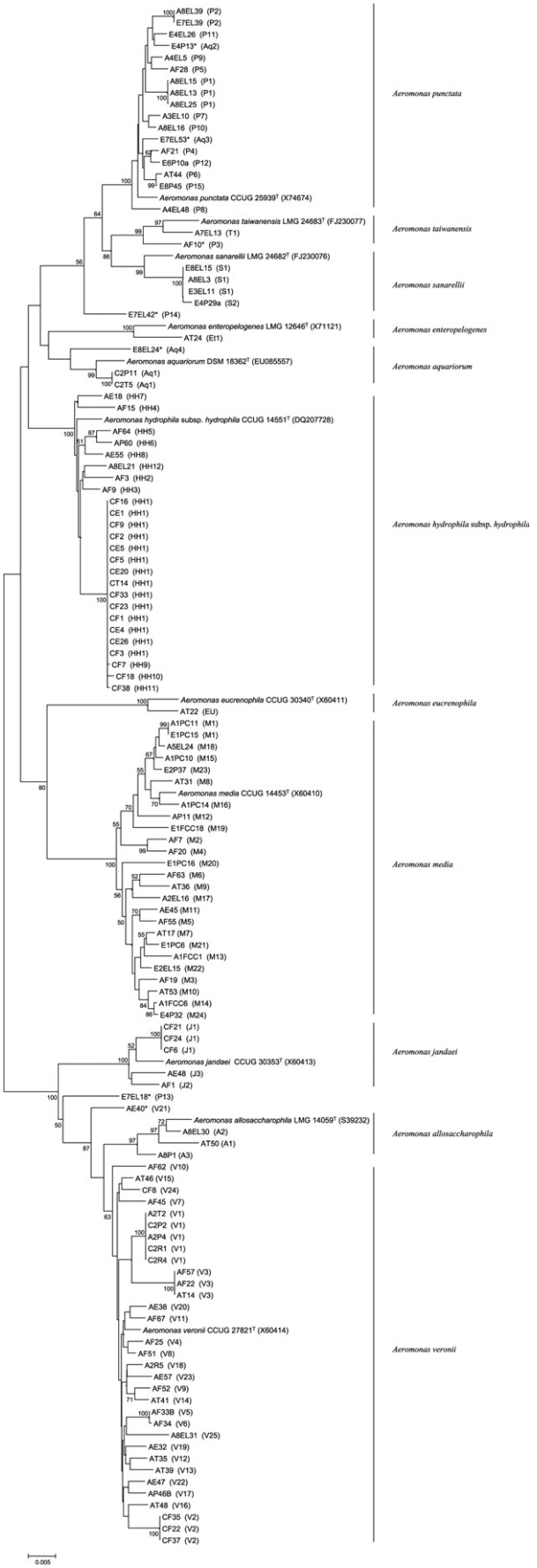
2.6. *Statistical analysis*

The chi-squared test was used to compare the prevalence values of antibiotic resistance phenotypes or genotypes and sequence types in different water sampled sites (SPSS 19.0 for Windows, SPSS Inc., Chicago, IL).

3. Results

3.1. *Species diversity and intra-species variation*

A collection of 741 Gram-negative cytochrome c oxidase-positive isolates recovered on the culture media and conditions described above was screened for the presence of



Aeromonas. On basis of 16S rRNA gene sequence analysis, among these isolates, 121 (80 from the WTP, 32 from WWTP and nine from WWTP isolated in the presence of ciprofloxacin) were identified as belonging to 11 species of the genus *Aeromonas*. The other strains were affiliated to genera such as *Pseudomonas*, *Ralstonia*, *Comamonas*, *Acidovorax*, *Brevundimonas*, *Cupriavidus*, *Chryseobacterium*, *Achromobacter* and to the family *Sphingomonadacae*.

The group of *Aeromonas* spp. isolates comprised 80 from the drinking water treatment plant (51 from raw surface water - SC and 29 recovered after water ozonation - PO) and 32 from the waste water treatment plant (17 from raw - RWW and 15 from treated waste water - TWW). Additionally, nine aeromonads were isolated in the presence of ciprofloxacin (five from RWW and four from TWW). Aeromonads were not detected in ground water samples, neither downstream the chlorination tank of the WTP, including in tap water (Table 2, Fig. 1).

Aeromonas spp. identification to the species level (Table 2) was supported by the 16S rRNA gene sequence analysis. Most of the times (114/121), these identifications were the same as those determined based on the analysis of the genes *gyr*B or *cpn*60. Only for seven isolates (five from TWW and two from SC) of the species *Aeromonas aquariorum*, *A. punctata* and *A. veronii*, the genes *gyr*B or *cpn*60 would lead to a different identification. Among the eleven species, eight were detected in raw surface water (Fig. 1, site A) and only four after the

Fig. 2 – Neighbour-joining dendrogram based on 16S rRNA, *cpn*60 and *gyr*B concatenated nucleotide sequences. Bootstrap values (≥50%) generated from 1000 replicates are indicated at branch points. Bold circles indicate branches recovered by the maximum-likelihood method. Sequence types (ST) and16S rRNA GenBank accession numbers are indicated in parenthesis. GenBank accession numbers for *cpn*60 and *gyr*B sequences are, respectively, for *A. allosaccharophila* EU306795 and AY101777, *A. aquariorum* FJ936120 and EU268444, *A. enteropelogenes* EU306837 and EF465526, *A. eucrenophila* EU306803 and AY101776, *A. hydrophila* subsp. *hydrophila* EU306804 and AY101778, *A. jandaei* EU306807 and AY101780, *A. media* EU306808 and AY101782, *A. punctata* EU306800 and AY101783, *A. sanarellii* (JF920655) and FJ807277, *A. taiwanensis* (JF920656) and FJ807272, *A. veronii* EU306839 and AY101795.* Strains for which 16S rRNA based identification differed from that given by the genes *gyr*B and *cpn*60. Strains designation: Isolates from the drinking water treatment plant were generically designated as SxMn, with S standing for site of isolation (A, raw surface water; C, after ozonation), x for the sampling date (2, second sampling date), M, for the culture medium of isolation (F, mFC agar; T, Tergitol-7 agar; P, Pseudomonas isolation agar; R, R2A agar; E, Bile Esculin agar), and n for the number of the isolate. Isolates from the raw or treated wastewater were generically designated as AxMn or ExMn, respectively (A relative to RWW and E to TWW); x for the sampling date; M for the culture medium of isolation (P, PCA; PC, PCA with ciprofloxacin; EL, m-endo-agar-LES; FC, mFC agar with ciprofloxacin); and n for isolate number of the isolate.

Table 2 – Diversity and percentage (number of isolates, number of sequence types) of *Aeromonas* species in the different types of water.

Species % (n)	Raw surface water (51)	After Ozonation (29)	Raw Waste water (22)	Treated Waste water (19)
A. allosaccharophila (3)	2.0% (1, 1)	–	9.1% (2, 2)	–
A. aquariorum (5)	–	6.9% (2, 1)	–	15.8% (3, 3)
A. enteropelogenes (1)	2.0% (1, 1)	–	–	–
A. eucrenophila (1)	2.0% (1, 1)	–	–	–
A. hydrophila subsp. *hydrophila* (25)	13.7% (7, 7)	58.6% (17, 4)	4.5% (1, 1)	–
A. jandaei (5)	3.9% (2, 2)	10.3% (3, 1)	–	–
A. media (25)	19.6% (10, 10)	–	36.4% (8, 8)	36.8% (7, 7)
A. punctata (18)	7.8% (4, 4)	–	36.4% (8, 6)	31.6% (6, 6)
A. sanarellii (4)	–	–	4.5% (1, 1)	15.8% (3, 2)
A. taiwanensis (1)	–	–	4.5% (1, 1)	–
A. veronii (33)	49.0% (25, 22)	24.1% (7, 3)	4.5% (1, 1)	–

No aeromonads were isolated from ground water or from any sampling point after water chlorination, including in 11 household taps.

ozonation process (Fig. 1, site C). Whereas in raw surface water the species *A. veronii* and *A. media* predominated, after ozonation, the species *A. hydrophila* subsp. *hydrophila* represented more than half of the isolates. The species *A. media* and *A. punctata* were not detected after the ozonation process. These same two species, *A. media* and *A. punctata*, prevailed in raw and in treated waste water (Table 2).

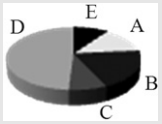
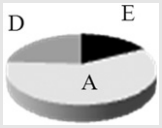
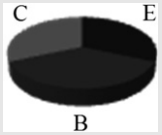
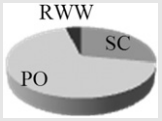
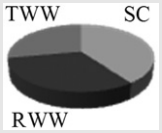
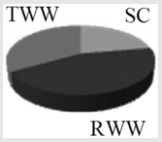
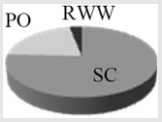
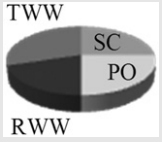
In order to infer about intra-species variability and to track bacteria in the different water samples, bacterial isolates were compared on basis of the nucleotide sequences of the genes 16S rRNA, *cpn*60 and *gyr*B. This procedure allowed the identification of 91 sequence types (Table 2). For sake of simplicity, the relationship of the isolates was represented in a dendrogram based on the comparative analyses of the 16S rRNA, *cpn*60 and *gyr*B concatenated sequences (Fig. 2). Not surprisingly, the same sequence type was not detected in non-directly-communicating water compartments, i.e, in surface and waste water. In contrast, the same sequence types were observed occasionally in communicating zones, separated by water treatment, i.e. ozonation or waste water treatment. In the drinking water treatment plant, the same sequence type of *A. veronii* (V1) was detected in raw surface water and after ozonation. In spite of this, water ozonation seemed to impose a serious bottleneck on strain diversity with the reduction of 48 sequence types in raw surface water to only nine in ozonated water. Moreover, the sequence types of *A. hydrophila* subsp. *hydrophila* detected in ozonated water (isolates C of sequence type HH1, Fig. 2) were, most of the times, distinct from those detected in raw surface water, suggesting that minor population representatives may have gained advantage under the oxidative stress imposed by ozone. In the waste water treatment plant, the same sequence types of the species *A. media* (M1), *A. punctata* (P2) and *A. sanarellii* (S1) were detected in raw and in treated waste water (Fig. 2) and P2 and S1 were also present in waste water samples collected in different dates.

3.2. *Antibiotic resistance phenotypes*

Amoxicillin resistance was observed in all isolates except in an *Aeromonas enteropelogenes* strain, suggesting that aeromonads are intrinsically resistant to this beta-lactam. For the other antibiotics, in general, the highest resistance prevalence values were observed for the penicillin ticarcillin and for the cephalosporin cephalothin, reaching percentages superior to 40%, irrespective of the type of water. Resistance phenotypes to the cephalosporin ceftazidime and the carbapenem meropenem were observed at low rates, exclusively in surface water (Table 3). Ceftazidime resistance was observed in a single isolate of *A. media* of raw surface water. Meropenem resistance was observed in three isolates of *A. veronii*, before and after water ozonation. Colistin resistance was another rare phenotype, detected only after the ozonation process in two isolates of *Aeromonas jandaei* with the same sequence type, presumably representing the same strain.

Nalidixic acid resistance was about five times more prevalent among waste water isolates (90.6%) than in surface water (17.6%, $p < 0.001$) and was not detected after ozonation (Table 3). In waste water, quinolone resistance was mainly related to the species *A. media* and *A. punctata,* which predominated in that type of water. Curiously, these two species were not observed after water ozonation, a fact that may explain the apparent efficiency of that disinfection process on quinolone resistance elimination. The potential of *A. media* as reservoir of quinolone resistance in waste water was confirmed by the fact that nine waste water strains (five from RWW and four from TWW) isolated in the presence of 4 mg L^{-1} of ciprofloxacin were all members of this species. These nine isolates, represented by eight distinct sequence types, were, not surprisingly, resistant to nalidixic acid and had at least an intermediary resistance phenotype to ciprofloxacin (four resistant and five intermediary). Six out of these nine strains were resistant to at least three different classes of antibiotics (R3) (gentamycin, tetracycline and sulfamethoxazole/trimethoprim), exhibiting resistance phenotypes rare among the aeromonads isolated in the absence of ciprofloxacin. Multi-resistance was also frequent among *A. punctata* (six out of 18 isolates) (Table 3). In contrast, none of the 33 *A. veronii* isolates presented resistance to three different classes of antibiotics, probably due to the fact that most of these isolates were recovered from raw surface or ozonated water. Multi-resistance (R3) did not differ significantly between raw surface and ozonated water. In contrast, R3 was significantly ($p < 0.05$) higher in waste water than in raw surface water.

Table 3 – Antibiotic resistance prevalence (%) in the different sampled sites and *Aeromonas* species.

	NA	CIP	TIC	CP	CEF	MER	STR	SXT	TET	GEN	CT	R3	
Type of water													Species distribution
SC ($n = 51$)	17.6	2.0	68.6	51.0	2.0	3.9	54.9	0	2.0	0	0	5.9	
PO ($n = 29$)	0	0	44.8	44.8	0	3.4	44.8	0	3.4	0	6.9	6.9	
RWW ($n = 22$)													
- without CIP ($n = 17$)	94.1	0	52.9	70.6	0	0	23.5	5.9	5.9	0	0	23.5	
- with CIP ($n = 5$)	100.0	40.0	100.0	80.0	0	0	100.0	20.0	20.0	40.0	0	100.0	
TWW ($n = 19$)													
- without CIP ($n = 15$)	86.7	0	40.0	66.7	0	0	26.7	20.0	13.3	0	0	20.0	
- with CIP ($n = 4$)	100.0	50.0	100.0	100.0	0	0	25.0	25.0	25.0	25.0	0	25.0	
Species													Water types distribution
A. *A. hydrophila* subsp. *hydrophila* ($n = 25$)	12.0	0	32.0	52.0	0	0	24.0	0	0	0	0	8.0	
B. *A. media* ($n = 25$)													
–without CIP ($n = 16$)	43.8	6.3	87.5	81.3	6.3	0	12.5	6.3	0	0	0	6.3	
–with CIP ($n = 9$)	100.0	44.4	100.0	88.9	0	0	66.7	22.2	22.2	33.3	0	66.7	
C. *A. punctata* ($n = 18$)	88.9	0	22.2	72.2	0	0	44.4	16.7	11.1	0	0	33.3	
D. *A. veronii* ($n = 33$)	3.0	0	81.8	21.2	0	9.1	78.8	0	0	0	0	0	
E. Other ($n = 20$)	55.0	0	50.0	75.0	0	0	35.0	0	15.0	0	10.0	15.0	

NA, nalidixic acid; CIP, ciprofloxacin; TIC, ticarcillin; CP, cephalothin; CEF, ceftazidime; STR, streptomycin; SXT, sulphamethoxazole/trimethoprim; TET, tetracycline; GEN, gentamicin; CT, colistin sulphate; MER, meropenem. R3 represents isolates resistant to 3 or more distinct antibiotic classes (except AML). SC, surface water captation; PO, post ozonation; RWW, raw waste water; TWW, treated waste water.

3.3. *Genetic determinants of quinolone resistance and* cphA *gene distribution*

Given the significantly higher prevalence of quinolone resistance in waste- than in surface water and in the species *Aeromonas puntacta* and *A. media* than in the others ($p < 0.001$) it was decided to investigate if similar mechanisms of resistance were present in both types of water and in the different species. Irrespective of the type of water or *Aeromonas* species, nalidixic acid resistance was associated with mutations in the gene *gyr*A ($n = 45$) and sometimes also on the gene *par*C ($n = 15$ and a silent mutation) (Table 4). The most common *gyr*A mutations were transversions in the position 83 (AGC → ATC, in 31 isolates or AGT → ATT, in six isolates) corresponding to the substitution of a serine for an isoleucine residue. In two isolates, one of *A. allosaccharophila* and one of *A. jandaei*, it was not possible to achieve a successful amplification of the gene *gyr*A, even using alternative primer sets and protocols (n.d. in Table 4). Among the plasmid-mediated quinolone resistance, only the genes *qnr*S and *aac(6')-ib-cr* were detected. Although being found exclusively in the species *A. media* (Table 4), these genes were observed in different strains (different sequence types). The gene *qnr*S was detected in strains of both surface and waste water, isolated either in the presence or in the absence of ciprofloxacin. The *qnr*S positive strains isolated in the presence of 4 mg L^{-1} ciprofloxacin harboured also the gene *aac(6')-ib-cr* which, in contrast to *qnr*S, was associated with a resistance or intermediary phenotype for ciprofloxacin. The gene *aac(6')-ib-cr* was detected exclusively in strains isolated on ciprofloxacin-supplemented medium, suggesting that these strains represent a minor fraction of the bacterial population, which can gain advantage in the presence of selective pressure. One isolate from surface water harboured the gene *aac(6')-ib*, but not the *cr* variant that confers resistance to ciprofloxacin. The *cr* variant of the gene *aac(6')-ib* presented mutations in the position 102, with an arginine residue (AGG or, in one RWW isolate, CGG) instead of tryptophan (TGG), on position 117, with a leucine residue (TTA) instead of serine (TCA) and on position 179, with a tyrosine residue (TAT) instead of an aspartate (GAT).

The most common metallo-beta-lactamase expressed by *Aeromonas* spp. is encoded by the chromosomal gene *cph*A, reported mainly in the species *A. hydrophila*, *A. veronii* and *A. jandaei* (Janda and Abbott, 2010). The presence and diversity of this gene was screened in an attempt to identify a differential pattern between isolates from the drinking and waste water treatment plants or between different species. The gene *cph*A was detected in the species *Aeromonas allosaccharophila* (one isolate from SC and one isolate from RWW), *A. aquariorum* (2 isolates from PO), *A. hydrophila* subsp. *hydrophila* (in all except in one isolate from SC and one from PO), *A. jandaei* (in all isolates) and *A. veronii* (in all except in one isolate from SC, seven from PO and one from RWW). Unexpectedly, it was also detected in a raw waste water isolate of the species *A. media*, recovered from ciprofloxacin-supplemented medium. The nucleotide sequences of the gene *cph*A were different among these isolates. However, those differences corresponded to silent mutations, as the amino acid sequences were identical among the waste- and drinking water treatment plant isolates (data not shown). Nevertheless, a noticeable contrast was found in terms of prevalence. The gene *cph*A was significantly ($p \leq 0.001$) more prevalent among surface water (65%) than in ozonated water (97%) isolates. It was also significantly ($p < 0.001$) less prevalent (18%) in the waste water treatment plant than in the drinking water treatment plant (76%). None of the treated waste water isolates harboured the gene *cph*A (Fig. 3). The plasmid related beta-lactamase gene bla_{TEM} was detected in a single raw waste water strain of *A. media* isolated on culture medium supplemented with 4 mg L^{-1} of ciprofloxacin, and which harboured also the gene *aac(6')-ib-cr*.

4. Discussion

This study was based on the hypothesis that *Aeromonas* spp. can serve as vehicle for antibiotic resistance dissemination within the urban water cycle. The experimental planning comprised the detection, diversity typing and determination of antimicrobial resistance patterns of aeromonads within different parts of the urban water cycle. In respect to detection, *Aeromonas* spp. were isolated from raw and treated waste water, as well as, from surface water, including after ozone disinfection. In contrast, culturable aeromonads were not detected in locations with pristine or disinfected water, ground and tap water, respectively. Apparently the drinking water treatment, mainly water chlorination, could remove aeromonads to, at least, less than one CFU in 100 mL of water. Although the failure to detect aeromonads in tap water could be attributed to low bacterial densities, in some taps, heterotrophs reached 10^1–10^4 CFU mL^{-1} (Fig. 1). The presence of *Aeromonas* spp. in drinking water is undesirable, as may have implications for user health, mainly via contact transmission (WHO, 2008). Nevertheless, aeromonads have been detected in different types of drinking water, namely tap, mineral bottled and wells (Kühn et al., 1997; Biscardi et al., 2002; Pablos et al., 2009). Some authors referred to the seasonality of aeromonads, which increase may coincide with the raise in the environmental temperature (Janda and Abbott, 2010). In this study, a priori, the failure to detect *Aeromonas* spp. in ground and tap water cannot be attributed to such seasonality, given the fact that these samples were collected in Summer and Winter (ground water) or in Spring, Summer and Autumn (taps). It is noteworthy that the absence of culturable *Aeromonas* spp. in tap water contrasts to what was observed in the same samples for other bacterial groups. For example, it was observed that sphingomonads, pseudomonads, and *Acinetobacter* spp., in spite the sharp decrease of total heterotrophs observed after water chlorination, were present in tap water (Vaz-Moreira et al., 2011; our data unpublished). The fact that the examined taps had a low usage rate (one to four times a month) may be part of the possible explanation for the absence of *Aeromonas* in tap water, as stagnancy of water in pipes is described as promoting bacterial community rearrangements (Lautenschlager et al. 2010). However, a deeper study would be needed to confirm such hypothesis. Thus, the apparent reduced risk of *Aeromonas* spp. to contribute for

Table 4 – Diversity, antibiotic resistance phenotypes, origin and genetic determinants of quinolone resistance of nalidixic acid resistant isolates.

Species (n)	ST (n)	Origin (n)	Resistance phenotype					Mutations		Quinolone resistance genes
			CIP	TIC	CP	STR	Other	*gyrA*	*parC*	
A. allosaccharophila (2)	A2 (1)	RWW (2)						GAA (Glu)[87]	-	-
	A3 (1)							n.d.	AGA (Arg)[80]	-
A. aquariorum (3)	Aq2 (1)	TWW (3)						ATC (Ile)[83]	-	-
	Aq3 (1)							ATC (Ile)[83]	ATC (Ile)[80]	-
	Aq4 (1)						TET	ATC (Ile)[83]	-	-
A. hydrophila subsp. *hydrophila* (3)	HH7 (1)	SC (2)						ATT (Ile)[83]	ATT (Ile)[80]	-
	HH8 (1)							ATT (Ile)[83]	-	-
	HH12 (1)	RWW (1)						ATC (Ile)[83]	ATC (Ile)[80]	-
A. jandaei (1)	J2 (1)	SC (1)						n.d.	-	-
A. media (16)	M1 (2)*	RWW (1)					GEN TET	CGC (Arg)[83]	AGT (Ser)[80]	*aac(6')-ib-cr* bla_{TEM}
		TWW (1)						ATC (Ile)[83]	-	*aac(6')-ib-cr*
	M6 (1)	SC (3)					CAZ	ATC (Ile)[83]	-	*aac(6')-ib qnrS*
	M7 (1)							AGA (Arg)[83]	-	-
	M10 (1)							ATC (Ile)[83]	-	-
	M12 (1)	RWW (6)						ATC (Ile)[83]	-	-
	M13 (1)*						GEN	ATC (Ile)[83]	ATC (Ile)[80]	*aac(6')-ib-cr*
	M14 (1)*						SXT TET	ATC (Ile)[83]	-	*aac(6')-ib-cr qnrS*
	M15 (1)*							GTC (Val)[83]	-	*aac(6')-ib-cr qnrS*
	M16 (1)*							ATC (Ile)[83]	-	*aac(6')-ib-cr*
	M18 (1)							ATC (Ile)[83]	-	-
	M19 (1)*	TWW (5)						ATC (Ile)[83]	-	*aac(6')-ib-cr*
	M20 (1)*							ATC (Ile)[83]	ATC (Ile)[80]	*aac(6')-ib-cr*
	M21 (1)*						SXT TET GEN	ATC (Ile)[83]	ATC (Ile)[80]	*aac(6')-ib-cr*
	M23 (1)						SXT TET	ATC (Ile)[83]	CGC (Arg)[80]	-
	M24 (1)							AGA (Arg)[83]	-	*qnrS*
A. punctata (16)	P1 (3)	RWW (3)						ATC (Ile)[83]	-	-
							TET	ATC (Ile)[83]	-	-
							SXT TET	ATC (Ile)[83]	-	-
	P2 (2)	RWW (1)						ATC (Ile)[83]	-	-
		TWW (1)						ATC (Ile)[83]	-	-
	P3 (1)	SC (3)						ATC (Ile)[83]	ATC (Ile)[80]	-
	P4 (1)							AGA (Arg)[83]	AAA (Lys)[84]	-
	P6 (1)							ATC (Ile)[83]	AAA (Lys)[84]	-
	P7 (1)	RWW (5)						AGG (Arg)[83]	-	-
	P8 (1)						SXT	ATC (Ile)[83]	-	-
	P9 (1)							AGA (Arg)[83]	-	-
	P10 (1)							ATC (Ile)[83]	-	-
	P12 (1)	TWW (4)						ATC (Ile)[83]	-	-
	P13 (1)						SXT	ATC (Ile)[83]	ATC (Ile)[80]	-
	P14 (1)						SXT	ATC (Ile)[83]	ATC (Ile)[80]	-
	P15 (1)							ATC (Ile)[83]	AAA (Lys)[84]	-
A. sanarellii (4)	S1 (3)	RWW (1)						ATT (Ile)[83]	-	-
		TWW (2)						ATT (Ile)[83]	-	-
								ATT (Ile)[83]	-	-
	S2 (1)	TWW (1)					TET	ATT (Ile)[83]	-	-
A. taiwanensis (1)	T1 (1)	RWW (1)						ATC (Ile)[83]	ATC (Ile)[80]	-
A. veronii (1)	V25 (1)	RWW (1)						ATC (Ile)[83]	-	-

n.d., not determined; (shadowing; black, resistant; grey, intermediary; white, susceptible) .
* isolated in medium supplemented with 4 mg L^{-1} of ciprofloxacin.

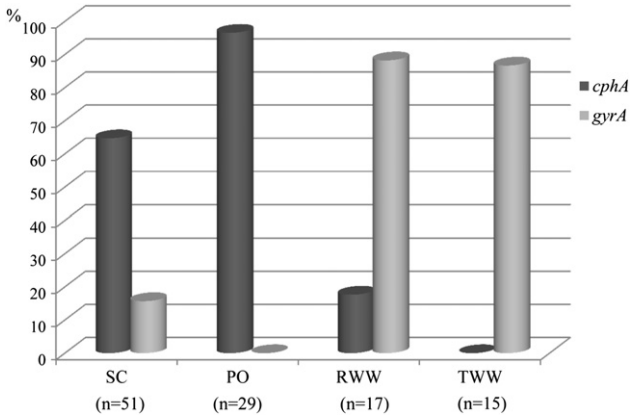


Fig. 3 – Percentage of isolates of each type of water that harbored the *cph*A gene or the *gyr*A mutation.

antimicrobial resistance dissemination via tap water, observed in this study, must be interpreted with precaution.

In respect to diversity, it was observed that the pattern of *Aeromonas* species was distinct in the different types of water. Although *A. media* was abundant in both raw surface and waste water, these types of water differed on the predominance of *A. veronii* and *A. punctata* (Table 2). Nevertheless, the percentage of distinct sequence types was not significantly different in surface and waste water. Apparently, and in spite the low number of isolates, waste water treatment did not lead to a significant reduction of the number of sequence types. In contrast, water ozonation seemed to impose a bottleneck both in the number of species and of sequence types (Fig. 2). Indeed, the species *Aeromonas hydrophyla* subsp. *hydrophyla* became over represented after ozonation with a significant ($p < 0.001$) reduction in the percentage of distinct sequence types. Moreover, in general, the sequence types detected after ozonation were different from those found in raw surface water, suggesting some kind of rearrangement in the aeromonads population due to water disinfection (Fig. 2).

A. media and *A. punctata* were the species in which quinolone resistance presented the highest prevalence ($p < 0.001$) and the predominance of these species in waste water contributed to explain the elevated rates of nalidixic acid resistance in waste water, significantly ($p < 0.001$) higher than in surface water. Similarly, sulfamethoxazole/trimethoprim resistance found exclusively in those two species, was observed only in waste water. In contrast, ceftazidime and meropenem resistance were detected only in surface water, although it is acknowledged that these resistance phenotypes could have been detected also in waste water if a larger number of isolates had been examined.

The search for genetic determinants related to quinolone resistance showed that the *gyr*A mutations were the primary, even not the unique, mechanism (Table 4). The higher prevalence of these mutations in waste water isolates in comparison with the prevalence values observed in surface water (Fig. 3), can be supported by previous studies which demonstrate that quinolone resistance may arise from the contact with mutagenic substances, widely found in the environment (Miyahara et al., 2011). In fact, in waste water the occurrence of such potential mutagens is much more probable than in uncontaminated waters. Additionally, some effect of selective pressure may take place in waste waters, in which the detection of quinolones is common, with concentrations of ciprofloxacin up to 0.7 μg/L detected in Portuguese municipal waste water treatment plants (Seifrtová et al., 2008; our data for the same plant, unpublished). It is not possible to know the relevance of *de novo* mutation or of selection (vertical transmission) for the observed chromosomal mutations associated with quinolone resistance. But, although probably both forms can contribute for resistance spreading, *de novo* events may be frequent as *gyr*A or *par*C gene mutations were observed in different strains (sequence types). In any case, the species *A. punctata* and *A. media* seem to play an important role on this form of dissemination. These results are in agreement with the work of Goñi-Urriza et al. (2000), who assessed the impact of an urban effluent on antibiotic resistance of *Aeromonas* spp. in a riverine area. As in the current study, nalidixic acid resistance was observed in the majority of the aeromonads (72%), most of them of the species *A. punctata* (*A. caviae*), and was exclusively chromosomally encoded. The conclusion reached by Goñi-Urriza et al. (2000), applies also to the present study – urban effluents are responsible for the increase of quinolone resistance in the receptor water courses.

Among the quinolone resistance determinants associated with mobile genetic elements, only the genes *qnr*S and *aac(6')-ib-cr* were detected and only in the species *A. media*. In both cases, these genetic determinants were found in different strains (sequence types), as expected if horizontal gene transfer is equated. Both determinants, and mainly *aac(6')-ib-cr* which was detected only in isolates recovered in the presence of ciprofloxacin, were rare in the analysed samples. Nevertheless, the gene *qnr*S is apparently widespread in waters (waste water, rivers, aquaculture), detected not only from total DNA and but also from cultures of aeromonads and *Enterobacteriaceae* (Cattoir et al., 2008; Picão et al., 2008; Szczepanowski et al., 2009; Ishida et al., 2010; Cummings et al., 2011). Similarly, the gene *aac(6')-ib-cr* is found in different types of water (lake water, river sediments, aquaculture), either in total DNA or in bacterial isolates (aeromonads and *Enterobacteriaceae*) (Picão et al. 2008; Ishida et al., 2010; Cummings et al., 2011). The presence of these genes, although conferring low-levels of resistance, can favour and complement the selection of other resistance mechanisms (Rodríguez-Martínez et al., 2010). The fact that the determinants *qnr*S and *aac(6')-ib-cr* were detected only in *A. media* suggests that this species may represent an important vector of quinolone resistance. Ceftazidime resistance was also detected only in this same species in surface water. Recent evidences that water *A. media* can colonize humans (Khajanchi et al., 2010) may give additional relevance to this species on the dispersal of resistance. Although the number of isolates examined was too low to strongly support this conclusion, the data suggested that water ozonation may promote the reduction of *A. media*. For instance, no quinolone or ceftazidime resistance were observed downstream of this point. In contrast, meropenem resistance, in this study associated to the species *A. veronii*, was observed also in ozonated water. Nevertheless, the data gathered in this study suggests that water chlorination may contribute to control resistance propagation by aeromonads via drinking water.

In general, the results obtained suggest that different aeromonads populations and antibiotic resistance determinants prevail in different parts of the urban water cycle. The clearest example of this was the distribution of the gene *cph*A and of the *gyr*A mutation, observed in the majority of the surface and waste water isolates, respectively (Fig. 3). As expected, the gene *cph*A was predominant among the species *A. hydrophila* subsp. *hydrophila*, *A. veronii* and *A. jandaei*, also the most prevalent in the drinking water treatment plant. In the same way, the *gyr*A mutations prevailed in *A. media* and *A. punctata*, the predominant species in waste water. The contrast observed in the distribution of both genetic determinants is mainly due to the patterns of species occurring in both types of water and which, probably, are due to the environmental conditions and selective pressures imposed in both types of habitat. This also demonstrates that, in each type of water, aeromonads may represent a source of distinct types of antibiotic resistance.

The importance of a given *Aeromonas* species for the antimicrobial resistance patterns in each type of water, observed in the current work, confirm previous studies conducted with other bacterial groups. Figueira et al. (2011) studied different populations of waste water *E. coli* and concluded that variations on the prevalence of quinolone resistance were correlated with the dynamics of some population sub-sets. Vaz-Moreira et al. (2011) characterizing the patterns of antimicrobial resistance in sphingomonads from tap water and cup fillers of dental chairs also concluded that antibiotic resistance patterns were often species- rather than site-related. Nevertheless, in the current work, and in contrast to what was suggested by other authors studying *A. salmonicida* from fish farms and environmental samples (Giraud et al., 2004; Kim et al., 2011), no clonal spreading of antibiotic resistance was observed. In contrast, rarely were observed the same sequence types in different water samples. This suggests that the acquisition of a specific resistance type, either by horizontal gene transfer or by adaptive mutation, may take place preferentially in a given habitat, in which a species is prevalent or has a higher fitness than the others. In other words, the success of resistance acquisition may depend on the fitness of the target bacterium (receptor of horizontal gene transfer or mutant) in a specific environment.

5. Conclusions

The patterns of *Aeromonas* species and antimicrobial resistance varied over different parts of the urban water cycle;

In each type of water, the antimicrobial resistance patterns were primarily function of the prevailing species;

In raw surface and waste water no strong evidences for clonal dissemination of antimicrobial resistance were detected;

Water ozonation imposed a bottleneck on species diversity, with evidences of clonal selection, and promoted a significant reduction of quinolone resistance and the increase of *cph*A metallo-beta-lactamase;

Waste water aeromonads, particularly *A. media* and *A. punctata*, were confirmed as relevant environmental harbours of quinolone resistance, either chromosomally (*gyr*A mutation) or plasmid encoded (*qnr*S and *aac*(6′)-*Ib-cr*).

Water aeromonads were confirmed as relevant agents for antimicrobial resistance spreading in the environment, which presence in tap water could be significantly reduced by water chlorination.

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