Ciprofloxacin Resistance in Domestic Wastewater Treatment Plants

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Abstract The potential of domestic wastewater treatment plants to contribute for the dissemination of ciprofloxacin-resistant bacteria was assessed. Differences on bacterial counts and percentage of resistance in the raw wastewater could not be explained on basis of the size of the plant or demographic characteristics of population served. In contrast, the treated effluent of the larger plants had significantly more heterotrophs and enterobacteria, including ciprofloxacin-resistant organisms, than the smaller ($p<0.01$). Moreover, longer hydraulic retention times were associated with significantly higher percentages of resistant enterobacteria in the treated effluent ($p<0.05$). Independently of the size or type of treatment used, domestic wastewater treatment plants discharged per day at least 10^{10}–10^{14} colony forming units of ciprofloxacin-resistant bacteria into the receiving environment.

Introduction

Wastewater has been considered an important environmental reservoir of antibiotic-tolerant bacteria (D'Costa et al. 2007; Baquero et al. 2008; Kümmerer 2009a). These antibiotic-tolerant populations comprehend both intrinsically resistant organisms and bacteria that acquired genetic determinants able to confer resistance (Alonso et al. 2001; D'Costa et al. 2006). It has been suggested that nutrient rich environments as sewage and wastewater offer optimal conditions to promote horizontal gene transfer processes, frequently involving the passage of plasmids and transposons encoding antibiotic resistance (Tran and Jacoby 2002; Summers 2006; Kelly et al. 2009). For this reason, wastewater treatment facilities, where high doses of antibiotic susceptible and tolerant bacteria are mixed together, are considered hot spots for antibiotic resistance spreading (Alonso et al. 2001; Summers 2006; Kim and Aga 2007; Baquero et al. 2008; Kümmerer 2009b). This reasoning finds support on previous studies, which demonstrated that domestic wastewater treatment plants (WWTP) may supply permanently to the environment antibiotic-resistant bacteria and antibiotic resistance determinants (Guardabassi et al. 2002; Reinthaler et al. 2003; Tennstedt et al. 2003; Schwartz et al. 2004; Costa et al. 2006; Ferreira da Silva et al. 2006, 2007; Watkinson 2007a; Faria et al. 2009; Zhang et al. 2009).

On basis of a recent study, fluoroquinolones, in which ciprofloxacin is included, hold the fourth position in the European market of antibiotics, with tendency to increase in ten of 21 countries included in the study (Sande-Bruinsma et al. 2008). The correlation between fluoroquinolone consumption in ambulatory care and resistance increase is also demonstrated in that study (Sande-Bruinsma et al. 2008). Un-metabolized ambulatory care antibiotics end up in domestic WWTP, justifying the need to study fluoroquinolone resistance in these systems. In previous studies we demonstrated that in an activated sludge wastewater treatment plant, treated wastewater presented significantly higher percentages of *Escherichia coli* and *Enterococcus* spp. resistant to the fluoroquinolone ciprofloxacin than the raw influent (Ferreira da Silva et al. 2006, 2007). Besides, fluoroquinolones have been detected in domestic wastewaters in concentrations up to 1,000–600 ng/L, hinting the possible occurrence of selective pressures and the consequent selection of resistant bacteria (Batt et al. 2007; Seifrtová et al. 2008; Gros et al. 2009; Kümmerer 2009b). The present study was designed to compare ciprofloxacin resistance prevalence in heterotrophic and enterobacteria in five WWTP situated in urban and in semi-urban areas, with different dimensions and using different secondary treatment processes. Specifically it was intended to assess if: (1) the raw wastewater in urban or semi-urban areas contain similar loads of ciprofloxacin-resistant bacteria; (2) higher hydraulic retention times, more common in smaller plants, may promote the increase of the percentage of antibiotic-resistant bacteria in the treated effluent; and (3) higher bacterial removal rates, even if associated with longer hydraulic retention times, may contribute to attenuate the dissemination of antibiotic-resistant bacteria per WWTP.

Material and methods

Wastewater Treatment Plants and Sampling

Five wastewater treatment facilities treating domestic residues were analyzed in this study (Table 1). WWTPs 1 (=AS) and 3 (=SAF) are situated in Northern Portugal (41°13′58.31″ N, 8°37′17.63″ W and 41°31′58.34″ N, 8°46′51.65″ W, respectively). WWTP 2 (=TF), 4 (=AL), and 5 (=AnL) are located 100 km south, in the central region of the country (40°12′26.18″ N, 8°25′21.93″ W; 40°12′42.52″ N, 8°39′00.34″ W; and 40°09′08.58″ N, 8°42′42.62″ W, respectively).

These facilities differ on dimensions, type of sewage received, secondary treatment process, and demographic characteristics of the region (Table 1). AS and TF, the larger facilities studied, are located in two towns where services sector is the main activity and industry represents, respectively 15.3% and 11.2% of the total urban area. SAF is located in a touristic village, near the Atlantic cost, with 9.0% of the urban area dedicated to industry and 6.5% to tourism. AL and AnL dist about 10 km and are located in two different parochial regions of the same municipality, which is mainly devoted to services and agriculture, with industry representing only 3.4% of the total urban area.

In all the studied WWTP the influent sewage undergoes a preliminary treatment to remove voluminous solids, but only in the larger plants (AS and TF) this treatment is followed by the removal of settleable solids in a primary settling tank. In AS the settled sewage is biologically treated through an activated sludge process. In TF and SAF the biological treatment occurs in fixed film reactors: trickling filter (TF) and submerged aerated filter, which constitutes approximately 50% of the volume of the biological tank (SAF). In these three WWTP, the treated wastewater from the secondary settling tank is discharged without any further treatment into a natural water course. In AL crude wastewater treatment is conducted in an aeration lagoon, with oxygen provided mechanically, followed by a treatment in a secondary facultative pond, where oxygenation is provided by the photosynthetic activity of algae, and where sedimentation occurs. AnL only differs from AL because wastewater is initially treated in an anaerobic lagoon.

Between February and July 2008, four independent sampling campaigns were carried out in AS, SAF, and AL and three in TF and AnL. Twenty-four hours composite samples were collected from the wastewater entering the biological treatment, herein referred as raw wastewater, and from treated wastewater, which is ready to be discharged into the natural receiving water

Table 1 Characteristics of wastewater treatment plants examined in this study

WWTP		AS	TF	SAF	AL	AnL
Population[a]		138,226	137,212	35,358	24,820	24,820
Population density (inhab/km^2)[a]		1,662	430	370	108	108
Population served		100,000	150,000	8,700	4,200	650
Type of sewage		Domestic (70%) and pre-treated industrial (30%)	Domestic (~85%) and pre-treated hospital (~15%)	Domestic	Domestic	Domestic
Biological treatment		Activated sludge	Trickling filter	Submerged aerated filter	Aeration lagoon	Anaerobic lagoon
Hydraulic retention time (h)		12	9	24	230	360
Range of COD in WW (mgO_2/L)[b]	Raw	291–745	497–625	ND	311–1,242	242–891
	Treated	71–126	126–138	92–187	39–50	60–182
Range of BOD_5 in WW (mgO_2/L)[b]	Raw	167–400	247–312	ND	110–720	85–440
	Treated	16–35	38–40	4–6	6–14	10–45
Treated outflow (m^3/d)		18,000	25,000	650	890	200
Site of WWTP discharge		Water stream	River, through an 2–3 km drain	Water stream, 500 m from the sea	Water stream	Water stream

ND not determined

[a] Source: INE (www.ine.pt)

[b] Data from the WWTP

streams. Samples were refrigerated, transported to the lab, and analyzed within 12 h.

Enumeration of Total Cultivable and Antibiotic-Tolerant Bacteria

Bacteriological analyses were performed using the membrane filtration method as described before (Ferreira da Silva et al. 2006). Briefly, 1 mL serial dilutions of water samples were filtered and the membranes (cellulose nitrate, 0.45 mm pore size, 47 mm diameter, Albet, Barcelona, Spain) were placed onto plate count agar (PCA, Pronadisa) for enumeration of total heterotrophic bacteria and m-FC Agar (m-FC, Difco) for enumeration of enterobacteria, and on the same media supplemented with 4 mg/L ciprofloxacin. Previous studies established a correlation between this concentration of ciprofloxacin and resistance phenotype, and thus in the present study we designate as resistant the organisms that were able to grow in the presence of this concentration of antibiotic, disregarding the clinical concept of resistance (Watkinson et al. 2007b). After an incubation period of 24 h at 30°C, the number of colony forming units (CFU) was registered on basis of filtering membranes with 10–80 colonies. Values of CFU/mL were registered for each culture medium and the percentage of resistance and bacterial removal rates were calculated for each WWTP.

Data analysis

Resistance percentage was estimated for each sampling campaign and wastewater type (raw or treated) as the ratio between the CFU/mL observed on each medium supplemented with 4 mg/L of ciprofloxacin and on the same medium without antibiotic. The bacterial removal rate was estimated for each sampling campaign as the ratio between the CFU/mL observed on PCA or on m-FC from treated and raw wastewater samples and was expressed as one minus that value. Resistant CFU per day and per inhabitant were determined as the ratio between the CFUs produced per day, considering the volume of wastewater treated daily in each WWTP, and the population served by each facility. Data on CFU/mL on each medium, resistance percentage in raw and treated wastewater, removal rate of total and antibiotic-tolerant bacteria, resistant CFU per day per inhabitant, and the effect of climate conditions or of the sampling month were compared in each or the five plants on basis of analysis of variance and post hoc test of tukey (SPSS 16.0 for windows).

Results

In general, the bacterial densities (CFU/mL) in raw and in treated wastewater varied significantly ($p < 0.05$) over different sampling campaigns of the same WWTP. In raw wastewater, the average of total heterotrophic bacteria and enterobacteria ranged, respectively, 6.3×10^6–4.0×10^5 and 1.8×10^6–1.2×10^5 CFU/mL (Fig. 1a). The values of CFU/mL of heterotrophs were not significantly different in the five plants examined. In contrast, enterobacteria counts in raw wastewater were divided into three homogeneous subsets, with the plants TF, AL, and AnL presenting significantly ($p<0.01$) lower CFU/mL of enterobacteria than the others. In respect to ciprofloxacin-resistant bacteria in the raw wastewater, AnL presented significantly lower ($p<0.05$) CFU/mL of heterotrophs than plants TF and SAF, whereas no significant differences were found for CFU/mL of ciprofloxacin-resistant enterobacteria in the five plants (Fig. 1a).

Ciprofloxacin resistance percentages of heterotrophs and enterobacteria in raw wastewater ranged, respectively, 1.7–4.4% and 0.7–3.5% (Table 2). As observed for the CFU/mL values, also the percentages of resistance varied in different sampling campaigns, as can be inferred from the range of values and standard deviations presented in Table 2. In spite of this, it was possible to detect significant differences among the five plants examined. TF, the largest plant studied, presented significantly higher ($p<0.05$) percentages of resistant heterotrophs in the raw wastewater than AS, AL, and AnL and a lower ($p<0.01$) resistance percentage of enterobacteria than AL and AnL (Table 2). Although significant differences were observed, the comparative analysis of the raw wastewater of the five plants studied did not allow a clear differentiation between the size and demographic characteristics and the densities (CFU/mL) or percentage of total or resistant heterotrophs or enterobacteria.

The biological treatment permitted in all the WWTP studied the reduction of the bacterial loads in the treated effluent (Fig. 1a). The highest average bacterial removal rates ranged 98.2±0.5%–99.9±0.2% and were observed in the three smaller plants (SAF, AL, and AnL). In AS the rates were slightly but not significantly lower (92.0±10.1%–97.0±1.0%). TF presented significantly ($p<0.001$) lower bacterial removal rates than the other plants, with average values ranging 39.5±19.3%–66.2±30.9%.

The bacterial density in the treated effluent was influenced by the removal rates. TF, the largest plant studied and with the lowest removal rate, had significantly higher counts (CFU/mL) of total heterotrophs and enterobacteria in the treated effluent than the other plants ($p<0.0001$). In the same way, AS, the second largest plant examined, presented significantly lower ($p<0.0001$) counts than TF, but higher than the other three smaller plants ($p<0.001$) (Fig. 1a). The discharge of resistant bacteria followed a similar pattern, with TF discharging significantly more CFU/mL of resistant heterotrophs ($p<0.0001$) and of enterobacteria ($p<0.0001$) than all the other plants. AS, when compared with the smaller plants, discharged significantly more CFU/mL of resistant enterobacteria ($p<0.0001$) but not heterotrophs (Fig. 1a).

The percentages of ciprofloxacin-resistant heterotrophs were not significantly different in the five plants studied. In contrast, ciprofloxacin-resistant enterobacteria were significantly more prevalent in the treated effluent of the lagoons (AL and AnL) than in the other plants (Table 2). In other words, the lagoons released lower numbers (CFU/mL) of resistant enterobacteria, although these were significantly ($p<0.01$) more prevalent in the treated effluent. The wide range of values of enterobacterial resistance percentage in the raw wastewater of the smaller plants suggests that antibiotic-resistant bacteria may be discharged sporadically into these WWTP. Nevertheless, the percentages of resistant enterobacteria observed in the treated wastewater of the lagoons, which have a hydraulic retention time of 9.5 and 15 days, showed higher values and much narrower ranges (Table 2). In fact, whereas for different sampling dates, evident variations were observed in the raw wastewater, such an effect was not observed in the treated effluent, suggesting that independently of the resistance percentage in the raw wastewater, the prevalence in the treated effluent is more homogeneous. In AS the average percentage of resistance in heterotrophic bacteria increased significantly ($p=0.01$) from 2.2±1.0% in the inflow to 3.4±1.1% in the treated effluent. This result was supported on the observation of a significantly ($p=0.03$) lower removal of resistant (92.6±3.9%) than of total heterotrophs (95.6±2.1%).

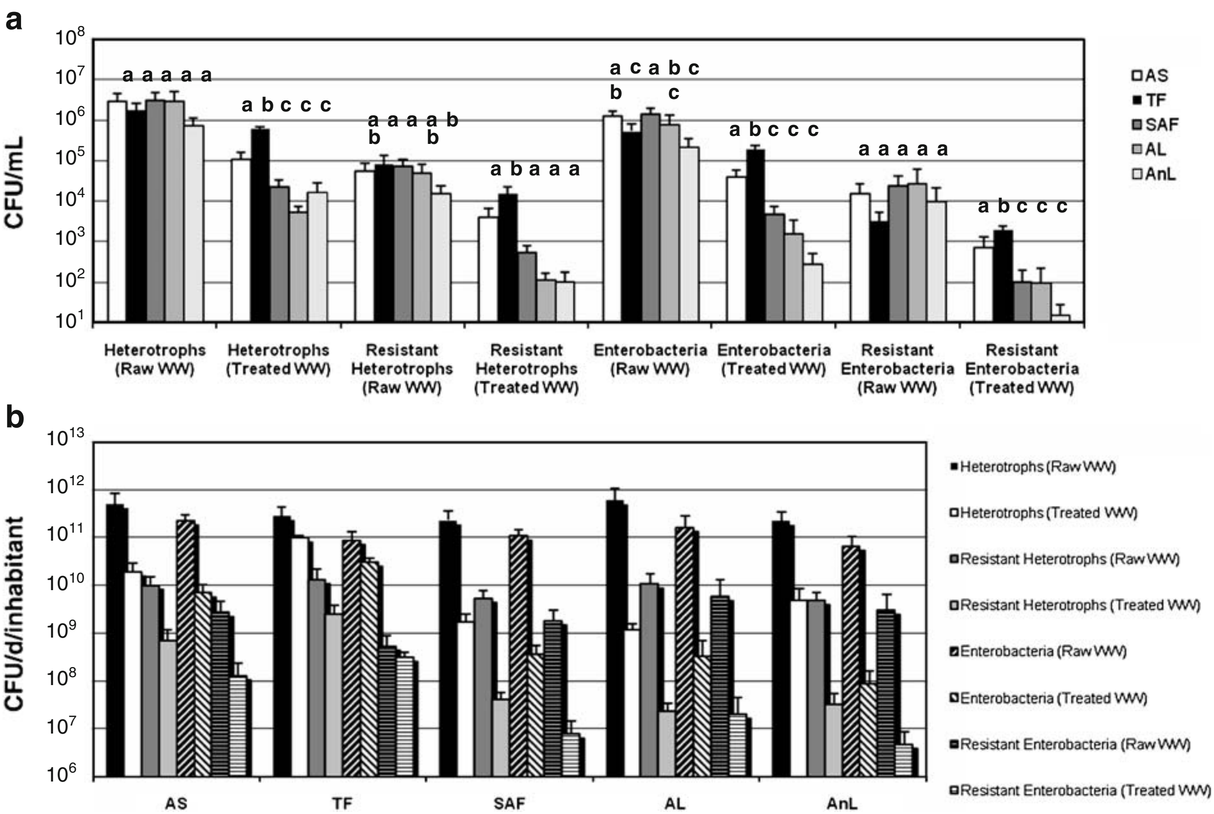

Fig. 1 Numbers of total and resistant heterotrophs and enterobacteria entering in the biological treatment (raw wastewater) and leaving (treated wastewater) each wastewater treatment plant. **a** CFU per milliliter. **b** CFU per day and per inhabitant. *a*, *b*, and *c*, homogeneous subsets on basis of Tukey test

When the counts of enterobacteria and heterotrophs were normalized as a function of volume of wastewater treated per day and population served, the distribution was very similar to that observed in terms of CFU/mL. One of the differences observed was that TF received significantly ($p<0.05$) more resistant heterotrophs per day per inhabitant than SAF, something that was not observed when analyzing the bacterial densities (CFU/mL) (Fig. 1b, homogeneous subsets not shown) and that may be due to the fact

Table 2 Range and average percentage values of bacteria able to grow in the presence of 4 mg/L of ciprofloxacin in the raw inflow and in the treated effluent

	% resistant heterotrophs				% resistant enterobacteria			
	Raw wastewater		Treated wastewater		Raw wastewater		Treated wastewater	
	Range	Average ± SD	Range	Average ± SD	Range	Average ± SD	Range	Average ± SD
AS	1.1–3.6	2.2±1.0 a	2.5–4.6	3.4±1.1 a	0.4–2.5	1.3±0.9 a, b	1.1–2.2	1.7±0.8 a
TF	3.0–7.3	4.4±2.5 b	1.1–3.9	2.6±1.4 a	0.3–1.0	0.7±0.5 a	0.5–1.5	0.9±0.6 a
SAF	1.2–3.6	2.6±1.1 a, b	2.1–4.0	2.6±0.9 a	0.4–4.2	2.0±1.5 a, b	0.7–5.2	2.8±2.2 a
AL	1.1–2.3	1.7±0.5 a	1.2–2.9	1.9±0.7 a	0.7–6.3	3.5±2.7 b	3.9–6.6	5.4±1.3 b
AnL	1.1–3.0	1.9±0.9 a	0.1–2.8	1.9±1.6 a	1.0–6.6	3.5±2.7 b	4.3–6.3	5.1±1.0 b

SD standard variation

Letters represent homogeneous subsets on basis of Tukey test that this plant receives hospital effluents. Another discrepancy was that AL showed a higher number ($p < 0.05$) of resistant enterobacteria per day per inhabitant than TF, when those numbers expressed as CFU/ml were not significantly different. This may hint the input of resistant enterobacteria in AL from other sources than those expected in a domestic effluent.

In the treated outflow, the pattern of bacterial counts per day per inhabitant was the same as observed for CFU/mL, evidencing that higher bacterial loads are released by larger WWTP, independently if the calculations are performed in terms of CFU/mL or of CFU/day/inhabitant. This rule applies also to antibiotic-resistant bacteria. For example, TF releases 100 times more resistant heterotrophic bacteria than the lagoons and 50–100 times more enterobacteria than the three smaller plants studied. AS discharges daily five to 10 more resistant bacteria per inhabitant than SAF, AL, or AnL.

Discussion

The variations of bacterial densities observed in raw and in treated wastewater over different sampling campaigns limited a straightforward interpretation of the fate of ciprofloxacin-resistant bacteria during wastewater treatment. Such variations could not be explained on basis neither of sampling month nor of rainy or dry weather. When any of these parameters was used as analysis of variance factor, no significant differences were observed neither for CFU/mL in raw or treated wastewater, nor for resistance percentages.

This study showed that larger WWTP discharge higher densities (in CFU/mL or CFU per inhabitant) of bacteria than the smaller facilities. In principle, this might be due to the nature of the raw wastewater, to shorter hydraulic retention times, or to the use of different treatment processes. According to our data, the nature of the raw wastewater seems to have little influence on the load of bacteria released, as plants in which the raw influent belonged to the same Tukey homogeneous subset the treated effluents were divided into different groups of significance—for instance, heterotrophs in every plant studied or enterobacteria in plants TF, AL, and AnL. Even the fact that plants AS and TF receive, respectively, pre-treated industrial or hospital effluents did not make the raw wastewater of these plants different from those receiving only domestic effluents. However, when calculations are made on basis of daily input per inhabitant, external sources of resistant bacteria may be envisaged and in this respect, such type of analysis may be more elucidative.

Given the data obtained, hydraulic retention time, which is highly dependent on the type of treatment used, may have influence on the microbiological quality of the treated effluent. In fact, it was observed that plants with shorter hydraulic retention time presented higher loads of heterotrophs and enterobacteria in the treated effluent. It is also worthy of note the difference between AS and TF, as in the first the biological treatment (activated sludge) takes about 8 h, whereas in the second the passage through the trickling filter may take less than half a hour. This fact may explain the low removal rates observed for TF and also its differentiation from AS.

Ciprofloxacin-resistant heterotrophs represented around 2–4% of the total heterotrophic bacteria in the raw wastewater. The fact that TF receives pre-treated hospital effluents may explain the higher range of percentage of ciprofloxacin resistance heterotrophs observed in its raw wastewater. Nevertheless, the percentages of resistant heterotrophs were not significantly different in the treated effluent of the five plants examined. Except for AS, wastewater treatment did not lead to an increase on heterotrophic ciprofloxacin resistance percentage. The significantly lower ($p=0.03$) removal rate for resistant heterotrophs than for total heterotrophs observed in AS corroborate this finding and may hint the selection and/or occurrence of horizontal gene transfer among heterotrophic bacteria. In previous studies, we detected ciprofloxacin resistance determinants widely associated with horizontal gene transfer in aeromonads and related bacteria isolated from AS (our results, unpublished).

Ciprofloxacin-resistant enterobacteria were in percentages around 1–4% of the total enterobacteria in the raw wastewater and around 1–5% in the treated effluent. When compared with the other WWTP, the percentages of resistant enterobacteria were significantly higher in treated wastewater of the two lagoons. In these plants, where wastewater treatment implies longer hydraulic retention times, biological treatment may be accompanied by an increase of the ciprofloxacin resistance percentage. However the wide variations observed in the raw wastewater hamper the drawing of a well-sustained conclusion. The occasional discharge of fluoroquinolone-resistant enterobacteria to the municipal sewage collector may be a possible explanation to this observation, and would be more evident in plants with a lower inflow volume.

Wastewater treatment did not lead to significant increases of ciprofloxacin resistance percentages in enterobacteria. In a previous study, it was demonstrated a significantly higher ciprofloxacin resistance percentage in *Escherichia coli* isolated from the treated effluent of AS than in the respective inflow (Ferreira da Silva et al. 2007). However, both results may not be comparable, as Ferreira da Silva et al. (2007) studied a collection of bacteria that were isolated and purified in the absence of antibiotic and tested for ciprofloxacin resistance using the disk diffusion method, where resistance corresponded to inhibitory concentrations above 1 mg/L. In the present study, CFU were enumerated, without isolation and purification, on culture medium supplemented with 4 mg/L of ciprofloxacin. Given the antibiotic concentration used in the present study, ciprofloxacin resistance corresponded to a higher tolerance to the antibiotic than in the report of Ferreira da Silva et al. (2007). Besides, the fact that resistance percentages were determined on basis of CFU growing on 4 mg/L of ciprofloxacin rather than on isolates that were purified, maintained in lab conditions, and characterized for their resistance phenotype, may explain why in the current study no significant differences were observed between the raw and treated wastewater. The method used by Ferreira da Silva et al. (2007) disregards bacteria that loose viability and/or the resistance phenotype after lab manipulation and may be, in this respect, more accurate.

Another fact that may hamper the observation of a possible increment or decrease of antibiotic resistance percentages after wastewater treatment is the significant variation of CFU/mL determined in different sampling campaigns. This effect, which has been demonstrated in other studies (Guardabassi et al. 2002; Reinthaler et al. 2003; Ferreira da Silva et al. 2006, 2007) may mask the real variations. Hence, the examination of the resistance phenotypes of pure isolates may be preferred when the objective is to determine overall increases or decreases of resistance percentages after wastewater treatment.

Some studies have tried to ascertain whether domestic WWTP may promote an increase in the prevalence of antibiotic-resistant bacteria. A few studies that compared the prevalence of resistance before and after wastewater treatment demonstrate an increase for some antibiotics, whereas for others resistance percentage does not vary significantly (Guardabassi et al. 2002; Reinthaler et al. 2003; Ferreira da Silva et al. 2006; Zhang et al. 2009). In some cases, there are also some dissonant results, with studies conducted in different regions or dates reaching different conclusions. For instance, Reinthaler et al. (2003) found resistance against ciprofloxacin only in WWTP receiving hospital effluents whereas others have observed this resistance phenotype consistently in domestic wastewater (present study; Ferreira da Silva et al. 2006, 2007; Zhang et al. 2009). In this respect, the establishment of standard international guidelines to control environmental antibiotic resistance might represent a step forward for further risk analysis.

Despite the possible methodological or regional drawbacks that may hamper solid conclusions on the effect of water treatment on antibiotic resistance, all the studies end up in a consensus conclusion: WWTP supply continuously antibiotic-resistant bacteria or genetic determinants to the environment (Guardabassi et al. 2002; Reinthaler et al. 2003; Tennstedt et al. 2003; Costa et al. 2006; Ferreira da Silva et al. 2006, 2007; Watkinson et al. 2007a; Sabate et al. 2008; Zhang et al. 2009).

According to our data, the size and capacity to remove bacteria may represent a critical aspect on the potential environmental impact of a WWTP. In the smaller plants examined in this study about 10^7 CFU of ciprofloxacin-resistant bacteria were released per day per inhabitant whereas these values were ten to 100 times higher in the larger plants examined. This finding leads to the recommendation that larger plants should be equipped with efficient wastewater disinfection prior to its release into the environment. However, also the disinfection process should be analyzed carefully, as previous evidences have suggested that disinfection may also promote the selection of antibiotic-resistant bacteria (Murray et al. 1984; Kümmerer 2009a).

When disinfection is not available, the achievement of bacterial removal rates close to 99%, as those observed in the present study in the three smaller plants, seems also to be an important factor to minimize the release of antibiotic-resistant bacteria.

However, this conclusion should be analyzed with precaution, because such high removal rates may be associated with processes with longer hydraulic retention times where horizontal gene transfer may be favored (Tran and Jacoby 2002; Summers 2006; Kelly et al. 2009). The data obtained for both lagoons, which have hydraulic retention time of 9 and 15 days, suggest that even when the inflow presents low levels of ciprofloxacin-resistant enterobacteria the wastewater treatment may favor the release of higher percentages of such organisms.

This study demonstrates that WWTP with bacterial removal rates close to 99% may have an attenuated impact on the spreading of antibiotic-resistant bacteria. However, the longer hydraulic retention time necessary to achieve higher removal rates may contribute to the establishment of resistant bacterial populations in the treating biomass leading to a progressive increase of the prevalence of antibiotic resistance in the treated effluent. A compromise between both types of system and the use of wastewater disinfection may be part of the solution to minimize the spreading of antibiotic-resistant bacteria.

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