Antibiotic resistant bacteria in urban sewage: Role of full-scale wastewater treatment plants on environmental spreading

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The presence of antibiotic resistant bacteria (ARB) in wastewater was investigated and the role of wastewater treatment plants (WWTPs) in promoting or limiting antibiotic resistance was assessed. Escherichia coli (E. coli) and total heterotrophic bacteria (THB) resistance to ampicillin, chloramphenicol and tetracycline was monitored in three WWTPs located in Milan urban area (Italy), differing among them for the operating parameters of biological process, for the disinfection processes (based on sodium hypochlorite, UV radiation, peracetic acid) and for the discharge limits to be met. Wastewater was collected from three sampling points along the treatment sequence (WWTP influent, effluent from sand filtration, WWTP effluent).

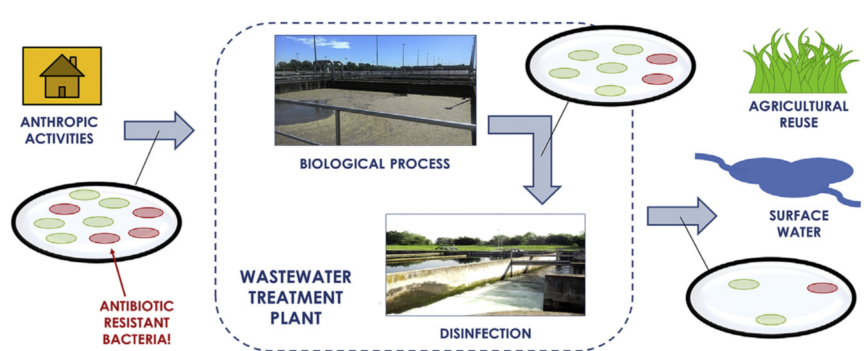
Antibiotic resistance to ampicillin was observed both for E. coli and for THB. Ampicillin resistant bacteria in the WWTP influents were 20e47% of E. coli and 16e25% of THB counts. A limited resistance to chloramphenicol was observed only for E. coli, while neither for E. coli nor for THB tetracycline resistance was observed. The biological treatment and sand filtration led to a decrease in the maximum percentage of ampicillin-resistant bacteria (20e29% for E. coli, 11e21% for THB). However, the conventionally adopted parameters did not seem adequate to support an interpretation of WWTP role in ARB spread. Peracetic acid was effective in selectively acting on antibiotic resistant THB, unlike UV radiation and sodium hypochlorite. The low counts of E. coli in WWTP final effluents in case of agricultural reuse did not allow to compare the effect of the different disinfection processes on antibiotic resistance.

Keywords: Antibiotic resistance, Biological process, Disinfection process, E. coli, Total heterotrophic bacteria (THB)

H I G H L I G H T S

- Resistance to 3 antibiotics was assessed in 3 municipal wastewater treatment plants.
- *E. coli* and total heterotrophic bacteria (THB) were selected as microbial indicators.
- A relevant quote of resistant *E. coli* and THB was observed only for ampicillin.
- Biological treatment and sand filtration influenced the presence of resistant bacteria.
- Peracetic acid reduced resistant THB, unlike UV radiation and sodium hypochlorite.

G R A P H I C A L A B S T R A C T



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1. Introduction

Micropollutants and emerging contaminants are nowadays commonly present in surface waters, in wastewater and in treated effluents at trace concentrations (ng/L to μg/L) (Luo et al., 2014; Petrie et al., 2015). This kind of pollution is currently undergoing intense monitoring and the removal performance of water and wastewater treatment processes which had not been specifically designed to remove these contaminants is the subject of many research works (Gaffney et al., 2014; Margot et al., 2015).

Among emerging contaminants, antibiotics are known for their relevant environmental and sanitary concern, due to their widespread use in agricultural, veterinary and clinical applications and the input of treated wastewater in rivers (Martinez, 2009; Michael et al., 2013). Antibiotic exposed bacteria can develop antibiotic resistance (AR) (Berendonk et al., 2015; Rizzo et al., 2013b; Sharma et al., 2016), mainly due to the horizontal transfer of genetic material, such as plasmids, integrons and transposons (Bouki et al., 2013; Gao et al., 2015), allowing them to survive to concentrations over the inhibition or toxicity thresholds. Keen and Montforts (2012) suggested that AR can also be found where antibiotics are not found, due to the environmental transport and dispersion of antibiotic resistant genes (ARGs). That's why ARGs have also been defined as environmental contaminants themselves (Hsu et al., 2015; Miranda et al., 2016).

ARB and ARGs represent a serious threat for human health, as diseases caused by ARB cannot be faced by standard therapies, as recently highlighted by World Health Organization (WHO, 2014). An important issue in AR spread into the environment is related to the role of wastewater treatment plants (WWTPs), which are at present considered as potential sources for ARB and ARGs input to aquatic ecosystems (Baquero et al., 2008; Ferro et al., 2017). Antibiotic residues, ARB and ARGs in wastewater (Guardabassi et al., 2002; Manaia et al., 2010) reach WWTPs, where the biological process can act as promoter of AR phenomenon, exerting a selective pressure on microorganisms (Novo et al., 2013). In fact, the presence of low antibiotic concentrations and the long contact times (both in the sewer and in the biological reactors) set favorable conditions for the onset of ARGs and their transfer among bacteria in WWTPs (Zhang et al., 2009). Moreover, in biological reactors the conditions are suitable for active microbial growth: high dissolved oxygen concentration, substrate and nutrient availability lead to high bacterial density (Luczkiewicz et al., 2011), increasing the probability of transfer of genetic material. According to Munir et al. (2011), the different kinds of biological processes and reactors (activated sludge, membrane bioreactor, oxidative ditch and rotatory biological contactor) can contribute differently to AR development. On the other hand, the biological process could also determine an attenuation in AR development, due to the degradation of antibiotics and ARGs (Chen and Zhang, 2013) and to the potential ARB predation by other microorganisms. Actually, some authors reported the presence of ARB in the final effluents of WWTPs (Figueira et al., 2011; Luczkiewicz et al., 2011) and in the aquatic environment (Leonard et al., 2015; Munir et al., 2011).

Disinfection plays an important role in limiting ARB release into surface waters (Dunlop et al., 2015; McKinney and Pruden, 2012) and, under appropriate operating conditions, it permits also to control ARGs presence in treated effluents. For instance, chlorine-based compounds (Huang et al., 2011), UV radiation (Rizzo et al., 2013a) and peracetic acid (Huang et al., 2013) were proved to be effective towards ARB and ARGs spread. Anyway, despite several research works have been published on this topic in the past, no clear, unambiguous conclusions have been drawn so far on the role of disinfection in AR spread (Di Cesare et al., 2016; Turolla et al., 2017).

In addition, it is important to stress that the majority of the published articles is focused on *Escherichia coli* (*E. coli*), that is the conventionally adopted indicator of faecal contamination and that was also suggested as possible indicator to assess the spread of AR in aquatic environment (Watkinson et al., 2007). However, poor information can be found about the characteristics and species of antibiotic-resistant heterotrophic, non-coliform bacteria (Zhang et al., 2015), which are prevailing in activated sludge population and could play a major role in AR development and transfer.

The aim of the present research was to investigate the presence of ARB in the sewage from Milan urban area (Italy) and to assess if three locally operating WWTPs, differing among them for treatment scheme, could promote a selective action on ARB. Milan urban area is particularly significant for the ongoing research topic because it is subjected to important anthropogenic pressures. Including over 5 million people, a high number of hospitals and health care facilities, it is an important source of antibiotics which are variably and often poorly removed by local WWTPs, and are discharged in surface water (Calamari et al., 2003; Zuccato et al., 2010).

Our research investigated the resistance of *E. coli* and total heterotrophic bacteria (THB) to three antibiotics (ampicillin, chloramphenicol and tetracycline) in samples collected from different points along the treatment sequence (WWTP influent, effluent from sand filtration, WWTP effluent). The effect of the configuration of the biological treatment and sand filtration, of the disinfection process and of the disinfectant dose (in the case of UV radiation) has also been evaluated. THB include all bacteria using organic matter for their metabolism, being aerobic or facultative aerobic, including pathogenic and/or opportunistic species and strains (e.g., *E. coli*, *Klebsiella* spp., *Enterobacter* spp., *Citrobacter* spp., *Serratia* spp.). Among THB there are, for instance, biofilm-forming bacteria whose proliferation is supported by residual organics in treated effluents, even if stringent limits are enforced for discharge in surface water or agricultural reuse. THB are usually numerous in wastewater, but just a small portion of them can be effectively incubated by plate counts techniques (Mezzanotte et al., 2003). At 37 °C incubation temperature (as stated by official analytical procedures) bacteria hosted by animals and humans are cultured better than environmental ones. However, among 37 °C growing THB many species and strains colonize any environmental matrix, independently from faecal contamination (Allen et al., 2004). That's why both *E. coli* and THB were analyzed to assess the role of wastewater treatment plants in AR development and spread. Concerning the three selected antibiotics, ampicillin, chloramphenicol and tetracycline have been commonly used for years in the clinical treatment of bacterial infections and belong to three different classes of compounds, while resistance mechanisms were identified for *E. coli* (Pignato et al., 2009) and THB (Huang et al., 2012). The present research work gives further insights on the occurrence of AR *E.coli* previously reported by Zanotto et al. (2016) for one of the investigated WWTPs.

2. Material and methods

2.1. WWTP characteristics

Samples were collected from three WWTPs located in Milan urban area: WWTP1 (1,250,000 Population Equivalent, PE), WWTP2 (1,050,000 PE) and WWTP3 (720,000 PE). These WWTPs receive wastewater from similar drainage basins, typically urban with a limited industrial contribution (34% as COD only in WWTP3). Milan urban area is densely populated and hosts several hospitals and health care facilities with over 7100 beds. 12 of them discharge their wastewater to WWTP1, 5 to WWTP2 and 5 to WWTP3.

As shown in Fig. 1, the three WWTPs are based on biological treatment (activated sludge for nitrification and denitrification), carried out at sludge retention time (SRT) of 30, 30 and 9 days in WWTP1, WWTP2 and WWTP3, respectively. The treatment sequence includes also sand filtration and disinfection. Disinfection is performed by peracetic acid (PAA; average dose: 2 mg/L, dry weather contact time: 45 min) at WWTP1, by UV radiation at WWTP2 and by sodium hypochlorite (NaOCl; average dose: 1.8 mg/L as active chlorine, dry weather contact time: 35 min) at WWTP3. WWTP1 effluent has to comply with the limit for agricultural reuse (max. *E. coli* count: 10 CFU/100 mL) all over the year. The same condition applies to a fraction of WWTP2 effluent, which is treated by UV at high dose (150–300 mJ/cm^2) from May to October. The remaining fraction of WWTP2 effluent (which is thus treated by low UV dose: 50–80 mJ/cm^2, on average) and the effluent from WWTP3 are discharged into surface water and have to meet the standard of 5000 *E. coli* CFU/100 mL. The Italian regulation on harmful disinfection by-product indicates a maximum concentration for total trihalomethanes in the effluent of 30 μg/L in case of agricultural reuse (Nurizzo et al., 2005), implying that chlorination is commonly used only for discharge in surface water. Only WWTP3 includes primary settling. The main operating parameters for the three WWTPs are summarized in Table 1.

The monitoring campaign lasted eighteen months, during which samples were collected in three (WWTP1 and WWTP2) and four (WWTP3) points along the treatment sequence in dry weather conditions (Fig. 1): wastewater inflow after preliminary treatments as screening and grit removal (and also after primary settling for WWTP3), outflow from sand filtration, and final outflow. Samplings were repeated 5 times for WWTP1 and WWTP2 and 7 times for WWTP3.

2.2. Reagents

Ampicillin, chloramphenicol, tetracycline and sodium thiosulfate were all purchased from Sigma Aldrich (USA). Ethanol (99%) was purchased from Carlo Erba Reagenti (Italy). Ampicillin and tetracycline stock solutions (50 g/L and 10 g/L, respectively) were prepared in deionized water, filtered on 0.45 μm cellulose nitrate membranes (Sartorius Stedim Biotech, Germany), and stored at −20 °C. Chloramphenicol stock solution (50 g/L) was prepared in ethanol and stored at 4 °C. The absence of toxicity on bacteria due to the use of ethanol was preliminary verified.

2.3. Sampling procedure, chemical and microbiological analyses

All samples were collected in 1 L sterile dark bottles, transported to the laboratory in refrigerated bags and processed within 4 h. Sodium thiosulfate was added when sampling the final effluents from WWTP1 and WWTP3 to quench residual PAA and NaOCl. Samples were analyzed for some physical-chemical parameters (TSS, COD, absorbance at 254 nm with 1 cm optical path) according to Standard Methods (APHA/AWWA/WEF, 2012). *E. coli* were enumerated by membrane filtration method according to Standard Methods (APHA/AWWA/WEF, 2012), using 0.45 μm pore size cellulose nitrate membranes (Sartorius Stedim Biotech) and chromogenic agar (EC X-GLUC agar, Biolife, Italy) as culture medium; inoculated plates were incubated at 44 °C for 24 h. THB were enumerated by plate count technique according to UNI EN ISO 6222:2001, by pouring 1 mL of sampled wastewater (eventually diluted in sterilized water) into 10 mL of nutrient agar (yeast extract agar, Biolife) and incubating inoculated plates at 37 °C for 48 h. *E. coli* and THB counts were expressed as Colony Forming Units (CFU) in 1 mL volume. Microbiological analyses were carried out at least in triplicate for each sample.

2.4. ARB determination

Antibiotic resistant *E.coli* and THB were enumerated according to the procedure reported by Manaia et al. (2010), based on the number of colonies developed on membranes or plates after spiking increasing antibiotic concentrations in the culture medium. As for antibiotic resistant *E. coli*, three concentrations of each antibiotic were tested (0, 8, 16 and 32 mg/L). As for THB, three concentrations of each antibiotic were tested for WWTP3 (0, 16, 32 and 64 mg/L), while for WWTP1 and WWTP2 a further intermediate concentration (48 mg/L) was also used.

Antibiotic concentrations were selected considering the EUCAST breakpoint tables, which classify *E. coli* as resistant to ampicillin, chloramphenicol and tetracycline when the minimum inhibitory concentration (MIC) is over values between 4 and 16 mg/L depending on the antibiotic (EUCAST website, http://www.eucast.org/, last access: August, 2017). The range of antibiotic concentrations selected for THB is in agreement with the research work carried out by Huang et al. (2012) using the same antimicrobial compounds. For each sample (WWTP, collection point) and antibiotic (type, concentration), *E. coli* and THB enumeration was

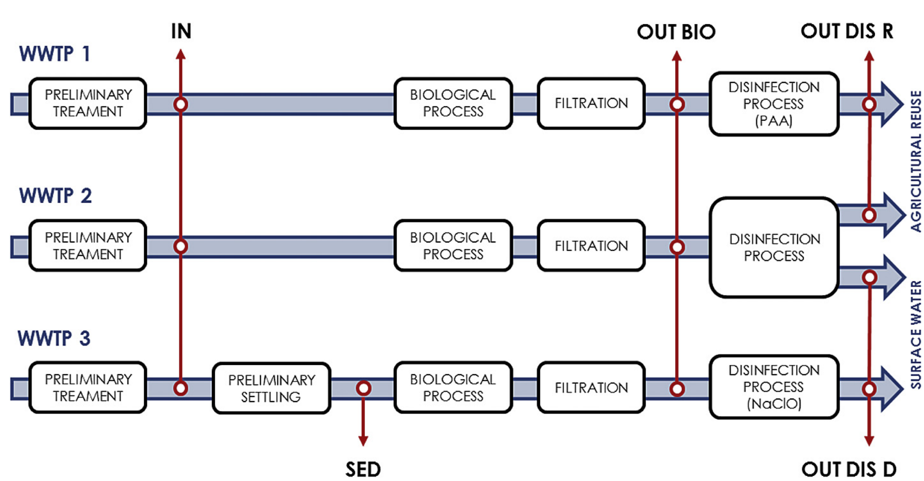

Fig. 1. Treatment scheme of the three WWTPs. The sampling points are indicated as: IN (WWTP influent after preliminary treatments, i.e. screening and grit removal); SED (effluent from the primary settling); OUT BIO (effluent from sand filtration); OUT DIS (final disinfected effluent to be reused in agriculture, OUT DIS R, or discharged into surface waters, OUT DIS D).

Table 1
Main operating parameters for the three WWTPs.

Operating parameter		WWTP1	WWTP2	WWTP3
Average Daily flow rate	m^3/d	432,000	345,600	177,000
Food to microorganisms ratio (F/M)	$kg_{COD}/(kg_{VSS}\,d)$	0.18	3.86–4.37	0.13–0.14
Sludge Retention Time (SRT)	d	28–30	30	9
Biomass concentration	kg_{VSS}/m^3	2.5	2.5	2.7

carried out at least in triplicate.

For each antibiotic concentration the percent *E. coli* and THB survival ratio ($S_{E.coli}$, S_{THB}) was determined as:

$$S_{E.coli(THB)} = \frac{N_C}{N_0} \cdot 100 \tag{1}$$

where N_C represents the number of *E. coli* or THB colonies survived after the exposure to the antibiotic at the concentration "C", while N_0 is the number of *E. coli* or THB colonies grown in the same sample but not exposed to the antibiotic. For each antibiotic the minimum (but not null) $S_{E.coli}$ and S_{THB} value observed for the maximum adopted antibiotic concentration represents the percentage of *E.coli* or THB resistant towards the selected antibiotic ($R_{E.coli}$, R_{THB}):

$$R_{E.coli(THB)} = min\left(S_{E.coli(THB)}\right) \text{ when } S_{E.coli(THB)} > 0 \tag{2}$$

2.5. Data processing

Statistical analyses were performed using the software Minitab 17. Since many factors (antibiotic, sampling point, WWTP) affected the survival ratios, an analysis of covariance (ANCOVA) on the whole dataset using antibiotic concentration as covariate could not be performed (Rutherford, 2011). Therefore, the analysis of variance (ANOVA) with two-sided 95% confidence interval was performed to assess the influence of the sampling point and of the WWTP on the survival ratios. The antibiotic concentration corresponding to ampicillin resistance, according to Equation (2), was taken as reference value. To minimize the differences between mean and median and to obtain a symmetrical distribution, ANOVA was performed on log-transformed survival ratio, which was assumed as the dependent variable. The assumptions of ANOVA (normality of residuals, homoscedasticity) were verified.

The experimental data obtained on disinfected effluents were neglected, due to the very low number of *E. coli* colonies counted in the samples.

3. Results

3.1. Wastewater characterization

The main wastewater physical-chemical and microbiological characteristics are reported in Table 2. Bacterial count in raw wastewater is moderately low in the three WWTPs, as it is typical in Milan urban area, where the flow of consumed and discharged water is very high and, historically, some small brooks have been integrated as part of the sewerage network. Consequently, the dilution of wastewater is great. *E. coli* and THB counts are comparable in raw (IN samples) and settled wastewater (SED samples), suggesting that the primary settling is not effective in reducing them.

Biological treatment and sand filtration reduce both *E. coli* and THB counts by three and two orders of magnitude, respectively, regardless of SRT or F/M values, suggesting a negligible role of operating parameters since SRT in WWTP3 is significantly shorter than in WWTP1 and WWTP2.

The effect of the different disinfection processes depends, of course, on the disinfectant and its applied dose, which is directly related to the targeted microbiological quality (*E. coli* count limit for discharge in surface water or for agricultural reuse). Experimental data from WWTP1 confirm the higher sensitivity of *E. coli* to PAA with respect to other microorganisms, as already discussed in Rossi et al. (2007). On the contrary, consistently with data reported in Antonelli et al. (2006), PAA seems to be scarcely effective in THB inactivation since their count moves just from $2 \cdot 10^4$ CFU/mL to $1 \cdot 10^4$ CFU/mL. In WWTP2 *E. coli* and THB reduction depends on the UV applied dose: the inactivation is about one order of magnitude for low UV doses 50–80 mJ/cm^2 (adopted when the effluent is discharged in surface water) and three orders of magnitude for high UV doses 150-300 mJ/cm^2 (adopted for the agricultural reuse of treated effluent). In WWTP3 NaOCl displays a strong disinfectant action for both *E. coli* and THB, whose counts are reduced by 3 and 2 orders of magnitude, respectively.

3.2. AR in E. coli and THB in the influent to WWTP

The results of analyses on ARB in the influents (after preliminary treatments and, for WWTP3, also after primary sedimentation) are reported in Fig. 2 as survival ratios of *E. coli* and THB exposed to growing concentrations of ampicillin, chloramphenicol and tetracycline. Data variability was high for both *E. coli* and THB counts, with coefficients of variation ranging mostly between 50 and 70%. The high variability of data, as well as the possibility of describing the occurrence of AR only for culturable bacteria, is an important constraint of the adopted method for ARB assessment. On the other hand, enumeration methods based on plate-count culturability can easily be adopted also in WWTP laboratories, permitting ARB monitoring to become a routine practice. Other methodologies based on molecular biology to assess AR in WWTPs have been reviewed by Rizzo et al. (2013b), where main benefits and drawbacks of these techniques are discussed.

Experimental data on ampicillin highlighted the presence of a relevant fraction of resistant bacteria.

Tetracycline was the most effective antibiotic against THB, whose survival ratios were close to zero (0.1–2.6%) for all concentrations, and was also effective against *E. coli*: in all samples bacterial count decreased regularly with increasing tetracycline concentration, with survival ratios between 3 and 7% at 32 mg/L.

The presence of chloramphenicol resistant *E. coli* was also observed, even if the resistance percentages were lower than for ampicillin (3% at WWTP1 and WWTP3, 6% at WWTP2). Chloramphenicol acted effectively on THB, determining very low survival ratios: survival ratios below 1% were observed for 64 mg/L dose. Further considerations on tetracycline and chloramphenicol are prevented by the low survival ratios and the high variability of data, making impossible to detect any significant variation in the mean values for different antibiotic concentration, sampling point and WWTP.

As for ampicillin, *E. coli* and THB survival ratios in raw and

Table 2
Main wastewater physical-chemical and microbiological characteristics (mean ± st.dev.). Absorbance at 254 nm is reported as UV_{254}. ND stands for Not Detected.

Sampling point	WWTP	TSS (mg/L)	COD (mg/L)	UV_{254} (−)	*E. coli* (CFU/mL)	THB (CFU/mL)
IN	WWTP1	159 ± 64.5	142 ± 45.6	0.14 ± 0.035	$(3 \pm 2.1)\cdot 10^4$	$(3 \pm 2.3)\cdot 10^6$
	WWTP2	98 ± 36.4	116 ± 30.1	0.14 ± 0.034	$(3 \pm 1.2)\cdot 10^4$	$(4 \pm 3.6)\cdot 10^6$
	WWTP3	66 ± 6.4	119 ± 54.9	0.17 ± 0.035	$(3 \pm 2.1)\cdot 10^4$	$(3 \pm 1.6)\cdot 10^6$
SED	WWTP3	76 ± 14.8	117 ± 59.9	0.22 ± 0.048	$(3 \pm 2.0)\cdot 10^4$	$(3 \pm 1.5)\cdot 10^6$
OUT BIO	WWTP1	2 ± 1.4	21 ± 3.3	0.07 ± 0.018	$(5 \pm 6.9)\cdot 10$	$(2 \pm 3.5)\cdot 10^4$
	WWTP2	2 ± 2.0	21 ± 4.5	0.08 ± 0.026	$(5 \pm 5.9)\cdot 10$	$(2 \pm 3.4)\cdot 10^4$
	WWTP3	5 ± 3.6	20 ± 4.9	0.13 ± 0.007	$(2 \pm 1.5)\cdot 10$	$(2 \pm 0.7)\cdot 10^4$
OUT DIS	WWTP1	2 ± 1.1	19 ± 1.6	0.07 ± 0.002	ND	$(1 \pm 1.1)\cdot 10^4$
	WWTP2 (reuse)	1 ± 1.1	18 ± 3.4	0.07 ± 0.006	ND	$(2 \pm 0.9)\cdot 10^2$
	WWTP2 (surface water)				3 ± 6.5	$(2 \pm 2.2)\cdot 10^4$
	WWTP3	5 ± 3.6	21 ± 4.9	0.14 ± 0.022	ND	$(5 \pm 3.4)\cdot 10^2$

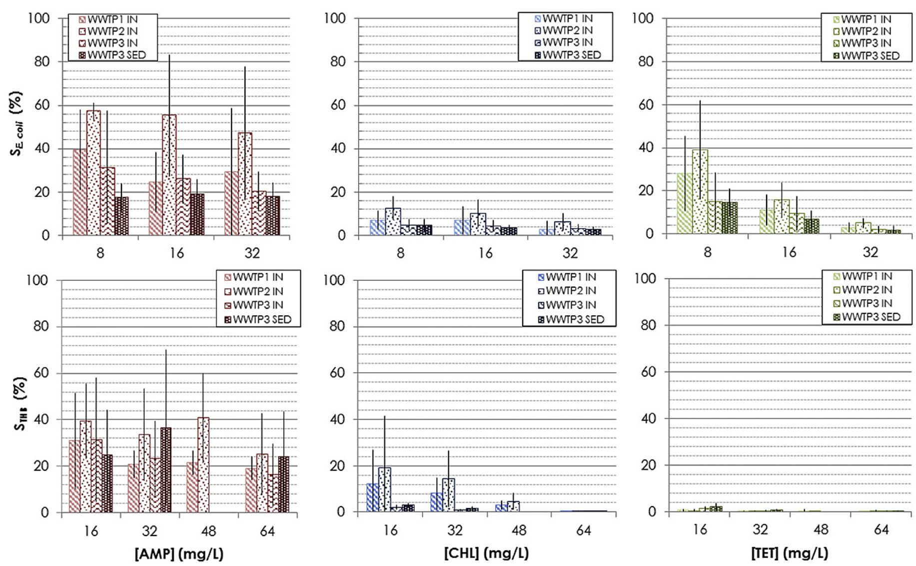

Fig. 2. Survival ratios (mean ± st.dev.) for *E. coli* ($S_{E. coli}$) and THB (S_{THB}) after exposure to ampicillin (AMP), chloramphenicol (CHL) and tetracycline (TET) in the influents to WWTPs as a function of antibiotic concentration. Values for mean and standard deviation are based on data from 5 to 7 samplings.

settled wastewater ranged in different intervals in spite of deriving from drainage areas with similar land use, population and industrial densities. *E. coli* survival ratios were 39–25% in WWTP1, 57-47% in WWTP2, 31-20% in WWTP3, while the ranges of THB survival ratios were 19–31% in WWTP1, 25–41% in WWTP2 and 16–31% in WWTP3. For both microbial indicators, a trend to decrease with increasing antibiotic concentration can be clearly observed, with 25% ampicillin-resistant *E. coli* of in WWTP1, 47% in WWTP2 and 20% in WWTP3. For THB, the percentages of ampicillin-resistant bacteria were 19%, 25% and 16% in WWTP1, WWTP2 and WWTP3, respectively.

This finding is in contrast with the number of hospitals in the drainage basin of each WWTP, which could be expected to account for the major contribution in discharging antibiotics into the public sewerage, as reported by Verlicchi et al. (2012): in fact, the WWTP1 drainage area includes the highest number of hospitals and the largest ones (about 4,100 beds), compared to WWTP2 and WWTP3 drainage areas (1,400 beds and 1,000 beds, respectively). As also reported by Li et al. (2015), antibiotic resistance in sewerage networks and WWTPs seems to be a more complex phenomenon, not only directly related to the use of antibiotics and, accordingly, to their release into wastewater. Several other aspects should be considered, such as the length of the drainage network, which is related both to the exposure time for bacteria and to the time available for antibiotic degradation, the type and efficiency of the treatments performed at the hospital before wastewater is discharged into the public sewer. No significant difference was observed in *E. coli* and THB counts between IN and SED samples in WWTP3, as confirmed by ANOVA (#data = 49, p-value = 0.831, F-value = 0.050 for *E. coli*, #data = 47, p-value = 0.638, F-value = 0.230 for THB), suggesting that primary settling has no selective influence on ARB.

3.3. Influence of biological treatment and sand filtration on ampicillin resistance

In the samples collected after biological treatment and sand filtration a change in survival ratios was observed with respect to IN samples, both as absolute values and as trends over ampicillin concentration.

As shown in Fig. 3, the survival ratios of *E. coli* exposed to ampicillin after biological treatment and sand filtration were in the range 20–37% at WWTP1 and 31-28% at WWTP3, with a trend to decrease with increasing ampicillin concentration.

A slight decrease with respect to the WWTP influent was observed at WWTP1 (ampicillin-resistant *E. coli* from 25% to 20%), while a slight increase took place at WWTP3 (ampicillin-resistant *E. coli* from 20% to 28%). On the contrary, despite the same process (activated sludge) and SRT values at WWTP1 and WWTP2, WWTP2 biological treatment and sand filtration led to a significant decrease in survival ratios, with a reduction in ampicillin-resistant *E. coli* colonies from 47% (influent to biological reactor) to 23%.

The ranges of THB survival ratios were 11–52%, 17–41% and 21–40% in WWTP1, WWTP2 and WWTP3 respectively. In WWTP1 and in WWTP2, decreasing trends with increasing antibiotic concentration were observed in OUT BIO samples (collected after biological treatment and sand filtration), which were not so evident in IN samples. In WWTP1 survival ratios at 16, 32 and 48 mg/L of ampicillin in OUT BIO samples (36–52%) were higher than in IN samples at the same antibiotic concentrations, while at 64 mg/L a sharp reduction to 11% was observed. A decrease in ampicillin resistance was then observed in WWPT1 (from 19% to 11%) and WWTP2 (from 25% to 17%) with respect to raw wastewater, but not in WWTP3, where ARB slightly increased (from 16% to 21%). For WWTP3 the decreasing trends with increasing antibiotic concentration in OUT BIO and IN samples were similar.

The statistical significance of the influence of biological treatment and sand filtration on the amount of ampicillin-resistant colonies in the microbial community was confirmed by ANOVA only for *E. coli* in WWTP2 (#data = 50, p-value = 0.006, F-value = 8.230), while in the other cases the high variability of experimental data probably prevented to highlight a statistical significance.

Actually, biological process causes a modification in the microbial population, which seems to modify also the presence of ampicillin resistant bacteria. Nevertheless, the role of the biological treatment and sand filtration in determining ampicillin resistance cannot be univocally correlated to the operating parameters conventionally used to define the process performance, such as F/M ratio or SRT. In fact, biological process in WWTP1 and WWTP2, which is characterised by high SRT and low F/M ratios, seems to reduce ampicillin resistance, contrarily to biological process in WWTP3, which is ineffective. Anyway, the reduction extent of ampicillin resistance is comparable for THB, but not for *E. coli*. Once more, the development of resistance is confirmed to be a composite phenomenon and, more likely, conventional indicators should be integrated with ecological variables to describe the complex ecosystem that is the activated sludge reactor. In fact, just considering these conventional parameters (see Table 1), WWTP1 and WWTP2 should behave the same. Hence, more thorough and extensive studies are required for assessing the modifications involving bacterial populations and their resistance to antibiotics. Interesting results were obtained by Yuan et al. (2016) who analyzed the influence of five biological reactors on six groups of ARB and corresponding ARGs. They concluded that MBRs (membrane biological reactor) and SBRs (aerobic sequencing batch reactor) were the most effective in reducing ARB abundances, with maximum values of 3.54 log and 3.13 log, respectively, while aerobic activated sludge systems achieved 2.06 log as maximum removal.

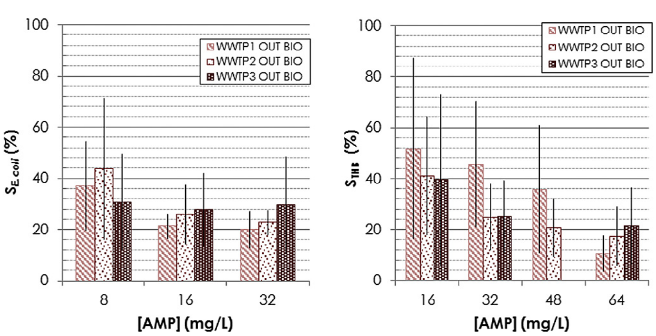

Fig. 3. Survival ratios (mean ± st.dev.) for *E. coli* ($S_{E.\ coli}$) and THB (S_{THB}) after exposure to ampicillin (AMP) as a function of antibiotic concentration, after biological treatment and sand filtration. Values for mean and standard deviation are based on data from 5 to 7 samplings.

3.4. Influence of disinfection on ampicillin resistance

The survey of various disinfection processes is useful for understanding how this stage can modify the presence of ARB in microbial population and to optimize the choice also in order to reduce the presence of viable ARB or of ARGs in WWTP effluent and, thus, their spread in the environment.

Although many recent scientific studies address the problem of antibiotic resistance in WWTPs, the differences in the used methods, in the organisms considered, as well as in the disinfection processes applied, make difficult to compare the reported results.

Regarding our study, the presence of *E. coli* in OUT DIS samples was highly variable, due to the very low final counts and, thus, to the small number of plates (not exposed to antibiotics) where colonies developed. This makes hard to interpret the results obtained in the presence of antibiotics and to draw definite conclusions on the influence of different disinfection processes. Only in the case of disinfection by UV radiation aimed at discharging in surface water (OUT DIS D) at WWTP2, *E. coli* count allowed to state that the disinfection process has not a substantial impact on ARB. A decreasing trend of survival ratios was observed, resulting in a resistance to ampicillin of about 19%, not significantly different from the percentage for the sand filtration effluent (23%), as also confirmed by ANOVA (#data = 58, p-value = 0.067, F-value = 2.350). Such results are in agreement with experimental data reported in Pang et al. (2016).

Fig. 4 reports the survival ratios of THB to ampicillin after disinfection. PAA and NaOCl had the same effect and the final counts decreased with increasing ampicillin concentration, but their effectiveness against ampicillin-surviving bacteria was different: the ranges of THB survival ratios were 3–20% for PAA and 23–34% for NaOCl, respectively. PAA seemed to act selectively on ampicillin-resistant bacteria, whose presence decreased after the disinfection process with respect to the effluent from sand filtration. In contrast, NaOCl did not act selectively on ampicillin-resistant bacteria, displaying its disinfecting action with the same intensity on the whole bacterial community present in OUT BIO samples, being their ratio unchanged.

THB survival ratios after UV radiation, independently from the applied UV dose, displayed a parabolic trend (16–28% for reuse, 6–15% for surface water discharge) with ampicillin concentration increase, suggesting a selective abatement of ampicillin-resistant THB, even if the survival ratios (15% and 22% for reuse and discharge, respectively) for the highest applied ampicillin dose are comparable with those in OUT BIO samples. Concerning UV treatment, our results show removal rate higher than reported by Lee et al. (2017). Also Zheng et al. (2017) reported a greater tolerance to UV of ARB than of non-ARB, even if they studied different heterotrophic bacteria and antibiotic; the same conclusion was drawn for NaOCl. On the contrary, Yuan et al. (2015) showed effective inactivation of all ARB after chlorination. ANOVA indicated that the influence of PAA on the amount of ampicillin-resistant THB is significant (#data = 66, p-value = 0.002, F-value = 12.340), unlike for NaOCl (#data = 57, p-value = 0.267, F-value = 1.280) and UV radiation (#data = 53, p-value = 0.956, F-value = 0.000).

Therefore, such analyses can be considered as a confirmation of the selective action operated by PAA on ampicillin-resistant THB and, conversely, of the negligible effect of UV radiation in selectively reducing ARB. However, such considerations are based on very low and highly variable counts and are thus to be held as preliminary indications.

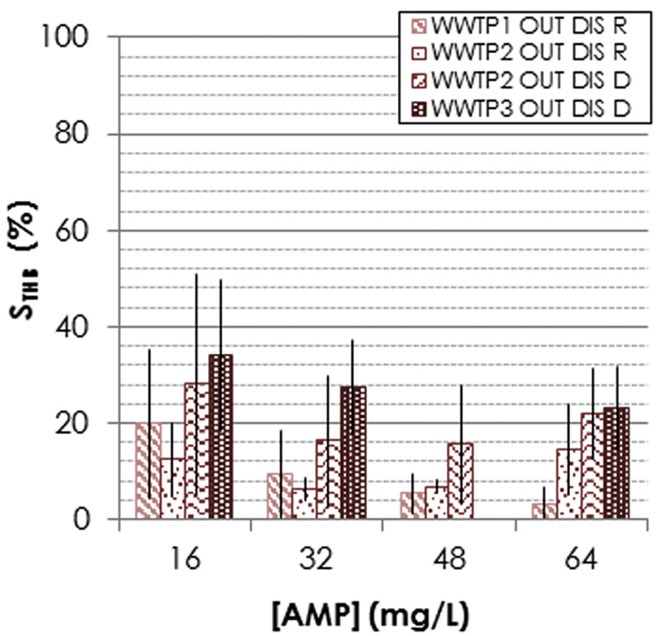

Fig. 4. Survival ratios (mean ± st.dev.) for THB (S_{THB}) after exposure to ampicillin (AMP) as a function of antibiotic concentration, after disinfection treatment. Values for mean and standard deviation are based on data from 5 to 7 samplings.

4. Discussion

Microbiological quality standards are usually based on fecal indicators, even if they represent a minor part of the total bacteria in wastewater. Nevertheless, considering AR spread in the environment, expanding microbiological monitoring to other bacterial strains seems very important, due to the potentially relevant role they could have in the transfer of genetic material.

THB count in raw wastewater is two orders of magnitude higher than *E. coli* count, while treatment processes are less effective against THB than against *E. coli*. In raw wastewater and in the effluent from sand filtration the trend of survival ratios over ampicillin concentration is the same for *E. coli* and THB, as also confirmed by ANOVA. In the six statistical analyses p-values significantly over 0.05 were obtained, indicating the absence of a difference between *E. coli* and THB behavior and suggesting that the development of AR is comparable for the two indicators.

Potential hotspots of ARB, as hospitals and health care facilities, appear as not the unique responsible of antibiotic load to sewerage and in AR development in complex urban area such as Milan urban area is, suggesting that the common domestic use of antibiotics plays an important (and maybe prevailing) role. The residence time in sewerage network is also important in the development of AR in sewerage. On the contrary, biological reactors characterized by long SRT probably cause a reduction of AR, if this is measured on thermo-tolerant bacteria, which are incubated at 37 °C. This latter consideration leads to suggest that AR development should be assessed also monitoring psychrophilic indicators, which can better survive both in biological reactors and in natural aquatic environment.

In samples disinfected by low UV dose (for discharge in surface water) *E. coli* and THB behaved at the same way, as confirmed by statistical analysis (# data = 55, p-value = 0.070, F-value = 3.450). On such basis, we could assume that *E. coli* and THB behave the same also after the other disinfection processes (PAA, NaOCl and high dose UV radiation), even if the counts were too low to provide reliable experimental data for *E. coli*. So, we could suppose that only PAA acts selectively on resistant microorganisms, behaving as an effective barrier against ARB spread into the environment. In addition, as reported by Biswal et al. (2014), also ARGs can be effectively reduced by PAA rather than by UV disinfection.

An important point is that stronger oxidizing agents, as NaOCl, leading to higher log-inactivation values (about 3-log for *E. coli* and 2-log for THB), could imply the release of resistant genes from damaged cells. In fact, in previous studies the disrupting effect of NaOCl on cells at conventionally adopted concentrations was proved, while the disinfecting action of PAA did not induce severe cell damages, even at very high and unfeasible concentrations (Mezzanotte et al., 2007). Moreover, also considering the use of ozone as disinfectant, the common dose applied in WWTPs could not significantly eliminate the antibiotic resistance (Ben et al., 2017).

Finally, the disinfecting efficiency of high dose UV radiation and NaOCl on *E. coli* and THB was comparable, but UV causes no cell damage, and thus less concern about ARG release.

5. Conclusions

Experimental results highlighted the presence of ARB in wastewater from Milan urban area. In particular, a relevant fraction of *E. coli* (20–47%) and THB (17–25%) in WWTP influent flows was resistant to ampicillin. Concerning both *E. coli* and THB a limited resistance to chloramphenicol was observed, while tetracycline was highly effective on the whole bacterial community. A consistent relation with the presence of specific hotspots, as hospitals and health care facilities, in the drainage area of WWTPs was not observed, suggesting that the common domestic use of antibiotics is an important source of wastewater pollution for such kind of compounds and that the development of AR is not directly and only related to the use of antibiotics and their discharge in wastewater.

The biological treatment and sand filtration determined a change in the presence of ampicillin-resistant bacteria, leading to resistance ranging from 20% to 28% for *E. coli* and from 11% to 21% for THB, although with different extent depending on WWTP. In addition, biological treatment and sand filtration induced a modification of the survival ratio trends over antibiotic concentration in WWTP1, unlike in WWTP2 and WWTP3, with respect to the influent. Anyway, these changes are not clearly correlated to the values of parameters conventionally adopted to define the operation of these processes, as SRT or F/M ratio in case of biological process.

Concerning disinfection, no conclusions of general validity can be drawn about *E. coli*, due to the high reduction percentages in absence of ampicillin. Otherwise, disinfectants seemed to have different influence on the inactivation of ampicillin-resistant THB, acting PAA selectively and limiting the ARB presence in the effluent, unlike UV radiation and NaOCl.

In conclusion, the AR development and spreading is a complex phenomenon, which requires an extensive monitoring to be faced. In this sense, the adoption of easy-to-implement methodologies, such as plate-count-based techniques, can be a valid support in data collecting from full-scale WWTPs, in which sophisticated instruments are usually unavailable. On the other hand, these monitoring campaigns, that could be carried out continuously at WWTPs on a selected pool of antibiotics, should be integrated more sporadically with molecular-based techniques for the accurate characterization of the abundance of resistance genes and potential gene transfer.

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