Identification of antibiotic-resistant *Escherichia coli* isolated from a municipal wastewater treatment plant

Carlo Zanotto[a], Massimiliano Bissa[b^], Elena Illiano[b], Valeria Mezzanotte[c], Francesca Marazzi[c], Andrea Turolla[d], Manuela Antonelli[d], Carlo De Giuli Morghen[e]*, and Antonia Radaelli[b,f]*

[a]Department of Medical Biotechnologies and Translational Medicine, University of Milan, Via Vanvitelli, 32, 20129 Milan, Italy.

[b]Department of Pharmacological and Biomolecular Sciences, University of Milan, Via Balzaretti, 9, 20133, Milan, Italy.

[c]Department of Environmental and Regional Sciences (DISAT), University of Milan Bicocca, Piazza della Scienza 1, 20126 Milan, Italy

[d]Environmental Section, Department of Civil and Environmental Engineering (DICA), Milan Polytecnic, Piazza Leonardo da Vinci 32, 20133 Milan, Italy

[e]Catholic University "Our Lady of Good Counsel", Rr. Dritan Hoxha, Tirana, Albania

[f]Cellular and Molecular Pharmacology Section, National Research Council (CNR) Institute of Neurosciences, University of Milan, Via Vanvitelli, 32, 20129 Milan, Italy

^Present address: National Cancer Institute (NCI), National Institutes of Health (NIH), Basic Res Lab, AMRVS, Bethesda, MD, USA.

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***Corresponding authors, co-last authors**

Department of Pharmacological and Biomolecular Sciences

University of Milan, Via Balzaretti, 9,

20133, Milan, Italy.

Tel: +39-02-50317061

Fax: +39-02-50317065

Carlo Zanotto - carlo.zanotto@unimi.it

Massimiliano Bissa - massimiliano.bissa@nih.gov; bissa.massimiliano@gmail.com

Elena Illiano - elena.illiano@unimi.it

Valeria Mezzanotte - valeria.mezzanotte@unimib.it

Francesca Marazzi - francesca.marazzi@unimib.it

Andrea Turolla - andrea.turolla@polimi.it

Manuela Antonelli - manuela.antonelli@polimi.it

Carlo De Giuli Morghen - carlo.degiulimorghen@unimi.it

Antonia Radaelli - antonia.radaelli@unimi.it

Abstract

The emergence and diffusion of antibiotic-resistant bacteria has been a major public health problem for many years now. In this study, antibiotic-resistance of coliforms and *Escherichia coli* were investigated after their isolation from samples collected in a municipal wastewater treatment plant in the Milan area (Italy) along different points of the treatment sequence: inflow to biological treatment; outflow from biological treatment following rapid sand filtration; and outflow from peracetic acid disinfection. The presence of *E. coli* that showed resistance to ampicillin (AMP) and chloramphenicol (CAF), used as representative antibiotics for the efficacy against Gram-positive and Gram-negative bacteria, was evaluated. After determining *E. coli* survival using increasing AMP and CAF concentrations, specific single-resistant (AMP^R or CAF^R) and double-resistant (AMP^R/CAF^R) strains were identified among *E. coli* colonies, through amplification of the β-lactamase Tem-1 (*bla*) and acetyl-transferase catA1 (*cat*) gene sequences. While a limited number of CAF^R bacteria was observed, most AMP^R colonies showed the specific resistance genes to both antibiotics, which was mainly due to the presence of the *bla* gene sequence. The peracetic acid, used as disinfection agent, showed to be very effective in reducing bacteria at the negligible levels of less than 10 CFU/100 ml, compatible with those admitted for the irrigation use of treated waters.

Keywords: Antibiotic resistance; WWTPs; *Escherichia coli*; *bla* and *cat* genes

1. Introduction

Antimicrobial agents are successfully used to treat animal and human diseases. After discovering bacterial innate resistance, such as the one of Enterobacteria to β-lactams (Sykes and Matthew, 1976), the extensive and unnecessary use of antibiotics has caused the selection of antibiotic-resistant bacteria (ARBs) (O'Neill, 2016). Over time, although new antibiotics were developed to contrast the emergence of ARBs, these have not been able to block the selection of strains resistant to these new antibiotics (Keen and Montforts, 2012).

To this purpose, different molecular mechanisms have been described by which bacteria become antibiotic resistant. The mechanisms include genetic mutations and horizontal transfer by conjugative plasmids or trasposons of mobile antibiotic resistance genes (ARG) (Alanis, 2005), easily identified by modern technologies, that may allow the survival also at high antibiotic concentrations (Meredith et al., 2015).

Antibiotic resistance has not been completely explored in the environment (Marti et al., 2014), but the continuous release of antibiotics in wastewater discharges, and their possible outflow in recycled water for agricultural purposes, may impair autochthonous bacteria and freshwater ecosystems (Roose-Amsaleg and Laverman, 2015). Sub-inhibitory antibiotic concentrations were also recently found in environmental settings, as those present in the aquatic environment (Kümmerer, 2009a; Kümmerer, 2009b), that may promote antibiotic resistance and select for ARBs (Chow et al., 2015; Gullberg et al., 2011).

Wastewater treatment plants (WWTP) can also be unable to effectively eliminate water contaminants (Pruden, 2014), which may be dangerous for human health and ecosystems (Richardson and Ternes, 2014). Many pharmaceuticals can persist in the environment and they are frequently detected in drinking water. Also, antibiotics are often administered for economic reasons in the production of food animals to prevent animal

infectious diseases, thus increasing their possible assumption with the diet by human beings (Silbergeld et al., 2008).

It is also known that ARG are not easily removed and ARBS may also increase inside WWTPs, since bacteria are often exposed to antibiotics in activated sludge (Proia et al., 2016; Rizzo et al., 2013), where microbial concentrations and diversity can also facilitate gene transfer (Zhang et al., 2009). Bacterial inactivation procedures may also be unable to deactivate intracellular genes (Dodd, 2012; Sharma et al., 2016), that can persist even after chlorination (Yuan et al., 2015) although the sequential use of chlorination and UV irradiation may improve ARG inactivation (Zhang et al., 2015).

WWTPs are therefore sites that need to be monitored, as they may be the recipients of waters that contain antimicrobials as well as human and animal metabolic waste (Kim and Aga, 2007) and they can provide a suitable environment for the spread of ARBs (Baquero et al., 2008; Marathe et al., 2013). Moreover, the fate of antibiotics and other pharmaceuticals can be strongly influenced not only by WWTP biological/chemical procedures (Sharma et al., 2013), but also by the design and type of treatment system and sewer network as well as by the efficiency of disinfection (Azzellino et al., 2011; Mezzanotte et al., 2007).

The aim of the present study was to detect ampicillin-resistant (AMP^R) and chloramphenicol-resistant (CAF^R) *E. coli* in the inflow and outflow from a municipal WWTP in the Milan area (Italy). AMP and CAF were used as representative antibiotics for the efficacy against Gram-positive and Gram-negative bacteria.

AMP and CAF were chosen as representative of the commonly used antibiotics in clinic, breedings and research laboratories. Although AMP is more generally used, CAF is more specific for Enterobacteria such as *E. coli*, which is the reference standard indicator of foecal contamination and used to monitor the spread of ARBs in recycled waters (Watkinson

et al., 2007). Antibiotic resistance was first determined by analysing *E. coli* survival after spiking their culture medium with increasing AMP and CAF concentrations.

The extensive use of antibiotics in both human and veterinary medicine can promote resistance inside WWTPs. The results of this study show that the biological process of this WWTP is effective in reducing ARB concentration. A prevalence of AMP^R versus CAF^R E. coli colonies was also observed, that can be explained by the larger use of β-lactam antibiotics. After disinfection, ARB removal is almost complete and residual bacteria are compatible with the irrigation use of treated waters.

2. Materials and methods

2.1. Wastewater treatment plant

The WWTP is located in an urban area of Milan (Italy), and it receives wastewater from the city (1,250,000 Inhabitant Equivalents, corresponding to 432,000 m^3/day average inflow (Pizza, 2014), which includes that from many hospitals. The within-plant treatment scheme (Fig. 1) starts with pretreatments (screening, sand and oil removal). This step is followed by the biological treatment with activated sludge, including pre-denitrification and biological oxidation (8 h hydraulic retention time, 30 day sludge retention time). After secondary settling, the biologically treated effluent undergoes rapid sand filtration to improve the removal of suspended solids and phosphorus.

The final disinfection is based on peracetic acid (about 2 mg/L, 45 min contact time in dry weather) and aimed at complying with the microbiological limits for the reuse of treated wastewater for agricultural purposes: 10 *E. coli* colony forming units (CFU)/100 mL.

2.2. Wastewater sampling and physicochemical characterisation

Wastewater samples were collected five times from 19 September to 17 October, 2012, from three different points in the plant: at the inflow to the biological treatment (i.e., IN-BIO samples), at the outflow from sand filtration (i.e., OUT-BIO samples), and at the outflow from disinfection (i.e., OUT-DIS samples) (Fig. 1). All of the samples were collected in sterile dark bottles, taken to the laboratory in refrigerated bags within 4 h, and immediately processed. Sodium thiosulphate was added to the WWTP effluent at the concentration required to reach a neutral pH to quench the residual peracetic acid (United States Environmental Protection Agency, 2012). The physicochemical characteristics of the samples were determined by measuring both the total suspended solids (TSS) using the APAT IRSA CNR method (HACH Lange, Lainate, Milan, Italy) and the chemical oxygen demand (COD) using specific analytical kits for organic pollutants (HACH) and following the manufacturer's specifications. The absorbance for aromatic and unsaturated compounds was determined at 254 nm (OD_{254}) by the DR 6000 spectrophotometer (HACH). All of these analyses were performed following the Standard Methods for the Examination of Water and Wastewater of the American Public Health Association, the American Water Works Association, and the Water Environment Federation (Rice et al., 2012).

2.3. Microbiological determination of total coliforms, E. coli, and AMP^R or CAF^R E. coli

Wastewater samples (100 ml) were filtered through 5-cm-diameter 0.45-µm nitrocellulose membranes (Sartorius Stedim Biotech, Goettingen, Germany). The membranes were then laid on plates containing chromogenic agar growth medium (EC X-GLUC agar; Biolife Italiana, Milan, Italy) to count total coliforms in the presence or absence of antibiotics, and to select the *E. coli* green colonies, which are positive for the β-glucoronidase activity. When the number of bacterial colonies was too high to be counted, the wastewater samples were diluted

in Ca^{2+}-free and Mg^{2-}-free phosphate buffered saline by 10^{-2} to 10^{-4} to have a reliable colony number. Since green colonies can be mostly ascribed to β-glucoronidase-positive Enterobacteria, and *E. coli* is by far the prevalent-one compared to *Salmonella* and *Shigella* (Fekadu et al., 2015), the latter-ones were not taken into consideration. Considering the EUCAST breakpoint tables, that classify Enterobacteria as AMP^R and CAF^R when the MIC is > 8 μg/mL (The European Committee on Antimicrobial Susceptibility Testing (2016; Choffnes et al., 2011), the *E. coli* colonies were grown in agar medium where increasing AMP and CAF concentrations had been added (0, 8, 16, 32 μg/mL; Sigma, St Louis, MO, USA), to selectively monitor and count the resulting AMP^R and CAF^R colonies.

The AMP stock solution (50 mg/mL) was prepared in deionised water, filter-sterilised through 0.45-μm cellulose nitrate membrane, and stored at -20 °C. The CAF stock solution (50 mg/mL) was prepared in 99% ethanol, and stored at 4 °C.

The plates with the *E. coli* colonies were incubated for 24 h at 44 °C, and the colony counts are expressed as CFU/100 mL. The percentage of ARBs was estimated as the ratio between the number of colonies growing in the presence and absence of the respective antibiotics. Enumeration of *E.coli* was carried out five times following the UNI EN ISO procedure 9308-1:2002 for each combination of three parameters (sampling point, dilution, and antibiotic concentration), for a total of 120 analyses.

2.4. Detection of AMP^R and CAF^R genes and single or double-resistant bla^+ and cat^+ E. coli colonies by PCR

From one of the five IN-BIO and five OUT-BIO samples, a maximum of 60 well separated *E. coli* green colonies were picked up from the filter membranes, and transferred to the master plates in the presence of AMP or CAF at 8, 16, 32 μg/mL on Luria Broth (LB)-agar (Biolife Italiana). The incubations were performed as described above.

All of the AMP^R and CAF^R *E. coli* colonies were grown and screened by PCR to determine the presence of the β-lactamase Tem-1 (*bla*) (Baraniak et al., 2005) gene sequence within the AMP^R colonies, and the CAF acetyl-transferase catA1 (*cat*) gene sequence (Maynard et al., 2003) within the CAF^R colonies, that are the prevalent resistance genes against these antibiotics. Primers were chosen to detect the *bla Tem-1* and *catA1* sequences and amplify 721 bp and 630 bp fragments respectively. For the *bla* gene, the designed forward V422 (5' TTG CTC ACC CAG AAA CGC TG 3') and reverse V423 (5' GTC GTG TAG ATA ACT ACG ATA CG 3') primers were based on the *Tem-1* gene (plasmid pBE135, accession no. NG_041180). For the *cat* gene, the designed forward V418 (5' CAC TGG ATA TAC CAC CGT TG 3') and reverse V419 (5' CAC TCA TCG CAG TAC TGT TG 3') primers were based on the *catA1* sequence (plasmid pCmGFP accession no. NC_011521). Amplifications were always carried out in mixtures containing 1 µM of each primer, 200 µM of each dNTP, and 0.025 U Taq DNA polymerase (Fermentas, Dasit Sciences, Milan, Italy). This was supplemented with at 2.5 mM $MgCl_2$ for the *bla* gene, and 1 mM $MgCl_2$ for the *cat* gene. For both the bla Tem-1 and catA1 sequences, the PCR conditions were 94 °C for 4 min, followed by 30 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 45 s. The final step was 72 °C for 7 min. The pcDNA3-CAT plasmid, containing both the AMP and CAF resistance genes, was used as a positive control for the amplification of *bla* and *cat* genes, respectively (Invitrogen Corp., Carlsbad, CA, USA).

To identify the AMP^R/CAF^R double-resistant *E. coli* colonies, the AMP^R colonies were also plated in LB-agar spiked with the different AMP or CAF concentrations (i.e., 8/8, 16/16, 32/32 µg/mL). The single- and double-resistant colonies were then screened by PCR for the presence of the *bla* and *cat* gene sequences, and also for both *bla* and *cat*.

2.5. Statistical analyses

Statistical analyses were performed using one-way ANOVA parametric tests and Bonferroni analysis of variance, using the Prism 5 software (GraphPad Software Inc., La Jolla, CA, USA). The significance was set as $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).

3. Results

3.1. TSS, COD, and OD_{254} values decrease in wastewater samples after the biological treatment and filtration

The analysis of the physical and chemical parameters of the samples showed that, after the biological treatment and filtration (OUT-BIO *versus* IN-BIO, Table 1), the TSS, COD, and OD_{254} values were reduced from 75 to 3 mg/L (96%), from 162 to 12 mg/L (93%) and from 0.40 to 0.07 OD_{254} (82.5%), respectively.

3.2. AMP^R E. coli colonies are already selected at 16 µg/mL AMP, and through the treatments their percentage numbers are only reduced after disinfection

In the absence of antibiotics, the number of *E. coli* colonies that grew on the chromogenic agar medium were 3×10^6 CFU/100 mL in IN-BIO samples, and were reduced to 1.5×10^3 CFU/100 mL after the biological treatment in OUT-BIO samples, with a decrease of >3-log units. A further reduction to <10 CFU/100 mL was found after the final disinfection process, resulting in >2-log units decrease in OUT-DIS samples.

The relative numbers of AMP^R and CAF^R *E. coli* colonies compared to the total *E. coli* colonies are shown in Figure 2, as an average of the 10 data obtained per each sampling point and each AMP (Fig. 2A) and CAF (Fig. 2B) concentration. At any of the AMP concentrations (Fig. 2A), no significant decrease of ARBs is observed through the biological treatment (OUT-BIO *versus* IN-BIO samples), whereas a slight decrease was found for both the IN-BIO

and OUT-BIO samples with the increase in the antibiotic from 8 µg/mL to 16 µg/mL, with no further significant changes when using 32 µg/mL. Although a decrease is always present after disinfection, only at the lowest AMP concentration (8 µg/mL), the treatment with peracetic acid significantly reduced the AMP^R *E. coli* colonies (3% *vs*. 37.2%; $p < 0.05$). In the case of the CAF^R *E. coli*, the colony numbers were always very low, with no significant changes seen (Fig. 2B).

3.3. AMP^R E. coli colonies increase with AMP concentration as well as bla^+ E. coli in OUT-BIO samples

To determine the presence of the *bla* and *cat* resistance genes in the *E. coli* colonies that survived the exposure to AMP or CAF (i.e., the AMP^R and CAT^R *E. coli*), the colonies grown on LB-agar plates were picked up and the specific *bla* and *cat* sequences were amplified by PCR. After electrophoretic separation of the DNA from the selected *E. coli* colonies, the *bla* and *cat* gene fragments appeared as 721-bp and 630-bp bands, respectively, as shown in the representative samples in Figure 3. The same fragments for *bla* and for *cat* were also amplified from pcDNA3-CAT plasmid, used as a positive control.

Based on the number of *E. coli* colonies grown on LB-agar in the presence of the antibiotics, the AMP^R total-coliform colonies showed a decrease of around 2-log units for the OUT-BIO *versus* IN-BIO samples at all three of the AMP concentrations; this was paralleled by the total *E. coli* colonies (Table 2). Here, after the biological treatment and filtration process (i.e., the OUT-BIO samples), at 32 µg/mL AMP, the total *E. coli* colonies represented 60% of the total coliforms, thus indicating that most of the *E. coli* AMP^R colonies were selected for. Among these total *E. coli* colonies, in the IN-BIO samples at 8, 16, 32 µg/mL AMP, the *bla* gene sequence was present (i.e., the bla^+ *E. coli*) in 97%, 92%, and 78% of the total AMP^R *E. coli* colonies, respectively, and in the OUT-BIO samples at the same

concentrations, in 80%, 87% and 91%. Although the CAF^R total-coliform colony numbers showed a similar trend as AMP^R total-coliform colonies, in that a decrease of 2-log units was observed at all CAF concentrations, the *cat* gene sequence was present (i.e., the cat^+ *E. coli*) at lower percentages (of CAF^R colonies) in IN-BIO samples in 50% and 38% of the total CAF^R *E. coli* colonies at 16 and 32 µg/mL CAF and in OUT-BIO samples in 67% and 33% at the same concentrations (Table 2).

3.4. AMP^R/CAF^R double-resistant E. coli colonies are mostly bla^+, while all cat^+ colonies are bla^+/cat^+

The AMP^R/CAF^R double-resistant *E. coli* colonies were identified by growing AMP^R colonies on the LB medium containing 8, 16, 32 µg/mL CAF. In the IN-BIO samples, the AMP^R/CAF^R double-resistant *E. coli* colonies showed as 33%, 32% and 19%, respectively, and in the OUT-BIO samples they decreased to 17%, 19% and 17% (Table 3). Of these AMP^R/CAF^R double-resistant colonies, the bla^+ colonies were always more represented than the cat^+ colonies, and in both the IN-BIO and OUT-BIO samples at all of the antibiotic concentrations, all of the cat^+ colonies were also bla^+/cat^+ (Table 3, two last columns).

4. Discussion

The removal of coliforms and their antibiotic-resistant strains by wastewater treatment represents an important issue to improve health safety and to reduce the potential horizontal transfer of genetic resistance factors among bacteria. In this study, the presence of total coliforms and *E. coli* AMP^R and CAF^R strains was investigated in samples from a municipal WWTP. The single and double-resistance to AMP and CAF were examined in *E. coli* colonies and the responsible genes were investigated after amplification of the *bla* and *cat* gene sequences. Although other genes, such as the *mecA, bla-AmpC, bla-Oxa-1, bla-SHV-1*

285 and *cmlA, flo*, have been identified for AMP and CAF resistance, respectively (Giedraitienë et
286 al., 2011), the *bla-TEM-1* (Livermore, 1995) and *catA1* (Maynard et al., 2003) sequences were
287 chosen as the most representative-ones.
288 These data demonstrate that: (1) the AMP^R total *E. coli* are already selected for at 16 µg/mL
289 AMP; (2) among AMP^R coliforms, AMP^R *E. coli* colonies are well-represented, but not CAF^R
290 *E. coli*; (3) both AMP^R and CAF^R total coliforms and *E. coli* show a 2-log decrease in OUT-
291 BIO samples at all antibiotic concentrations; (4) by increasing AMP concentration, the
292 percentage of AMP^R *E. coli* colonies increases *versus* total coliforms and the percentage of
293 bla^+ *E. coli* increases in OUT-BIO samples; (5) AMP^R/CAF^R double-resistant *E. coli* colonies
294 are mostly bla^+ and all of the cat^+ colonies are both bla^+ and cat^+.
295 The remarkable decrease in TSS, COD and OD_{254} values supports the efficiency of the
296 biological treatment and filtration, and the low counts of *E.coli* in the OUT-DIS samples
297 confirm the effectiveness of the peracetic acid disinfection.
298 When the relative numbers of AMP^R *E. coli* colonies were compared to total *E. coli* at
299 the different treatment stages, there were no further decreases when the AMP was increased
300 from 16 µg/mL to 32 µg/mL, thus demonstrating that the selection for AMP^R colonies was
301 already present with 16 µg/mL AMP. Conversely, the low number of CAF^R bacteria at all
302 treatment stages hampered any conclusions for CAF resistance. It is important to note that,
303 although AMP^R bacteria have drastically diminished after the OUT-DIS treatment, no
304 substantial variation was noticed in the percentage of AMP^R colonies by the OUT-BIO
305 treatment compared to IN-BIO. Since the OUT-BIO treatment reduced by 3 log the number of
306 *E. coli*, the persistence of almost the same percentage of AMP^R bacteria seems to indicate that
307 no further selection occurs in spite of the activated sludge treatment. This finding seems to be
308 in contrast with the increase in antibiotic-resistant bacteria in biological reactors observed in
309 other studies (Berglund et al., 2015; Marathe et al., 2013; Rizzo et al., 2013; Rodriguez-

310 Mozaz et al., 2015; Zhang et al., 2009), although at present we have no clear explanation for
311 this discrepancy.

312 In AMP-treated samples, the percentage of *E. coli* colonies *versus* total coliforms
313 increased by increasing AMP concentration both in IN-BIO and OUT-BIO samples as well as
314 the percentage of *E. coli* carrying the *bla* resistance gene in OUT-BIO, as if *bla* was the gene
315 mostly responsible for resistance. In particular, the relative numbers of bla^+ colonies grown at
316 32 µg/mL increased through the biological treatment and filtration process (i.e., IN-BIO
317 *versus* OUT-BIO) from 78% to 91%, as if the retention of bacteria in the biological reactors
318 favours the selection of AMP^R bla^+ *E. coli*. It can be hypothesised that the *bla* gene sequence
319 is not only responsible for AMP resistance, but can also make *E. coli* more resistant to the
320 biological treatment process. Although CAF^R *E. coli* colonies increase by increasing the
321 antibiotic concentration, CAF^R *E.coli* colonies represent a low percentage among CAF^R
322 coliforms. However, *E. coli* CAF resistance seems to be due to the *cat* gene, although the
323 percentage of cat^+ *E. coli* decreases by increasing CAF concentration as if a selection may
324 occur of bacteria producing higher amounts of enzyme, able to make them resistant to higher
325 antibiotic concentrations. We can also hypothesize that the increase at 16 µg/mL of cat^+ *E.*
326 *coli* in OUT-BIO samples may be due to a selection in favour of cells carrying this resistance
327 gene.

328 The decrease in the percentage of AMP^R/CAF^R double-resistant colonies to around
329 50% in the OUT-BIO samples only at low CAF concentrations, but not at 32 µg/mL CAF,
330 seems to indicate that the biological treatment could not be more effective in reducing the
331 percentage of double-resistant colonies, already selected in the influent (i.e., IN-BIO samples)
332 at high AMP concentration. As expected, the percentage of AMP^R/CAF^R double-resistant cat^+
333 colonies also carried the *bla* gene sequence, as they were first selected for AMP resistance.

Overall, the biological process of this WWTP appears to be effective for the reduction of the numbers of AMP^R and CAF^R total coliforms and *E. coli*, by about 2-log units and *E. coli* colonies seem to be the most resistant as AMP^R *E. coli* colonies increase compared to total coliforms with increasing AMP concentration. The high percentage of AMP^R *E. coli versus* total *E. coli* in the influent (i.e., the IN-BIO samples) also seems to correlate with the more than twenty hospitals and clinics in the Milan area from which the plant receives the wastewaters (Pizza, 2014)

Moreover, although the biological treatment selected for similar levels of AMP^R and CAF^R total coliforms, a prevalence of AMP^R *versus* CAF^R *E. coli* colonies was observed. AMP resistance was related to the presence of the *bla* gene, the product of which promotes hydrolysis of the β-lactam ring of AMP, whereas lower levels of cat^+ *E. coli* colonies were found, which suggests that CAF resistance might be related to mechanisms that do not involve the *cat* gene. The higher levels of AMP^R than CAF^R *E. coli* colonies *versus* total AMP^R coliforms can be explained by the more generalised use of β-lactam antibiotics (e.g., AMP) *versus* CAF in the treatment of humans and animals for prevention and control of bacterial diseases. In Europe, CAF was banned in the veterinary field for the treatment of food-producing animals in 1994 and its use is now limited to pets, whereas it is still indicated in humans for the therapy of a small number of life-threatening infections (Schwartz et al., 2006). Our data also show that the AMP^R and AMP^R/CAF^R double-resistant *E. coli* colonies are related to the presence of the *bla* gene, which is in agreement with the most frequent mechanism of AMP resistance in clinically relevant Gram-negative bacteria (Bush and Jacoby, 2010).

The extensive use of antibiotics in both human and veterinary medicine can promote resistance inside WWTPs, where bacteria can be exposed to antibiotic doses before being released into the aquatic environment and the increase of ARBs into the environment may be

favoured by inefficiently treated domestic and hospital wastewaters (Pruden et al., 2012). As already shown by other studies (Rodriguez-Mozaz et al., 2015), we have demonstrated that WWTPs can reduce ARB concentration, but our results seem to indicate that the percentage of AMP^R bacteria remains almost the same after the biological treatment, and thus does not increase AMP^R bacteria although a selection for the *bla* gene seems to occur. However, after the disinfection process ARB removal is almost complete and the number of the residual bacteria is compatible with regulatory guidelines for the irrigation use of treated waters.

Notes

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373 **References**

374

375 The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for
376 interpretation of MICs and zone diameters. Version 6.0, 2016. http://www.eucast.org. 2016.

377

378 Alanis, A.J., 2005. Resistance to antibiotics: are we in the post-antibiotic era? Arch. Med.
379 Res. 36**,** 697-705.

380 Azzellino, A., Antonelli, M., Canziani, R., Malpei, F., Marinetti, M., Nurizzo, C., 2011.
381 Multivariate Modelling of Disinfection Kinetics: A Comparison Among Three Different
382 Disinfectants. Desalination and Water Treatment 29**,** 128-139.

383 Baquero, F., Martínez, J.-L., Cantón, R., 2008. Antibiotics and antibiotic resistance in water
384 environments. Curr. Opin. Biotechnol. 19**,** 260-265.

385 Baraniak, A., Fiett, J., Mro´wka, A., Walory, J., Hryniewicz, W., Gniadkowski, M., 2005.
386 Evolution of TEM-Type Extended-Spectrum _-Lactamases in Clinical Enterobacteriaceae
387 Strains in Poland. Antimicrob. Agents Chemother. 49**,** 1872-1880.

388 Berglund, B., Fick, J., Lindgren, P.E., 2015. Urban wastewater effluent increases antibiotic
389 resistance gene concentrations in a receiving northern European river. Environ. Toxicol.
390 Chem. 34**,** 192-196.

391 Bush, K., Jacoby, G.A., 2010. Updated functional classification of beta-lactamases.
392 Antimicrob. Agents Chemother. 54**,** 969-976.

393 Choffnes, E.R., Relman, D.A., Mack, A. Antibiotic Resistance: Implications for Global
394 Health and Novel Intervention Strategies: Workshop Summary. National Academies Press.
395 2011.
396

397 Chow, L., Waldron, L., Gillings, M.R., 2015. Potential impacts of aquatic pollutants: sub-
398 clinical antibiotic concentrations induce genome changes and promote antibiotic resistance.
399 Front. Microbiol. 6**,** 803.

400 Dodd, M.C., 2012. Potential impacts of disinfection processes on elimination and deactivation
401 of antibiotic resistance genes during water and wastewater treatment. J. Environ. Monit. 14**,**
402 1754-1771.

403 Fekadu, S., Merid, Y., Beyene, H., Teshome, W., ebre-Selassie, S., 2015. Assessment of
404 antibiotic- and disinfectant-resistant bacteria in hospital wastewater, south Ethiopia: a cross-
405 sectional study. J. Infect. Dev. Ctries 9**,** 149-156.

406 Giedraitienë, A., Vitkauskienë, A., Naginienë, R., Pavilonis, A., 2011. Antibiotic resistance
407 mechanisms of clinically important bacteria. Medicina (kaunas) 47**,** 137-146.

408 Gullberg, E., Cao, S., Berg, O.G., Ilback, C., Sandegren, L., Hughes, D., Andersson, D.I.,
409 2011. Selection of resistant bacteria at very low antibiotic concentrations. PLoS Pathog. 7.

410 Keen, P.L., Montforts, M.H.M.M., 2012. Antimicrobial resistance in the environment. Wiley-
411 Blackwell.

412 Kim, S., Aga, D.S., 2007. Potential ecological and human health impacts of antibiotics and
413 antibiotic-resistant bacteria from wastewater treatment plants. J. Toxicol. Environ. Health B.
414 Crit. Rev. 10**,** 559-573.

415 Kümmerer, K., 2009a. Antibiotics in the aquatic environmentea reviewepart I. Chemosphere
416 75**,** 417-434.

417 Kümmerer, K., 2009b. Antibiotics in the aquatic environmentea reviewepart II. Chemosphere
418 75**,** 435-441.

419 Livermore, D.M., 1995. β-Lactamases in Laboratory and Clinical Resistance. Clin. Microbiol.
420 Rev. 8**,** 557-584.

421 Marathe, N.P., Regina, V.R., Walujkar, S.A., Charan, S.S., Moore, E.R.B., Larsson, D.G.J.,
422 Shouche, Y.S., 2013. A treatment plant receiving waste water from multiple bulk drug
423 manufacturers is a reservoir for highly multi-drug resistant integron-bearing bacteria. PLoS
424 One 8**,** e77310.

425 Marti, E., Variatza, E., Balcazar, J.L., 2014. The role of aquatic ecosystems as reservoirs of
426 antibiotic resistance. Trends Microbiol. 22**,** 36-41.

427 Maynard, C., Fairbrother, J.M., Bekal, S., Sanschagrin, F., Levesque, R.C., Brousseau, R.,
428 Masson, L., Lariviere, S., Harel, J., 2003. Antimicrobial Resistance Genes in Enterotoxigenic
429 Escherichia coli O149:K91 Isolates Obtained over a 23-Year Period from Pigs. Antimicrob.
430 Agents Chemother. 47**,** 3214-3221.

Meredith, H.R., Srimani, J.K., Lee, A.J., Lopatkin, A.J., You, L., 2015. Collective antibiotic tolerance: mechanisms, dynamics and intervention. Nat. Chem. Biol. 11**,** 182-188.

Mezzanotte, V., Antonelli, M., Citterio, S., Nurizzo, C., 2007. Wastewater Disinfection Alternatives: Chlorine, Ozone, Peracetic Acid and UV Light. Water Environ. Res. 79**,** 2373-2379.

O'Neill, J. Tackling drug-resistant infections globally: final report and recommendations. The review on antimicrobial resistance. 2016.

Pizza, F., 2014. Agricultural reuse of treated wastewater: the case of Milano-Nosedo municipal wastewater treatment plant. In: ECI Symposium Series (Ed.), Wastewater and Biosolids Treatment and Reuse: Bridging Modeling and Experimental Studies.

Proia, L., Von Schiller, D., Sànchez-Melsiò, A., Sabater, S., Borrego, C.M., Rodríguez-Mozaz, S., LuisBalcàzar, J., 2016. Occurrence and persistence of antibiotic resistance genes in river biofilms after wastewater inputs in small rivers. Environ. Pollut. 210**,** 121-128.

Pruden, A., 2014. Balancing water sustainability and public health goals in the face of growing concerns about antibiotic resistance. Environ. Sci. Technol. 48**,** 5-14.

Pruden, A., Arabi, M., Storteboom, H.N., 2012. Correlation between upstream human activities and riverine antibiotic resistance genes. Environ. Sci. Technol. 46**,** 11541-11549.

449 Rice, E.W., Baird, R.B., Eaton, A.D., Clesceri, L.S., 2012. Standard Methods for the
450 Examination of Water and Wastewater. American Public Health Association/American Water
451 Works Association/Water Environment Federation.

452 Richardson, S.D., Ternes, T.A., 2014. Water analysis: emerging contaminants and current
453 issues. Anal. Chem. 86**,** 2813-2848.

454 Rizzo, L., Manaia, C., Merlin, C., Schwartz, T., Dagot, C., Ploy, M.C., Michael, I., Fatta-
455 Kassinos, D., 2013. Urban wastewater treatment plants as hotspots for antibiotic resistant
456 bacteria and genes spread into the environment: a review. Sci. Total Environ. 447**,** 345-360.

457 Rodriguez-Mozaz, S., Chamorro, S., Marti, E., Huerta, B., Gros, M., Sanchez-Melsio, A.,
458 Borrego, C., Barcelo, D., Balcazar, J.L., 2015. Occurrence of antibiotics and antibiotic
459 resistance genes in hospital and urban wastewaters and their impact on the receiving river.
460 Water Res. 69**,** 234-242.

461 Roose-Amsaleg, C., Laverman, A.M., 2015. Do antibiotics have environmental sideeffects?
462 Impact of synthetic antibiotics on biogeochemical processes. Environ. Sci. Pollut. Res. 1-13.

463 Schwartz, T., Kohnen, W., Jansen, B., Obst, U., 2006. Detection of antibiotic-resistant
464 bacteria and their resistance genes in wastewater, surface water and drinking water biofilms.
465 FEMS Microbiol. Ecol. 43**,** 325-355.

466 Sharma, V.K., Johnson, N., Cizmas, L., McDonald, T.J., Kim, H., 2016. A review of the
467 influence of treatment strategies on antibiotic resistant bacteria and antibiotic resistance genes.
468 Chemosphere 150**,** 702-714.

469 Sharma, V.K., Liu, F., olan, S., ohn, M., im, H., turan, M.A., 2013. Oxidation of beta-lactam
470 antibiotics by ferrate(VI). Chem. Eng. J. 221**,** 446-451.

471 Silbergeld, E.K., Graham, J., Price, L.B., 2008. Industrial food animal production,
472 antimicrobial resistance, and human health. Ann. Rev. Pub. Health 29**,** 151-169.

473 Sykes, R.B., Matthew, M., 1976. The ß lactamases of Gram negative bacteria and their role in
474 resistance to ß lactam antibiotics. J. Antimicrob. Chemother. 2**,** 115-157.

475 United States Environmental Protection Agency. Alternative disinfection methods fact sheet:
476 peracetic acid. 2012.
477

478 Watkinson, A.J., Micalizzi, G.B., Graham, G.M., Bates, J.B., Costanzo, S.D., 2007.
479 Antibiotic-resistant Escherichia coli in wastewaters, surface waters, and oysters from an urban
480 riverine system. Appl. Environ. Microbiol. 73**,** 5667-5670.

481 Yuan, Q.B., Guo, M.T., Yang, J., 2015. Fate of antibiotic resistant bacteria and genes during
482 wastewater chlorination: implication for antibiotic resistance control. PLoS One 10.

483 Zhang, Y., Marrs, C.F., Simon, C., Xi, C., 2009. Wastewater treatment contributes to selective
484 increase of antibiotic resistance among Acinetobacter spp. Sci. Total Environ. 407**,** 3702-
485 3706.

Zhang, Y., Zhuang, Y., Geng, J., Ren, H., Zhang, Y., Ding, L., Xu, K., 2015. Inactivation of antibiotic resistance genes in municipal wastewater effluent by chlorination and sequential UV/chlorination disinfection. Sci. Total Environ. 512/513**,** 125-132.

Figure legends

Figure 1. WWTP scheme and sample collection points.

Figure 2. AMP^R and CAF^R *E. coli* colonies *versus* total *E. coli* colonies. The AMP^R (A) and CAF^R (B) *E. coli* colony numbers were determined at the different antibiotic concentrations, and are expressed as percentages of resistant *E. coli* colonies compared to total *E. coli* that grew on the chromogenic medium in the absence of the antibiotics. AMP^R E. coli colonies are already selected at 16 µg/mL AMP. No significant decrease in the percentage of ARBs is observed through the biological treatment and filtration (OUT-BIO *versus* IN-BIO samples), whereas disinfection significantly reduced the AMP^R *E. coli* colonies (OUT-DIS *vs* OUT-BIO, $p < 0.05$) at the lowest AMP concentration (8 µg/mL). Bars represent the standard deviation.

Figure 3. Representative electrophoresis gels for the PCR-amplified *bla* and *cat* gene sequences of the AMP^R and CAF^R *E. coli* colonies. The *bla* gene fragment appears as a 721-bp DNA band (A) and the *cat* gene fragment as a 630-bp DNA band (B). (+), positive colonies; (-), negative colonies; M, molecular weight markers. The pcDNA3-CAT plasmid was used as the positive controls for *bla* (C1) and *cat* (C2), respectively.

Table 1.

Main physicochemical characteristics of the wastewater according to the sampling points.

Sample	**Total suspended solids (TSS)** (mg/L)	**Chemical oxygen demand (COD)** (mg/L)	**Optical density (OD_{254})**
IN-BIO	75	162	0.40
OUT-BIO	3	12	0.07
OUT-DIS	3	<10	0.08

Table 2A.

AMP^R total coliform and *E. coli* colonies grown on LB agar in the presence of AMP.

Antibiotic (µg/mL)	Sample	Total coliforms (CFU /100 mL)	Total coliforms OUT-BIO *vs.* IN-BIO (% reduction)	*E. coli* (CFU /100 mL)	*E. coli* *vs.* total coliforms (%)	bla^+ *E. coli* (CFU /100 mL)	bla^+ *E. coli* *vs.* total *E. coli* (%)
AMP 8	IN-BIO	1.74×10^6	na	6.0×10^5	34	5.8×10^5	97
	OUT-BIO	2.25×10^4	98.7	6.0×10^3	27	4.8×10^3	80
AMP 16	IN-BIO	1.10×10^6	na	7.4×10^5	67	6.8×10^5	92
	OUT-BIO	1.56×10^4	98.6	6.2×10^3	40	5.4×10^3	87
AMP 32	IN-BIO	0.95×10^6	na	7.3×10^5	77	5.6×10^5	78
	OUT-BIO	1.19×10^4	98.7	7.1×10^3	60	6.4×10^3	91

Table 2B.

CAF^R total coliform and *E. coli* colonies grown on LB agar in the presence of CAF.

Antibiotic (µg/mL)	Sample	Total coliforms (CFU /100 mL)	Total coliforms OUT-BIO *vs.* IN-BIO (% reduction)	*E. coli* (CFU /100 mL)	*E. coli* *vs.* total coliforms (%)	cat^+ *E. coli* (CFU /100 mL)	cat^+ *E. coli* *vs.* total *E. coli* (%)
CAF 8	IN-BIO	4.58×10^6	na	5.0×10^5	11	nd	nd
	OUT-BIO	4.80×10^4	98.9	1.0×10^3	2	nd	nd
CAF 16	IN-BIO	3.28×10^6	na	2.0×10^5	6	1.0×10^5	50
	OUT-BIO	4.72×10^4	98.6	3.0×10^3	6	2.0×10^3	67
CAF 32	IN-BIO	0.80×10^6	na	2.1×10^5	26	0.8×10^5	38
	OUT-BIO	1.00×10^4	98.8	1.5×10^3	15	0.5×10^3	33

na, not applicable

nd, not determined

525 **Table 3**.

526 AMP^R/CAF^R double-resistant *E. coli* colonies first selected on LB agar with AMP and then in

527 LB agar with CAF at the same antibiotic concentration.

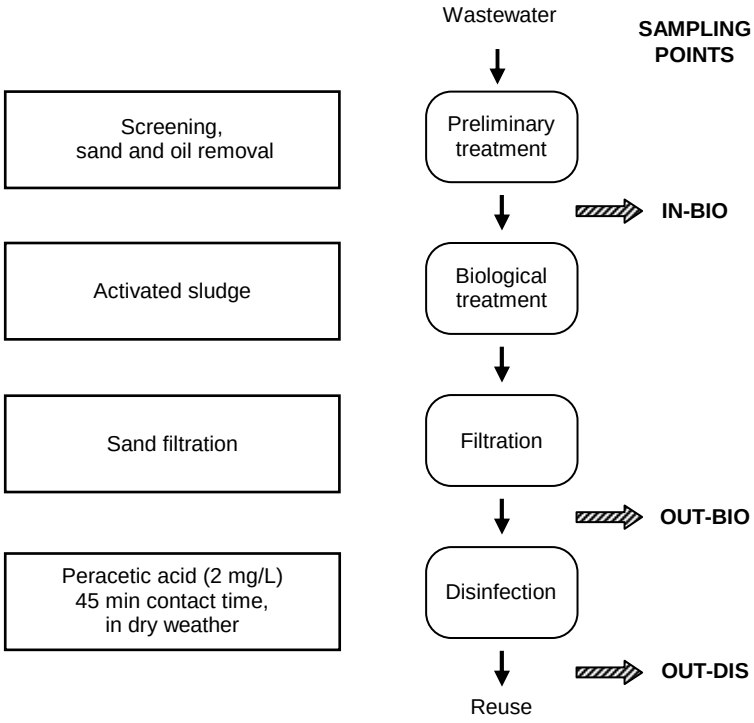
528

Antibiotics (µg/mL)	**Sample**	**AMP^R/CAF^R *vs.* AMP^R (%)**	***bla*$^+$ *vs.* AMP^R/CAF^R (%)**	***cat*$^+$ *vs.* AMP^R/CAF^R (%)**	***bla*$^+$/*cat*$^+$ *vs.* AMP^R/CAF^R (%)**
CAF 8	IN-BIO	$2.0x10^5$ (33)	$2.0x10^5$ (100)	$0.4x10^5$ (20)	$0.4x10^5$ (20)
	OUT-BIO	$1.0x10^3$ (17)	$1.0x10^3$ (100)	$0.6x10^3$ (60)	$0.6x10^3$ (60)
CAF 16	IN-BIO	$2.4x10^5$ (32)	$2.2x10^5$ (92)	$0.8x10^5$ (33)	$0.8x10^5$ (33)
	OUT-BIO	$1.2x10^3$ (19)	$1.0x10^3$ (83)	$0.8x10^3$ (66)	$0.8x10^3$ (66)
CAF 32	IN-BIO	$1.4x10^5$ (19)	$1.2x10^5$ (86)	$0.2x10^5$ (29)	$0.2x10^5$ (29)
	OUT-BIO	$1.2x10^3$ (17)	$1.2x10^3$ (100)	$0.8x10^3$ (66)	$0.8x10^3$ (66)

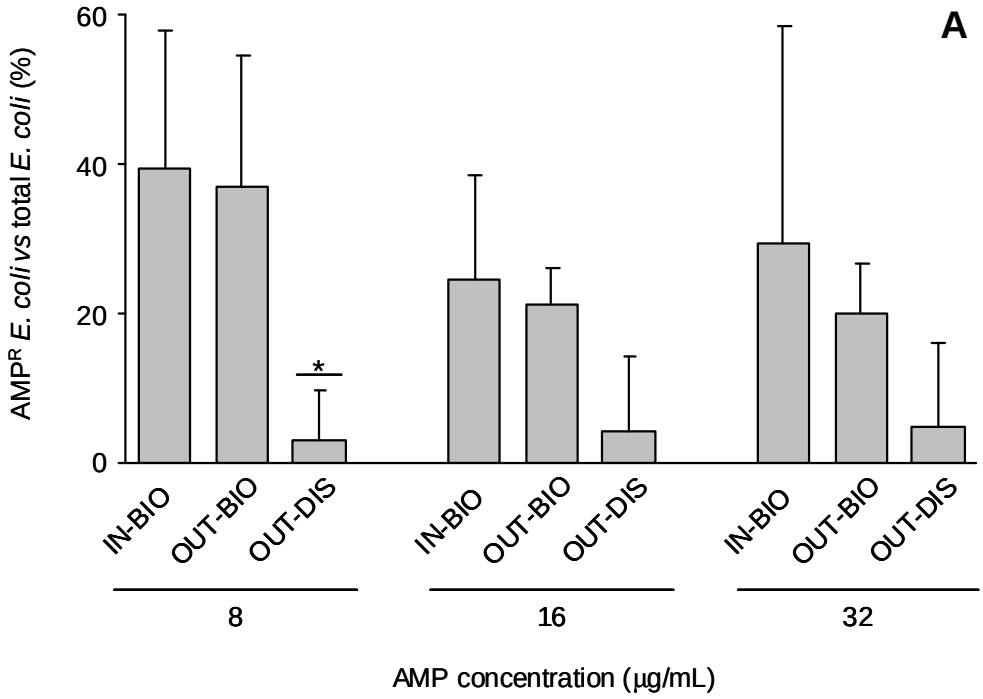
529

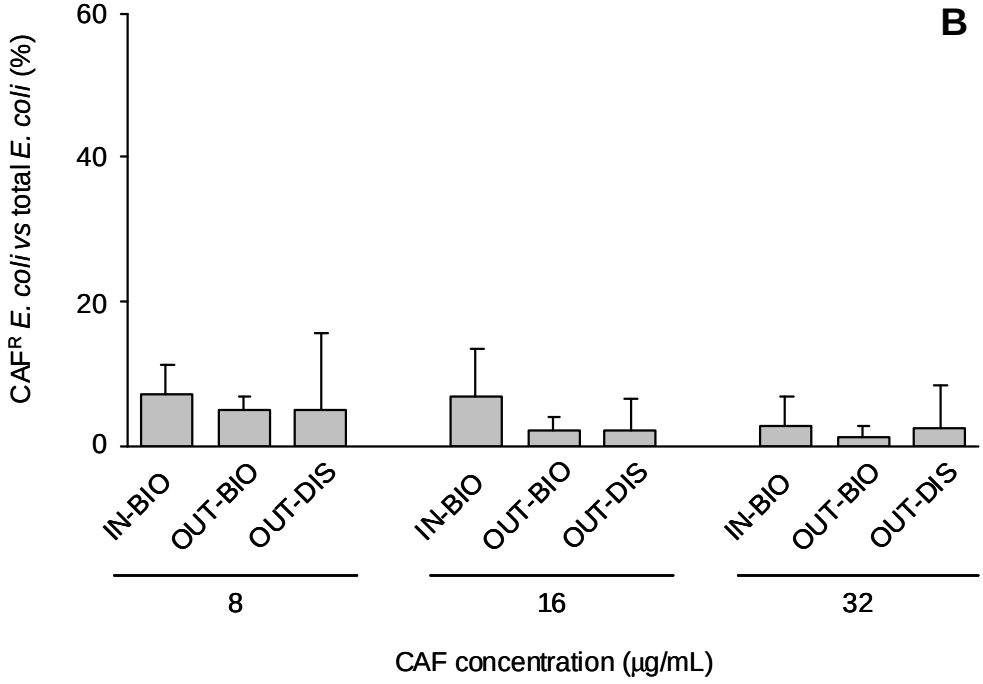
530

Fig. 1

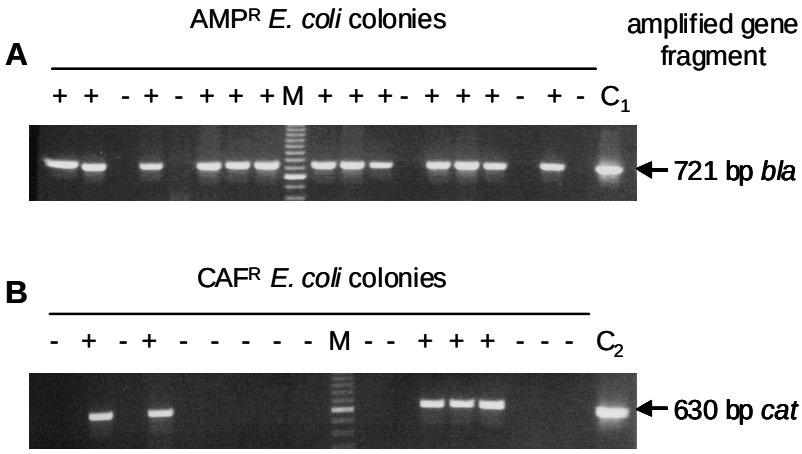

Wastewater
SAMPLING POINTS
Screening, sand and oil removal
Preliminary treatment
IN-BIO
Activated sludge
Biological treatment
Sand filtration
Filtration
OUT-BIO
Peracetic acid (2 mg/L) 45 min contact time, in dry weather
Disinfection
OUT-DIS
Reuse

531


A
60
40
20
0
AMP^R E. coli vs total E. coli (%)
*
IN-BIO
OUT-BIO
OUT-DIS
IN-BIO
OUT-BIO
OUT-DIS
IN-BIO
OUT-BIO
OUT-DIS
8
16
32
AMP concentration (µg/mL)


B
60
40
20
0
CAF^R E. coli vs total E. coli (%)
IN-BIO
OUT-BIO
OUT-DIS
IN-BIO
OUT-BIO
OUT-DIS
IN-BIO
OUT-BIO
OUT-DIS
8
16
32
CAF concentration (µg/mL)

532


AMP^R E. coli colonies
amplified gene fragment
A
+ + - + - + + + M + + + - + + + - + - C1
721 bp bla
CAF^R E. coli colonies
B
- + - + - - - - - M - - + + + - - - C2
630 bp cat