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Review

Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: A review

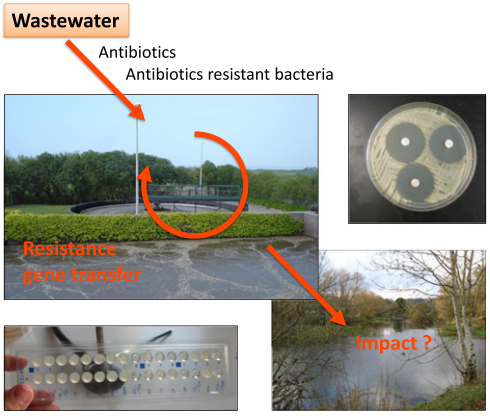
L. Rizzo [a,*], C. Manaia [b], C. Merlin [c], T. Schwartz [d], C. Dagot [e], M.C. Ploy [f], I. Michael [g], D. Fatta-Kassinos [g]

[a] Department of Civil Engineering, University of Salerno, 84084, Fisciano (SA), Italy
[b] CBQF/Escola Superior de Biotecnologia, Universidade Católica Portuguesa, R. Dr. António Bernardino de Almeida, 4200-072, Porto, Portugal
[c] Laboratoire de Chimie Physique et Microbiologie pour l'Environnement (LCPME), UMR 7564 CNRS-Université de Lorraine, 15 Avenue du Charmois, 54500 Vandoeuvre-les-Nancy, France
[d] Department of Microbiology at Interfaces, Karlsruhe Institute of Technology (KIT) – Northern Campus, Institute of Functional Interfaces (IFG), D-76344 Eggenstein-Leopoldshafen, Germany
[e] Université of Limoges, EA4330, GRESE, ENSIL, F87000, Limoges, France
[f] Université de Limoges, INSERM, UMR S1092, F-87000, Limoges, France
[g] Nireas, International Water Research Centre, University of Cyprus, 1 Panepistimiou Avenue, P.O Box 20537, 1678 Nicosia, Cyprus

HIGHLIGHTS

- UWTPs may positively affect ARB spread and selection as well as ARG transfer.
- Resistance integrons may be used to characterize ARG transfer.
- High trough technologies are a useful complementation of PCR technologies.
- Biological process effect on ARB and ARG transfer should be further investigated.
- Advanced treatments/disinfection effect should be further investigated too.

GRAPHICAL ABSTRACT



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ABSTRACT

Urban wastewater treatment plants (UWTPs) are among the main sources of antibiotics' release into the environment. The occurrence of antibiotics may promote the selection of antibiotic resistance genes (ARGs) and antibiotic resistant bacteria (ARB), which shade health risks to humans and animals. In this paper the fate of ARB and ARGs in UWTPs, focusing on different processes/technologies (i.e., biological processes, advanced treatment technologies and disinfection), was critically reviewed. The mechanisms by which biological processes influence the development/selection of ARB and ARGs transfer are still poorly understood. Advanced treatment technologies and disinfection process are regarded as a major tool to control the spread of ARB into the environment. In spite of intense efforts made over the last years to bring solutions to control antibiotic resistance spread in the environment, there are still important gaps to fill in. In particular, it is important to: (i) improve risk assessment studies in order to allow accurate estimates about the maximal abundance of ARB in UWTPs effluents that would not pose risks for human and environmental health; (ii) understand the factors and mechanisms that drive antibiotic resistance maintenance and selection in wastewater habitats. The final objective is to implement wastewater treatment technologies capable of assuring the production of UWTPs effluents with an acceptable level of ARB.

* Corresponding author. Tel.: +39 089 969334; fax: +39 089 969620.
E-mail address: l.rizzo@unisa.it (L. Rizzo).

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Contents

1. Introduction

The intensive use of antibiotics for human, veterinary and agricultural purposes, results in their continuous release into the environment (Batt et al., 2006; Brown et al., 2006; Díaz-Cruz et al., 2003; Kümmerer, 2009). The main concern for the release of antibiotics into the environment is related to the development of antibiotic resistance genes (ARGs) and bacteria (ARB), which reduce the therapeutic potential against human and animal pathogens (Kemper, 2008; X.X. Zhang et al., 2009b). The increasing interest of the scientific community and international institutions/organization for this problem is respectively testified by the number of publications reviewed in this paper and internationally/scientifically relevant initiatives, such as research projects (e.g., PHARMAS) and networking (e.g., COST Action DARE).

Effluents from urban wastewater treatment plants (UWTPs) are suspected to be among the main anthropogenic sources for antibiotics (no maximum contaminant levels have been set by EU and other international institutions/organizations), ARGs and ARB spread into the environment (Ferreira da Silva et al., 2006; Figueira et al., 2011a; Kümmerer, 2009; Lupo et al., 2012). The biological treatment process creates an environment potentially suitable for resistance development and spread because bacteria are continuously mixed with antibiotics at sub-inhibitory concentrations (Auerbach et al., 2007; Davies et al., 2006; Ferreira da Silva et al., 2006). The knowledge regarding the effects of sub-inhibitory concentrations of anti-microbials and their effects on environmental bacteria, is scarce and contradictory especially with respect to resistance (Kümmerer, 2009).

ARBs are mostly studied in bacteria belonging to the common indicators of faecal contamination, namely coliforms and enterococci (Araújo et al., 2010; Boczek et al., 2007; Figueira et al., 2011a; Martins da Costa et al., 2006; Reinthaler et al., 2003; Sabate et al., 2008). The search for agents associated with human "difficult-to-treat infections" such as methicillin resistant *Staphylococcus aureus*, vancomycin resistant *Entereococcus* spp., and Gram-negative bacteria (enterobacteria, pseudomonads, acinetobacter) resistant to fluoroquinolones, carbapenems and producers of extended spectrum beta-lactamase, has also been addressed in such studies. Although at low percentages, when compared to what is observed in the clinical settings, antibiotic resistance profiles are often detected in wastewaters and recreational waters; a fact that may represent a relevant public health issue (Araújo et al., 2010; Soge et al., 2009). However, the current knowledge on the prevalence and types of antibiotic resistance in the environment is barely sufficient. In contrast to what has been done over the last years in the standardization and organization of antibiotic resistance data of clinical origin (ECDC, 2012; EUCAST, 2011), information regarding resistance of environmental bacteria is still very fragmented.

UWTPs typically include different processes (e.g., mechanical, biological, physical, chemical and physical–chemical) which may affect the fate of antibiotics, ARB and ARGs in different ways and consequently the development and spread of resistance into the environment. Accordingly, the aim of the present paper is to critically review the fate of ARB and ARGs in UWTPs. In particular, the effect of different processes/technologies, namely biological processes, advanced treatment technologies and disinfection, is addressed. Moreover, methods for the characterization/evaluation of ARB, ARGs and genes transfer are presented and discussed in a comprehensive way to facilitate the understanding of the effect of wastewater treatment processes/technologies on ARB and ARGs.

2. Tools to assess antibiotic resistance in UWTPs

The relationship existing between antibiotic consumption and the emergence and development of resistances is now well documented (Davies, 2007). If the acquisition of mutations widely contributes to bacterial adaptation, the exchange and reshuffling of genetic material between bacteria involving mobile genetic elements are the main contributors explaining the rapid dissemination of antibiotic resistances (Courvalin, 2008). Identifying hotspots of possible ARG dissemination starts by determining the relative occurrence of ARB or ARG in the wastewater network. At this stage, two main approaches are followed, either culture-based or molecular-based, each of them exhibiting specific advantages and drawbacks. Lately the molecular-based approaches have been extended to the detection of genetic structures involved in ARG capture, namely integrons, enhancing the understanding of ARB and ARG dynamics in complex anthropogenic environments. Despite the recent progresses, demonstrating ARG transfer in UWTP remains a difficult task that should be studied on a case-by-case basis. Before addressing the effect of wastewater treatment processes on ARB and ARGs, the methods and tools for characterizing antibiotic resistance and genes transfer in the aquatic environment are presented first.

2.1. Antibiotic resistance characterization by cultivation-based methods

Antibiotic resistance testing is highly standardized worldwide, enabling laboratories to assist clinicians in bacterial infection therapy

(CLSI, 2007; WHO, 2008; Wiegand et al., 2008). Given the nature of the samples and diversity of bacteria involved, numerous modifications have been introduced in order to achieve reliable and accurate methods with a feasible application to surface or wastewaters (Galvin et al., 2010; Łuczkiewicz et al., 2010; Novo and Manaia, 2010; Watkinson et al., 2007). Although culture-independent approaches have been used, the determination of prevalence values and resistance patterns is more frequently based on culture-dependent methods (Czekalski et al., 2012; LaPara et al., 2011; Szczepanowski et al., 2009). These methods, although with several adaptations, are supported by guidelines developed for clinical and veterinary microbiology (e.g. EUCAST, 2011; CLSI; CA-SFM, 2011; Andrews, 2009; DANMAP, 2011). The membrane filtration method, commonly used for water microbiological analysis, is frequently adapted to isolate bacteria for further characterization of antibiotic resistance (APHA, 2005). The use of selective culture media allows the enumeration and isolation of specific bacterial groups. Mostly for this reason, the microbiological indicators of water quality coliforms and enterococci, are frequently the major targets of such analyses (APHA, 2005; EC, 1998). After purification, isolates can be identified and typed for their antibiotic resistance patterns, allowing the calculation of resistance rates or the definition of resistance profiles. Antibiotic resistance testing is frequently based on the disc diffusion or micro-dilution methods, which, according to standardized values, allow the distinction between resistant and susceptible organisms (e.g. CLSI, 2007; EUCAST, 2011). All procedures to assess antibiotic resistance susceptibility by disc diffusion or micro-dilution methods need to follow highly standardized conditions, available to the public (e.g. ECDC, 2012; EUCAST, 2011). In general, culture-based methods are highly laborious and time consuming (Ferreira da Silva et al., 2007; Figueira et al., 2011a,b; Łuczkiewicz et al., 2010; Martins da Costa et al., 2006), a fact that gave space for the development of easier alternatives, more adequate when routine monitoring analyses are to be implemented. One of such adaptations involves the use of selective culture media supplemented with antibiotics at concentrations similar to or above those reported as inhibitory for the target bacteria. In this case, the percentage of resistance can be estimated as the ratio between the number of bacteria growing in the presence and in the absence of antibiotic (Figueira et al., 2011b; Novo and Manaia, 2010; Watkinson et al., 2007). Watkinson et al. (2007), who originally proposed this method to estimate the prevalence of resistance of *E. coli* to ampicillin, tetracycline, ciprofloxacin and sulfamethoxazole, concluded about its great potential for a representative assessment of antibiotic resistance in *E. coli*, allowing the rapid characterization of a large number of samples. Novo and Manaia (2010) adapted this method to assess the resistance prevalence to amoxicillin, tetracycline and ciprofloxacin in heterotrophs, enterobacteria and enterococci, since it was a feasible approach to compare the resistance loads in the inflow and outflow of three UWTPs. This method also promotes the enrichment of ARB, and therefore can help on the detection of resistance genetic determinants. For example, in the work of Figueira et al. (2011b), characterizing quinolone resistance in Aeromonas isolated from water habitats, the gene *aac*A6-ib-cr was detected mainly in bacteria isolated in the presence of ciprifloxaxcin.

2.2. Molecular biology methods to study the diversity and the abundance of ARB/ARG in UWTPs

Molecular biology methods have been used for several years for the examination of water of different origins and qualities (Alexandrino et al., 2007; Szewzyk et al., 2000; Wimpeny et al., 2000). Molecular methods can be used to detect microorganisms that cannot be grown in the laboratory or that multiply very slowly but contribute to the resistance in specific compartments (Oliver, 2005, 2010; Trevors, 2011). In many applications, molecular methods are of limited use due to the lack of standardization and validation by round robin tests.

On the basis of the prevailing assumption that gene carriage equals resistance, genetic tests aim at detecting resistance genes or genetic elements contributing to horizontal gene transfer in bacterial isolates and communities by using DNA probes or PCR methods. Genetics-based tests are more accurate than antibiograms in tracing the epidemiological spread of relevant resistance genes in a hospital or wastewater community setting since beside phylogenetic DNA markers other genetic markers like antibiotic resistance and virulence genes could be detected in parallel. Conventional microbiology as well as molecular biology methods can be complementary for presence/absence analyses of pathogens and their genetic targets of antibiotic resistances (Fig. 1).

Furthermore, natural habitats harbour many non-culturable bacteria or bacteria of different physiological states, which limit cultivation-based quantitative analyses. Molecular biology techniques are applied to identify specific DNA targets without prior cultivation of their organisms. Quantitative PCR (qPCR) assays tracking various resistance genes originating from different pathogens revealed their spread in clinical and municipal wastewater systems, and their occurrence in surface water and drinking water biocoenoses (Lupo et al., 2012; Volkmann et al., 2004). Many studies have demonstrated the presence of clinically-relevant antibiotic resistance genes along the water cyle, with potential hotspots of wastewater discharge into other aquatic environments (Schwartz et al., 2006, 2003; Volkmann et al., 2004; Y. Zhang et al., 2009). As an example, the methicillin resistance gene *mecA* of staphylococci, the ß-lactam resistance gene *ampC* of *Enterobacteriaceae*, the carbapenem resistance gene *blaVIM* of *Pseudomonas aeruginosa*, and the vancomycin resistance gene *vanA* of enterococci as well as taxon-specific ribosomal DNA sequences for enterococci and *P. aeruginosa* have been chosen as targets to quantify their abundances in defined amounts of DNA extracts of different wastewater matrices (Schwartz, 2012; Volkmann et al., 2004). The antibiotic resistance genes *ampC*, *vanA* and *blaVIM* detected in wastewater samples of three German cities (Schwartz, 2012) are summarized in Table 1.

In case of the ampicillin resistance gene *amp*C the abundance of the gene in 100 ng of total DNA isolated from the whole community was found to increase in urban wastewater as well as wastewaters receiving hospital effluents (Volkmann et al., 2004). In the cases of the vancomycin and imipenem resistance gene detection, it was revealed that their occurrence is linked with hospital wastewater due to the ARG abundances (Volkmann et al., 2004). Moreover, multi-resistant *P. aeruginosa* were frequently found in hospital wastewaters and in low abundances in wastewater from housing areas indicating hospitals

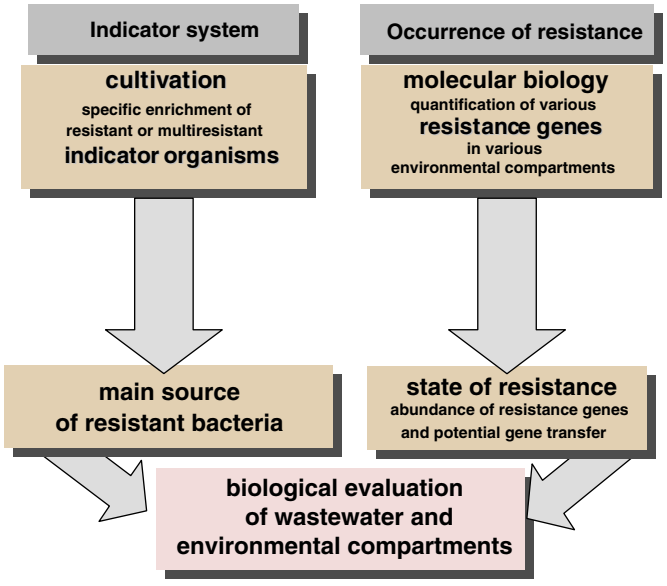

Fig. 1. Microbiological evaluation of resistance situations in wastewaters and environment.

as one hotspot for the dissemination of antibiotic resistant clinically-relevant bacteria (Schwartz, 2012). Since DNA is extracted from the whole community, followed by qPCR, this approach is useful for the detection and quantification of antibiotic resistance and pathogen targets even of non-culturable fractions of populations (Khan and Yadav, 2004). Extensive data of bacterial genes support a suitable primer and probe design for qPCR (X.X. Zhang et al., 2009a,b). In case of under-represented ARG in the metagenome of complex microbial communities, the process of nucleic acid extraction and its quality are very crucial for the subsequent PCR gene detection (Volkmann et al., 2007). Wastewater samples with a variety of undefined natural and anthropogenic substances appear to be difficult matrices for a microbiological analysis at the nucleic acid level. Despite of the specific extraction and purification techniques, inhibitors like humic substances, organic salts or detergents disturb the detection of specific bacterial species and functional genes (Volkmann et al., 2007; Weyant et al., 1990). Furthermore, even small numbers of specific DNA targets have to be detected in highly concentrated total DNA with unknown quantities of similar and dissimilar sequences. Apart from the exigency of an effective extraction method that reduces interfering impurities, further interactions of the sample matrix DNA with the PCR have to be considered when qPCR is applied to wastewater samples.

In order to evaluate the applicability of a number of primer and probe sets for qPCR on DNA extracted from wastewater communities, the effects of both the sample matrix and the DNA background on target quantification were studied and target detections quantifiable along up to seven decimal orders of magnitude were demonstrated (Volkmann et al., 2007). Therefore, the implementation of these PCR based sequence detection systems on wastewater samples using qPCR, is strongly influenced by varying properties of the sample matrices like chemical ingredients, microorganism population composition and concentration, salinity or pH (Gibson et al., 2012; Weyant et al., 1990). Reduced detection sensitivity in environmental samples may produce an underestimation of the amount of initial targets or false negative results. Thus, as a measure of PCR quality, the amplification efficiency must be determined by spiking the sample matrix with known concentrations of DNA templates (Volkmann et al., 2007).

New molecular high throughput (HTP) technologies are valuable tools to study natural microbial communities, their diverse genomic capacities, and gene expression activities (next generation sequencing). It has to be mentioned that metagenome and transcriptome analyses via next generation sequencing analyses need databank entries of reference bacteria genomes to compare gene sequences and gene activities. Such bacteria reference data are still limited to few isolates (mostly from clinical origin). In consequence, reference data should be extended with genome sequences from different kinds of origin including wastewater compartments. In contrast to the HTP approaches, the validity of more conventional techniques (PCR, qPCR) to assess the fate of genetic markers for taxonomy approaches, resistance genes, and mobile genetic elements are limited by the availability of specific primer sets and are more or less time consuming. Therefore, the application of the next generation sequencing technology and of functional genomics approaches would be very helpful for a deeper understanding of vertical and horizontal gene transfer events in wastewater systems for a verified evaluation of antibiotic resistance situations. To summarize, molecular based methods offer the great advantage of getting rid of biases associated to the non-cultivability of some ARB or the non-expression of ARG in some bacterial hosts (Oliver, 2005, 2010; Trevors, 2011). Still, these molecular methods do not allow the identification of the ARB of interest as culture-based approaches do, and should circumscribe their use to the identification of possible environmental hotspots of ARG. Furthermore molecular techniques do not allow the distinction between living and inactivated organisms. Table 2 presents some comments on the advantages and disadvantages of culture-based and molecular-based methods.

2.2.1. Methods to study gene transfer potential in UWTPs

The main challenge of studying the dynamics of ARG spread is to rely on global and sensitive detection techniques allowing the detection of possibly rare events/sequences, especially in treatment processes that do not favour the acquisition and spread of resistance genes in indigenous bacteria.

As discussed above, various approaches have been developed based on the systematic search and enumeration of either ARB by culture-based methods or ARG using molecular detection tools. These approaches remain limited to the resistance targeted, which does not preclude the existence of unforeseen resistances that may also disseminate. Therefore, identifying hotspots of ARG dissemination should also rely on the detection of the common genetic structures hosting the ARG (typically, mobile genetic elements) rather than the ARG themselves (Lupo et al., 2012). There are different types of mobile genetic elements, namely plasmids, transposons, bacteriophages, integrons, and combinations of them. Integrons are of particular interest because (i) are among the simplest elements involved in the mobility of gene cassettes, (ii) all share a common structure, (iii) can be associated to other mobile genetic elements, and (iv) are particularly efficient in trapping ARG. All these reasons make integrons good alternative targets for detecting possible ARG spread. Integrons are genetic elements able to acquire, exchange and express ARGs embedded within gene cassettes (GCs). By definition, an integron is structurally based on a functional platform composed of 3 elements: (i) the *intI* gene, encoding an integrase protein, (ii) a specific recombination site *attI*, and (iii) a promoter (Cambray et al., 2010; Hall and Collis, 1995). GCs which generally combine an open reading frame (ORF) with an *attC* site are integrated in or excised from the functional platform by a site-specific recombination mechanism catalysed by the IntI integrase.

Resistance integrons (RIs) and chromosomal integrons (CIs), also termed super-integrons, are the two major groups of integrons described in the literature. CIs are located on the chromosome of hundreds of bacterial species (Cambray et al., 2010). RIs have been described in a wide range of Gram-negative bacteria and a few times in Gram-positive bacteria (Martin et al., 1990; Nandi et al., 2004; Nesvera et al., 1998; Xu et al., 2010). RIs contain a limited number of GCs (to date, up to 9) and are located on mobile genetic

Table 1
Abundances of ampicillin (*ampC*), vancomycin (*vanA*), and imipenem (bla_{VIM}) antibiotic resistance genes in total genomic DNA from wastewater samples of three German cities (Schwartz, 2012). Numbers are cell equivalents/100 ng total DNA.

Wastewater pathways	City A			City B			City C		
	ampC	*vanA*	bla_{vim}	*ampC*	*vanA*	bla_{vim}	*ampC*	*vanA*	bla_{vim}
Housing area	8.9×10^4	–	3.0×10^0	1.8×10^4	–	1.2×10^1	8.8×10^3	–	–
Clinical wastewater	1.5×10^4	3.3×10^2	4.9×10^4	1.8×10^3	–	2.3×10^1	1.8×10^3	9.0×10^2	9.0×10^0
Inflow UWTP	4.6×10^4	+[a]	1.8×10^3	2.2×10^3	–	1.0×10^1	2.2×10^3	8.2×10^1	6.0×10^0
Effluent UWTP	1.6×10^3	–	1.6×10^4	3.9×10^2	–	–	3.9×10^2	1.0×10^0	1.8×10^1

[a] Detected, but not quantified.

Table 2
What do provide culture-based and molecular-based approaches on antibiotic-resistance surveys?

Non standardized molecular-dependent methods	Culture-dependent methods
Advantages	
High specificity and sensitivity	Direct proof of living and progeny capacities
Detection independent from physiological status of the bacteria	Comparable results over longer time scales and among different laboratories
Direct taxonomical affiliation coupled with functional genes and/or virulence factors	Easy to perform, robust, and reliable
Direct quantification of DNA targets (like ARB and ARG) in whole communities	Low costs
Diagnostic without any enrichment feasible	
Detection almost independent from other microflora	
Disadvantage	
Free DNA and DNA from dead microorganisms are detected in parallel	Physiological factors influence the quantification and detection
Low or no information about the microbial progeny	"Viable but not culturable bacteria" (VBNC) and injured bacteria are not detected
Cross reactions with uncharacterized microflora possible	Total microflora can influence results (overgrowth)
Inhibitors might influence the detection (internal controls and purification of DNA extracts are needed)	Laborious and time consuming
High costs and know how required (e.g. thermocycler, enzymes)	Isolation of bacteria from complex habitats are problematic
Up to now no standardized protocols	Detection of virulence factors is missing
Restricted to small sample volumes, otherwise time consuming sample preparations are needed	Cultivation and detection of some microorganisms is time consuming (e.g. mycobacteria, protozoa)

elements such as transposons and plasmids, which promote their dissemination among bacteria. Among the five classes of RIs outlined, based on the amino-acid sequence of the IntI protein (Cambray et al., 2010), classes 1, 2 and 3 are the most described. Class 1 and class 2 integrons are the most frequently found and have been detected in human, animal, and environmental samples and bacterial isolates (Stokes and Gillings, 2011). Class 1 RIs have been extensively studied and have mainly been described in multidrug-resistant bacteria (Cambray et al., 2010; Mazel, 2006; Stalder et al., 2012). To date, only four class 3 RIs have been described in clinical *Enterobacteriaceae* strains and in environmental *Delftia* isolates (H. Xu et al., 2007).

More than 130 GCs encoding resistance to almost all antibiotic families, including beta-lactams, aminoglycosides, trimethoprim, chloramphenicol, fosfomycin, macrolides, lincosamides, rifampicin and quinolones, besides antiseptics as quaternary ammonium compounds (QACs) have been described (Partridge et al., 2009). Thus detecting integron structures rather than specific ARG may provide a global perspective on antibiotic resistance and ARG spread. With this respect, recent reports unambiguously demonstrated the effect of direct or indirect antibiotic pressures in clinical, agricultural and environmental settings on the RIs prevalence in bacterial communities (Barlow et al., 2009; Daikos et al., 2007; Kristiansson et al., 2011; Luo et al., 2010; Skurnik et al., 2005). What is important to note is that studies on wastewater effluents are usually limited to class 1 integrons. Recently, a qPCR method enabling the amplification of the three main classes on integrons directly from complex DNA matrices was described (Barraud and Ploy, 2011). Thus, RIs may be useful to track the occurrence of resistance genes in the environment and their detection may be a good indicator of antibiotic resistance acquisition. However, this marker is global and do not describe precisely which ARGs are present within the RI.

RIs prevalence in environmental samples ranges from 0 to more than 90% of integron bearing isolates, depending on the environmental matrix considered (Stalder et al., 2012). Different environmental studies demonstrated that anthropogenic impact led to the enrichment of class 1 RIs (Gaze et al., 2011; Heuer et al., 2011; Rosewarne et al., 2010; Wright et al., 2008). More specifically, all factors leading to bacterial stress, such as antibiotics, QACs or high concentrations of heavy metals resulted in the selection of class 1 RIs-harbouring bacteria (Gillings et al., 2008; Skurnik et al., 2006). Agricultural manure amendment, UWTPs or industrial effluents have been identified as hotspots of class 1 RIs' dissemination (Stalder et al., 2012). Consequently, integrons seem to be a good indicator of the presence of antibiotic resistance within an environmental matrix, useful to quantify and assess the effectiveness of wastewater treatment processes. Class 1 RIs, mainly those associated to mobile elements, have also been found in different stages of the UWTP with the highest concentration values determined in raw effluents (Ferreira da Silva et al., 2007; Figueira et al., 2011b; Moura et al., 2007). Despite the fact that UWTPs can remove two to three log of the initial bacteria content, most often the treatment processes applied currently, do not affect specific antibiotic resistant bacteria and RIs-harbouring bacteria (Ferreira da Silva et al., 2007; Figueira et al., 2011b). Furthermore, hospital effluents may represent an important source of RIs, which are highly prevalent (58.9% and 48.4%) in antibiotic resistant *Enterobacteriaceae* (Guo et al., 2011).

2.3. Dynamics of ARG transfer in complex environmental matrices

Despite the evidences linking the ever-increasing occurrence of antibiotic resistance genes in bacterial communities to the presence and use of antibiotics (Davies, 2007; Davies and Davies, 2010), the dissemination of resistance genes in complex environments remains difficult to demonstrate. Indeed, the emergence of antibiotic resistance may result from either or both, the progressive enrichment of resistant bacteria, and the transfer of resistance genes between microbial members of a given community (Courvalin, 2008; David and Daum, 2010; Stokes and Gillings, 2011). On a practical point of view, determining the relative contribution of each phenomenon is a difficult question to address. As a matter of fact, an increasing occurrence of ARB or ARG does not demonstrate that gene transfer had occurred. Often, the emergence of resistances could be attributed to horizontal gene transfer by retrospective evidences. Mainly, there are three types of such retrospective evidences as already discussed by Top and Springael (2003) in another context: (i) the association of the acquired ARG with mobile genetic elements such as plasmids, phages, transposons (Partridge, 2011), (ii) the loss of synteny between the insertion site in the host and the acquired DNA which then appears as a variable "resistance islands" (Deurenberg and Stobberingh, 2008; Dobrindt et al., 2004; Miriagou et al., 2006; Novick et al., 2001), and (iii) finally the absence of congruence between the phylogeny of the supposedly transferred genes and the phylogeny of the corresponding hosts (Lal et al., 2008; Sørensen et al., 2005).

UWTP is one of these ecosystems that are believed to be probable hotspots for antibiotic resistance gene transfer because they combine several favourable factors, namely a high cell density sustained by a nutrient rich environment, and a recurrent contamination with both antibiotics and resistant bacteria (Dröge et al., 2000; Schlüter et al., 2007). With this respect, mobile elements such as plasmids carrying ARGs, have been detected/isolated repeatedly from wastewater or activated sludge (Dröge et al., 2000; Schlüter et al., 2008; Szczepanowski et al., 2008), but their direct involvement in mediating ARG transfer in UWTP remains to be demonstrated. The main reason for the lack of direct evidences regarding ARG transfer in UWTPs probably lies on the technical/methodological difficulties in studying such transfer from a given donor bacterium to the numerous possible indigenous recipient bacteria hosted by complex environmental matrices (Sørensen et al., 2004, 2005). To date, three main types of approaches have been

developed and used to monitor the transfer of mobile genetic elements by conjugation, mostly plasmids, in environmental matrices (Fig. 2): culture-based approaches, fluorescence-based approaches and molecular-based approaches. Each of them present their own advantages and drawbacks, starting by the fact that they all solely focus on the transfer of a given/specific mobile element rather than studying the various possible genetic exchanges that are likely to happen in complex microbial communities. Several studies have demonstrated gene transfer in environmental matrices involving mechanisms other than conjugation (e.g. natural transformation) but they remain relatively scarce if not inexistent in the context of natural ARG dissemination (Demanèche et al., 2001; Pontiroli et

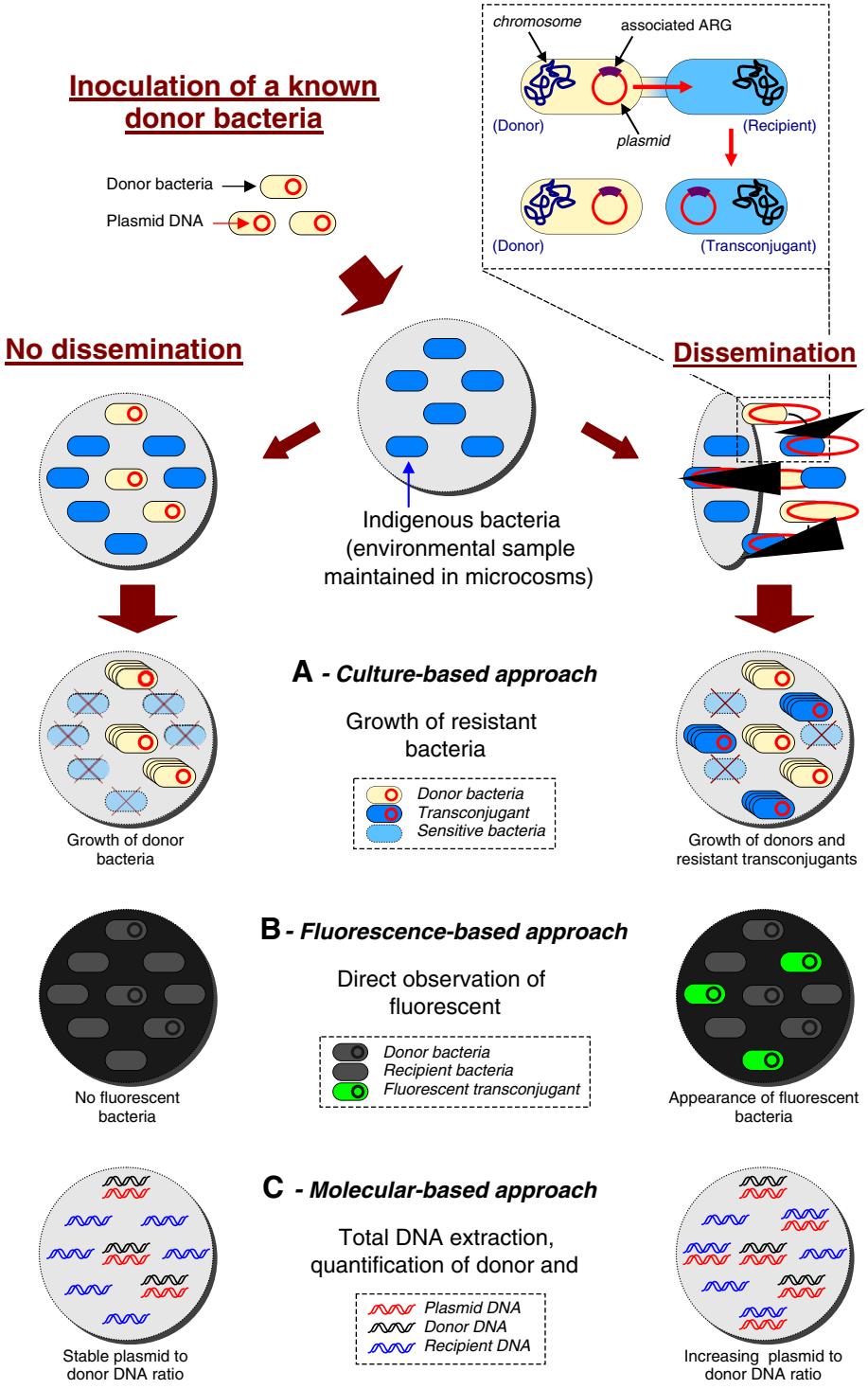

Fig. 2. Monitoring plasmid transfer in complex environmental matrices using different approaches. (A) The culture-based approach relies on the selection of microorganisms hosting and expressing the plasmid encoded ARG of interest. The other bacteria are supposedly counterselected (red cross). (B) The fluorescence-based approach makes use of a genetically engineered plasmid expressing a fluorescent protein once transferred. Only transconjugant should be visible by fluorescence microscopy (green cells). (C) The molecular-based approach relies on the fact that conjugation is a replicative process, meaning that the relative amount of plasmid increase while transferring (red DNA). Inset: plasmid transfer by conjugation leading to the acquisition of a new copy of plasmid in the transconjugant.

al., 2009; Rizzi et al., 2008). Hence, only plasmid transfer is discussed further below.

2.3.1. Methods to assess plasmid transfer in complex matrices

The culture-based approach is probably the most popular one, as it is the easiest to perform and therefore the more accessible. It mainly consists in inoculating an environmental sample with a known couple of donor (hosting a mobile genetic element of interest) and recipient bacteria chosen for their unambiguous selectable characteristics (Kowalchuk et al., 2004). After incubation under defined conditions the transconjugants are recovered and enumerated on appropriate selective media (Fig. 2A). The transfer frequency, calculated as a ratio of transconjugants to recipient or donor cells, indicates how permissive the conditions or the matrices regarding a particular genetic exchange are. In some instances, the indigenous bacteria can directly be used as recipient cells but in such cases the recovery of transconjugants is impaired by (i) the poor cultivability of environmental bacteria, considered to be a very limiting factor (less than 1% are culturable), and (ii) the host range in which the marker used for selection is expressed (Sørensen et al., 2005). However, culture-based approaches successfully pointed out various factors affecting plasmid transfer in environmental matrices such as sludge/wastewater, among which: (i) the bacterial species/strains involved (De Gelder et al., 2005; Gealt et al., 1985), (ii) the incompatibility group and host range of the transferred plasmid (Dröge et al., 2000), (iii) the sampling origin and physico-chemistry of the environmental matrix considered (sewage versus primary or secondary clarifier, temperature, pH, nutrient conditions, agitation, and presence of suspended matter) (Inoue et al., 2005; Mach and Grimes, 1982; Soda et al., 2008).

The fluorescence-based approach is an interesting alternative consisting of using plasmid derivatives that have been genetically modified to express a fluorescent protein once leaving the donor cell (Sørensen et al., 2005). It is assumed that all transconjugants should appear fluorescent when the community is observed under a fluorescent microscope (Fig. 2B). This approach advantageously provides information regarding the localization where the transfers took place in complex environmental matrices. For instance, it was demonstrated that in *Pseudomonas putida* biofilms, plasmid pWWO transfers mostly occur at the periphery of the cell aggregate (Haagensen et al., 2002), and fluorescent transconjugants could also be observed in environments as complex as activated sludge microcosms (Geisenberger et al., 1999; Sørensen et al., 2005). Apart from preserving the structure of the matrices, this approach provides the advantage of overcoming the cultivation of the indigenous bacteria (Dahlberg et al., 1998b; Geisenberger et al., 1999; Musovic et al., 2010). Nevertheless, the fluorescence-based approach also present limitations that have been pointed out in several occasions: (i) it requires the genetic modification of the plasmid which, when feasible, may impair unforeseen but important plasmidic functions, (ii) the detection of transconjugant directly relates to the expression level of the fluorescent protein, which may not be sufficient enough in some indigenous species, and finally (iii) background fluorescence from the matrix may interfere with the transconjugant fluorescence, especially when the level of expression is low (Bonot, 2010; Dahlberg et al., 1998a,b; Nancharaiah et al., 2003; Sørensen et al., 2005).

The molecular-based approach, the last one to have been developed, makes use of qPCR to monitor the dissemination of a given plasmid in the vastness of microbial community DNA (Bonot and Merlin, 2010). Because the conjugative transfer of the plasmid is a certain form of DNA replication, the plasmid to donor DNA ratio increases when the plasmid disseminates into the indigenous population (Fig. 2C). On a practical point of view, an environmental matrix is inoculated with a donor bacterium hosting the plasmid of interest, and incubated under controlled conditions in microcosms. The microcosms are sampled at intervals, the total community DNA is extracted and both, the plasmid and the donor DNA are quantified by qPCR. Then, a careful analysis of how the plasmid to donor DNA ratio evolves indicates whether or not the plasmid was transferred. The advantage of this method probably lies on the fact that because it is culture- and gene expression-independent, it allows the study of gene transfer on a wide range of the indigenous bacteria. Additionally the sensitivity of the qPCR approach allows working with very small initial inoculums, which is not the case with the two other approaches. This approach has successfully been used by Merlin et al. (2011) to compare different processes in UWTPs and to identify matrices that seem to promote the transfer of a model plasmid. Nevertheless, it is difficult to carry out and very specific sets of primers and probe for a non-ambiguous quantification donor and plasmid have to be designed (Bonot and Merlin, 2010). Finally, the major drawback of the molecular approach lies on the fact that because plasmid transfer is deduced from an increasing plasmid to donor ratio, persistent donor bacteria may occult a weak transfer rate.

In the present section we have been discussing the different methods available to quantify plasmid transfer in environmental matrices including wastewater or activated sludge. Even though each method individually offers advantages and drawbacks, it should be kept in mind that gene transfer is studied using model genetic elements, which are therefore not entirely representative of different behaviours associated to different kinds of mobile genetic elements. Additionally, and despite being informative, maintaining environmental matrices in a laboratory context can also bring some biases as the microbial communities involved may behave and evolve differently than when in their natural equivalent (Cadotte et al., 2005; Valentín-Vargas et al., 2012; Van den Broeck et al., 2009).

3. Antibiotic resistance in UWTPs

Over the last years a renewed interest on the antibiotic resistance phenotypes in UWTPs became apparent in the scientific literature (Baquero et al., 2008; Kümmerer, 2009; Manaia et al., 2012). Human and animal commensal bacteria and other of environmental origin have been the major focus of these studies on antibiotic resistance in wastewaters. Given their close contact with humans and easiness to isolate and identify, the indicators of faecal contamination, coliforms and enterococci, are among the most studied groups (Araújo et al., 2010; Boczek et al., 2007; Ferreira da Silva et al., 2007; Martins da Costa et al., 2006; Reinthaler et al., 2003; Sabate et al., 2008). In an attempt to establish a relationship between the most severe cases reported in clinical settings and environment, the search for the last generation antibiotic resistant determinants is also reported in UWTP studies (Araújo et al., 2010; Czekalski et al., 2012; Figueira et al., 2011a,b; Gajan et al., 2008; Parsley et al., 2010; Szczepanowski et al., 2009). In this category the methicillin resistant *S. aureus*, vancomycin resistant *Enterococcus* spp., Gram-negative bacteria producing extended spectrum beta-lactamase, among others are included.

Although the occurrence of antibiotic resistant superbugs in the effluents may be an issue of concern, the amount of common bacteria harbouring antibiotic resistances that are continuously discharged in receiving waters is impressive (Ferreira da Silva et al., 2007; Galvin et al., 2010; Łuczkiewicz et al., 2010; Martins da Costa et al., 2006). The final effluent of UWTP can discharge about 10^9–10^{12} Colony Forming Units (CFU) per day, per inhabitant equivalent (Novo and Manaia, 2010); among these, at least 10^7–10^{10} could have any kind of acquired antibiotic resistance. These numbers underline the importance of UWTPs on the accumulation and spread of antibiotic resistant bacteria in the environment. Moreover, in these estimates, only the culturable fraction of the bacterial population is being considered, and it can represent only 1% of the total. Indeed, the numerous unculturable bacteria thriving in wastewaters and related systems (sludge, biofilms) can host a myriad of antibiotic resistance genes (Szczepanowski et al., 2009; X.X. Zhang et al., 2009b). In a study conducted with wastewater samples in Germany, 140 clinically relevant genes, encoding resistance to the different classes of antibiotics (aminoglycosides, beta-lactams,

chloramphenicol, fluoroquinolone, macrolides, rifampicin, tetracycline, trimethoprim and sulfonamide as well as efflux pumps) were detected (Szczepanowski et al., 2009). Some of these have a recent history in clinical isolates, showing the rapid spread of ARB/ARG into wastewaters. One of the major driving forces for the maintenance and spread of ARB and their genes in wastewaters may be the occurrence of antimicrobial residues, which besides selecting resistance phenotypes may also disturb the indigenous bacterial communities (Novo et al., in press). The current knowledge about the relationship between antibiotics and the spread of ARG in microbial communities is mostly the result of retrospective evidences (see Section 2.3). In a recent paper Merlin et al. (2011) described the transfer of the antibiotic resistance plasmid pB10 in UWTP sludge maintained in microcosms exposed (or not) to sulfamethoxazole and amoxicillin at low concentrations. They showed that (i) some matrices such as biofilm reactor sludge could support better than the other transfer of the plasmid, and (ii) in that particular setting the antibiotic did not favour the dissemination of the plasmid. In the latter case, the effects of the antibiotic were rather visible as an increasing persistence of the plasmid donor bacteria used to inoculate microcosm.

3.1. Effect of biological processes on ARB and gene transfer

3.1.1. Culture dependent surveys of antibiotic resistance in UWTPs

In spite of the lack of systematic and conclusive studies on the effect of biological processes and their operating conditions (e.g., HRT, SRT, biomass concentration etc.) on antibiotic resistance, some studies available in the scientific literature show that biological processes may positively affect ARB strains' spread and selection as well as ARG transfer (Auerbach et al., 2007; Ferreira da Silva et al., 2006; Kim et al., 2007a; Łuczkiewicz et al., 2010; Marcinek et al., 1998). Table 3 summarizes data from different works available in the scientific literature dealing with the effect of biological process on antibiotic resistance in UWTPs.

Given the absence of standardized methods to assess antibiotic resistance in environmental samples, the resistance prevalence data in different world regions or bacterial groups can be hardly compared with accuracy. Nevertheless, it is possible to recognize some trends mainly in what concerns bacterial groups.

Enterococci are among the most investigated bacteria in UWTPs. Different authors, using distinct methodologies, indicated high resistance rates (20–44%) for tetracycline, erythromycin and quinolones and considerably lower values for aminopenicillins and sulfonamides (1–7%) (Ferreira da Silva et al., 2006; Łuczkiewicz et al., 2010; Martins da Costa et al., 2006). The UWTP studied by Ferreira da Silva et al. (2006) included a preliminary mechanical treatment, a primary settling tank and a secondary biological treatment (activated sludge process), with the treated wastewater from the secondary settling tank being discharged without any further treatment into a natural water stream. This wastewater treatment process was observed to conduce to a reduction of bacterial counts of 1.0–1.5 log, although it could not contribute to the reduction of antibiotic resistance rates. Although these authors did not found any vancomycin resistant enterococci,

Table 3
Antibiotic resistance in UWTPs after biological process.

UWTP[a]	Biological process	ARB	Resistance to antibiotics[b]	Ref.
UWTP (150,000 habitants, 11,000 m^3/day) in Beni Mellal (Morocco) receives sewage of essentially domestic origin.	Activated sludge	Faecal coliforms	111 strains isolated: overall resistance (resistance to at least one antibiotic) was 72.07%. The antibiotic resistance of FC was found to be comparable between raw (71.0%) and treated sewage (77.8%). The higher levels of antibiotic resistance were towards streptomycin (54.0%), ampicillin (42.3%), amoxicillin (42.3%) and amoxicillin–clavulanic acid (31.5%).	Fars et al. (2005)
UWTP (100,000 inhabitants) in northern Portugal, 70% domestic ww and 30% pretreated industrial ww.	Activated sludge	*Enterococcus hirae*, *Enterococcus faecium* and *Enterococcus faecalis*	Tetracycline (31/33%), gentamicin (50/50%), erythromycin (33/23%), ciprofloxacin (9/25%), amoxicillin (0/3%), sulfamethoxazole/trimethoprim (0/1%) and no resistance to vancomycin	Ferreira da Silva et al. (2006)
14 UWTPs in Portugal	N.A.	Enterococci	983 isolates: multidrug resistance (49.4%), rifampicin (51.5%), tetracycline (34.6%), erythromycin (24.8%), nitrofurantoin (22.5%), ciprofloxacin (14%), ampicillin (3.3), vancomycin (0.6%)	Martins da Costa et al. (2006)
Gdansk–Wschod' UWTP (700,000 PE) in Northern Poland, mainly domestic ww, only limited industrial (5%) and undisinfected hospital (0.17%) ww	Activated sludge process working in modified UCT (University of Cape Town) type system	Enterococci	199 isolates: nitrofuration (53%), erythromycin (44%), ciprofloxacin (29%), tetracycline (20%).	Łuczkiewicz et al. (2010)
		Escherichia	153 isolates: ampicillin (34%), piperacillin (24%), tetracycline (23%), levofloxacin (10/15%), Trimethoprim/Sulphamethoxazole (11%), ciprofloxacin (10%)	
UWTP (72,000 inhabitants) in Ireland under the effect of ww from four hospitals.	Activated sludge	*Escherichia*	Ampicillin (24.5/12.5%), streptomycin (16.5/0%), sulfamethoxazole (11.1/12.5%), tetracycline (12.4/39%), ciprofloxacin (7.15/0%), cefoxitin (0.11/2.6%)	Galvin et al. (2010)
UWTP (100,000 inhabitants) in northern Portugal, 70% domestic ww and 30% pretreated industrial ww.; 400 mg COD/L, 250 mg BOD/L	Activated sludge	*Escherichia*	Tetracycline (32.1/36.8%), amoxicillin (28/34.8%), sulfamethoxazole/trimethoprim (22.2/22.5%), cephalothin (10.5/20.5%), ciprofloxacin (2.5/9.7%)	Ferreira da Silva et al. (2007)
		Shigella	Tetracycline (0/25%), amoxicillin (20/12.5%), sulfamethoxazole/trimethoprim (0/12.5%), cephalothin (12/0%), ciprofloxacin (0/6.3%)	
		Klebsiella	Tetracycline (5/13%), amoxicillin (94.7/95.7%), sulfamethoxazole/trimethoprim (0/8.7%), cephalothin (0/5.9%), ciprofloxacin (0/4.4%)	
2 large-scale Danish UWTPs (240,000 and 500,000 inhabitants)	Activated sludge for N and P removal	*Acinetobacter*	442 isolates: ampicillin (27/22.3%), gentamicin (7.2/5.8%), tetracycline (11.5/12.9%), multidrug resistance (n.d./0.3%)	Guardabassi et al. (2002)
UWTP (210,700 PE) in Ann Arbor, Michigan, USA, mostly domestic ww, only limited industrial and untreated hospital ww	Activated sludge for N and P removal (+ ferric chloride P removal)	*Acinetobacter*	366 isolates: trimethoprim (92.2/100%), rifampin (63.1/77.5%), chloramphenicol (25.2/35%), amoxicillin/clavulanic (8.7/20%), sulfisoxazole (8.7/22.5%), ciprofloxacin (4.9/11.3%),	Y. Zhang et al. (2009)

[a] ww = wastewater.
[b] Between brackets prevalence of antibiotic resistance (%): data before and after (/) biological process where available.

they concluded on a positive selection of ciprofloxacin-resistant enterococci. Indeed, a significant increase in resistance prevalence ($p<0.05$) was observed in treated wastewater compared to the raw wastewater. Similar findings were reported by Łuczkiewicz et al. (2010), who studying 199 isolates, from a UWTP based on activated sludge process, working in modified UCT (University of Cape Town), observed an increase of *E. faecalis* and *E. faecium* resistant to fluoroquinolones ($p<0.05$) after wastewater treatment (73% and 78%, before and after, respectively). The high prevalence of antibiotic resistant enterococci in the final effluents of UWTP has been widely observed. For instance, Martins da Costa et al. (2006) investigated the occurrence of antibiotic resistant enterococci in 14 UWTPs in Portugal and concluded that almost 50% of the 983 isolates examined were resistant to different antibiotics. These are worrisome values considering that although wastewater treatment resulted in enterococci decreases of 0.5–4 log, these bacteria were at densities up to 10^3 CFU/mL in the final effluent.

Escherichia coli are another group of bacteria widely monitored to assess the environmental spread of antibiotic resistance. Probably because of its biology and ecology, the antibiotics for which high resistance rates in *E. coli* are observed, are different from those observed in enterococci. In this case, high resistance rates were observed for aminopenicillins, sulfonamides and tetracycline (10–40%), whereas comparatively lower rates are reported for quinolones or gentamycin (<10%) (Ferreira da Silva et al., 2007; Galvin et al., 2010; Łuczkiewicz et al., 2010). However, like for enterococci, wastewater treatment cannot reduce antibiotic resistance rates in coliform bacteria. Indeed, some studies report on the slight increase of resistance to antibiotics of different families (e.g. penicillins, fluoroquinolones, trimethoprim/sulphamethoxazole or tetracycline) (Ferreira da Silva et al., 2007; Łuczkiewicz et al., 2010). For instance, Ferreira da Silva et al. (2007) observed significantly higher prevalence of ciprofloxacin resistant *E. coli* in the final effluent ($p<0.0001$) than in the raw inflow of an UWTP. Although the mechanisms that lead to such variations are not well understood, Figueira et al. (2011a) suggested that this may be due to the selection of ciprofloxacin resistance and/or the more extensive elimination of fluoroquinolone susceptible *E. coli* during wastewater treatment. Carbapenem resistance, observed in enterobacteria of clinical origin, has not been reported in recent studies concerning wastewaters (Figueira et al., 2011a; Łuczkiewicz et al., 2010). However, in analogy with other resistance phenotypes, it may be argued that it will be a matter of time. Although at very low prevalence values, these studies reported the occurrence of *E. coli* resistant to third generation cephalosporins in wastewaters.

Acinetobacter spp., given their relevance in wastewater bacterial communities and the clinical importance of *Acinetobacter baumannii*, gained attention with respect to antibiotic resistance in the environment (Guardabassi et al., 2002; Y. Zhang et al., 2009). Studying an UWTP (i.e., raw influent, secondary treatment effluent, and final effluent) and the receiving water (upstream and downstream UWTP discharge), Y. Zhang et al. (2009) observed significant increases of resistance due to wastewater treatment. Based on a collection of 366 *Acinetobacter* isolates these authors observed that resistance to amoxicillin/clavulanic acid, chloramphenicol, rifampin, and multi-drug (three antibiotics or more) significantly increased ($p<0.01$) from the raw influent samples to the final effluent samples, and was significantly higher ($p<0.05$) in the downstream samples than in the upstream samples.

3.1.2. Antibiotic resistance genes in UWTPs

The use of culture-dependent approaches as those indicated in Table 3, overlooks a significant part of the bacterial communities. To overcome such limitations, the use of culture-independent approaches, targeting specific antibiotic resistance genes or genetic elements known to harbour and/or mobilize resistance determinants, is a good alternative. Table 4, which is far from being an exhaustive list, provides examples of some genes that have been detected in bacterial isolates or in total DNA wastewater samples of UWTP.

According to the information currently available (Table 4), genes encoding resistance to all classes of antibiotics are widespread in UWTP effluents. Among the diversity of genes found, it is possible to conclude that every mechanism available in nature to resist antibiotics (e.g., target protection, target modification, drug modification, reduced permeability or efflux) is capable of surviving wastewater treatment processes. Because of some methodological limitations, resistance due to mutation events, which modifies the antibiotic target, such as gyrase mutations conferring resistance to quinolones, often cannot be detected using culture-independent methods. Most of the times, studies based on total DNA analyses do not allow straightforward inferences on the relationship between the resistance genetic elements and the phlylogenetic lineages holding them (Szczepanowski et al., 2008, 2009). A good example of an exception is the study of Zhang et al. (2011), in which the predominant hosts of plasmid-related antibiotic resistance genes in activated sludge samples are indicated. Based on metagenomic analyses of activated sludge samples, these authors conclude that *Proteobacteria* of the classes *Alpha-*, *Beta-* and *Gamma-* and members of the genera *Bacillus*, *Mycobacterium* or *Nocardiopsis* are the most abundant antibiotic resistance plasmids harbourers. Moreover, Zhang et al. (2011) proposed (based on the number of metagenome reads) that tetracycline, macrolide and multidrug resistance genes are those more represented (~25% each), whereas other resistances, such as resistance to aminoglycosides, sulfonamides and bacitracin are less frequent. The putative mobilization of resistance genes in an UWTP may also be inferred from studies which demonstrate a clear abundance of transposase genes and other elements associated with gene transfer processes (Moura et al., 2010; Parsley et al., 2010; Sanapareddy et al., 2009). *Proteobacteria*, mainly of the classes *Alpha-*, *Beta-* and *Gamma-*, seem to be the predominant bacteria in the activated sludge of wastewater treatment plants (Sanapareddy et al., 2009; Zhang et al., 2012). In this respect, it is also important to note that genera such as *Enterococcus* or *Escherichia*, often targeted in antibiotic resistance culture-dependent studies in UWTP, are not detected among the most abundant groups. The current state-of-the-art shows that both, culture-dependent and culture-independent methods, provide different and valuable insights into the diversity and ecology of antibiotic resistance. The use on non-targeted approaches involving functional metagenome studies might potentially unveil other features of the environmental resistome.

3.1.3. Occurrence and transfer of antibiotic resistance in UWTPs

ARGs occurrence and transfer in UWTPs have also been investigated (Auerbach et al., 2007; Guillaume et al., 2000; Marcinek et al., 1998; Schwartz et al., 2003). Many resistance genes are located on mobile genetic elements such as plasmids, transposons and integrons. Marcinek et al. (1998) evaluated the ability of *Enterococcus faecalis* to transfer various genetic elements in the activated sludge process of two UWTPs in Germany. The transfer rate among different strains of *E. faecalis* was found to be at least 10^5-fold lower for the sex pheromone plasmids, at least 100-fold lower for a resistance plasmid possessing a broad host range for Gram-positive bacteria, and at least 10-fold lower for a conjugative transposon (which is transferred under laboratory conditions at low rates to Gram-positive bacteria and at very low rates to Gram-negative bacteria) compared to laboratory conditions. Transfer from *E. faecalis* to other bacterial species was not detected at all. Moreover, the authors estimated the maximum number of transfer events for some sex pheromone plasmids between different strains of *E. faecalis* in the UWTP ranging from 10^5 to 10^8 events per 4 h, indicating that gene transfer should take place under natural conditions. In subsequent studies, several resistance plasmids were isolated from UWTPs and found to confer resistance to different antibiotics (Bönemann et al., 2006; Heuer et al., 2004; Schlüter et al., 2007, 2003; Szczepanowski et al., 2007). Other studies addressed the occurrence of selected antibiotic-resistance

Table 4
Examples of antibiotic resistance genes detected in wastewater of UWTP.

Class	Mechanism (Type)[a]	Examples of genes[b]	Approach[c]	Reference[d]
Aminoglycosides	Modification by adenylylation (DM)	*aad*(A1, A2, A13, B)	CD	4; 8
	Modification by phosphorylation (DM)	*aph*(A, A-3, A-6, 2); str(A, B)	CI	11
		*str*B	CD	9
Beta-lactams	Beta-lactam ring cleavage (beta-lactamase production) (DM)	Class A: CTX, GES, NPS,PER, SHV, TEM,TLA, VEB	CI	3; 6; 11; 12
		Class B: IMP, VIM	CI	11
		Class C: *amp*C, CMY	CI	11
		Class D: OXA	CI	11
		Class D: OXA	CD	8
	Penicillin binding protein (TP)	*mec*A	CI	2; 3; 10
Glycopeptides	Modified peptodoglycan pentapeptide (TM)	*van*A	CD	1
			CI	10
Macrolides	Macrolide-efflux protein (DE)	*mel*	CI	11
	Erythromycin inactivation (DM)	*ere*A2	CI	11
	Modification by 23S rRNA methylation (TP)	*erm*B	CD	1
		erm(B, F)	CI	11
	Macrolide phosphotransferase (DM)	*mph*(A, B)	CI	11
Quinolones	Modification by acetylation (DM)	*aac*A6-*ib-cr*	CD	5
	DNA gyrase protection (TP)	*qnr*(A3, B1, B2, B4, B5, S2)	CI	11
		*qnr*VC; *qnr*S	CD	5; 12
Sulfonamides	Modified dihydropteroate synthase (TM)	*sul*2	CD	9
		sul(1, 2, 3)	CI	11
Tetracyclines	Other	*tet*U	CI	11
	Tetracycline efflux pump (DE)	*tet*A	CD	9
		tet(A, B, D, G, H, Y, 31, 35, 36, 39)	CI	2; 7; 11
	Ribosomal protection protein (TP)	*tet*M	CD	1
		tet(M, S)	CI	7; 11
	Tetracycline modification (DM)	*tet*X	CI	7; 11
Trimethoprim	Dihydrofolate reductase (DM)	*dfr*(A1, A12, 18)	CD	4; 8; 9
		dfr (II, V, VII, XII, 13, 16, 17, A19, B2, D); *dhfr* (I, VIII, XV)	CI	11
Multidrug	Multidrug resistance efflux pump (DE)	*acr*(B, D), *mex*(B, D, F, I, Y)	CI	11

[a] DM, drug modification; DE, drug efflux; TM, target modification; TP, target protection (Liu and Pop, 2009).
[b] This is not an exhaustive list; for simplicity gene variations are presented within parenthesis.
[c] CD, culture dependent method; CI, culture independent method.
[d] References: 1. Araújo et al. (2010); 2, Börjesson et al. (2009); 3, Colomer-Lluch et al. (2011); 4, Ferreira da Silva et al. (2007); 5, Figueira et al. (2011b); 6, Lachmayr et al. (2009); 7, LaPara et al. (2011); 8, Moura et al. (2007); 9, Okoh and Igbinosa (2010); 10, Schwartz et al. (2003); 11, Szczepanowski et al. (2009); 12, Xia et al. (2010).

genes, e.g. *vanA*, *ampC*, *mecA* (Schwartz et al., 2003; Volkmann et al., 2004), or genes conferring resistance to a specific class of antimicrobial compounds, e.g. tetracyclines (Guillaume et al., 2000; Smith et al., 2004). The occurrence of tetracycline resistance determinants (*tetR*) genes in samples from two activated sludge UWTPs was investigated to determine which *tetR* occurs in these systems (Auerbach et al., 2007); quantitative assays were conducted to determine different *tetR* gene concentrations. On a volumetric basis, *tetQ* abundances in influent samples were always higher than the levels observed in the activated sludge samples; on the opposite, the *tetG* was always more abundant in the activated sludge in all cases.

RIs have also been detected in activated sludge process of UWTP. In particular, 30% to 61% of the strains isolated in activated sludge harboured a class 1 RI in activated sludge, results that may vary depending on the methods of nucleic acids extraction as well as the primers used to detect the RIs (Ma et al., 2011; Moura et al., 2007; X.X. Zhang et al., 2009a). Moreover, it is found that 12% of plasmids isolated from UWTP sludge carried class 1 RIs, among which more than half are broad-host-range plasmids displaying very high transfer frequencies (Moura et al., 2007; Tennstedt et al., 2003). Class 2 RIs and class 3 RIs, seem to play a minor role in UWTP although some publications describe class 3 RIs in *Delftia* sp. (*D. acidovorans* and *D. tsuruatensis*) isolated from activated sludge (Moura et al., 2010; W. Xu et al., 2007). The activated sludge process does not seem to reduce the prevalence of RIs (Ferreira da Silva et al., 2007; Figueira et al., 2011a; Ma et al., 2011; Moura et al., 2007). That is demonstrated also by culture-independent methods (X.X. Zhang et al., 2009b). However, when using abundance normalized to the total DNA amount, it can be found that the effluent treatment process can decrease the RIs rate (X.X. Zhang et al., 2009a). The effect of sludge treatment processes on RI has also been investigated. While aerobic digestion at 35–37 °C did not reduce the relative abundance of RIs (Diehl and Lapara, 2010), thermophilic anaerobic digestion (between 50 °C and 60 °C) significantly reduced the relative abundance of class 1 RIs with removal efficiencies between 80 and 95% (Ghosh et al., 2009). However, other factors may influence the RIs occurrence during the sludge digestion (Ma et al., 2011): thermal hydrolysis pretreatment (150 °C/4.80 bar, 30 min) efficiently removed the RIs, nevertheless during following anaerobic or aerobic digestion steps a recurrence in numbers of RIs was observed, suggesting that horizontal gene transfer occurred during the sludge digestion.

3.1.4. Effect of the operational conditions on the antibiotic resistance fate in UWTPs

According to the above reviewed studies, the investigations have been mainly focused on the selection and relative prevalence of ARB and ARG transfer in UWTP irrespective of the biological process, technology and operating parameters. Studies on the effects of the operating conditions (Kim et al., 2007a,b) and different wastewater treatment technologies (Mezrioui and Baleux, 1994; Munir et al.,

2011) on the occurrence and release of ARGs and ARB was carried out. In particular, the fate of tetracycline resistant bacteria as a function of activated sludge organic loading rate and growth rate was investigated in lab scale sequencing batch reactors (SBRs) (Kim et al., 2007a). Increases in the organic loading (achieved by altering the influent wastewater flux) and growth rate (achieved by altering sludge retention time (SRT)) resulted both in the increased occurrence of tetracycline resistance. These trends were observed for activated sludge reactors loaded with typical municipal background tetracycline concentrations (1 μg L^{-1}) and those receiving inflow augmented with 250 μg L^{-1} tetracycline. When comparing 250 μg L^{-1}tetracycline fed SBRs with parallel SBRs having a background tetracycline concentration of approximately 1 μg L^{-1}, tetracycline fed reactors were found to have increased loads and production rates of tetracycline resistant bacteria, and higher net growth rates of resistant bacteria (Kim et al., 2007b). Antibiotic resistance of 870 *E. coli* strains isolated from domestic raw sewage, were comparatively investigated in the effluent from aerobic lagoons and activated sludge plants (Mezrioui and Baleux, 1994). The reduction of faecal coliforms was higher in the aerobic lagoon (99.99% in summer) than in the activated sludge system (91.30% in summer). The *E. coli* strains isolated from the effluent of the aerobic lagoon showed higher antibiotic-resistance (35%) than those isolated from domestic sewage (23%). In the activated sludge, the percentage of antibiotic resistant strains (resistance to at least one antibiotic) showed seasonal changes in the inflow and outflow wastewater samples. In authors' opinion, the increase of the percentage of antibiotic resistant strains of *E. coli* in the effluent of the aerobic lagoon is probably related to the selection of antibiotic resistant strains by this kind of treatment. Moreover, survival experiments comparing *E. coli* strains resistant to seven antibiotics and *E. coli* susceptible to 15 tested antibiotics showed that resistant bacteria had higher survival rates than susceptible ones in wastewater treated in lagoons. Munir et al. (2011) investigated the effect of different wastewater treatment technologies (namely membrane biological reactor (MBR), conventional activated sludge, oxidative ditch and rotatory biological contactors) on the occurrence and release of ARGs and ARB. ARGs and ARB abundance in the final effluent was found to be in the range of ND-2.33×10^6 copies $(100\ mL)^{-1}$ and 5.00×10^2–6.10×10^5 CFU $(100\ mL)^{-1}$, respectively. Consistently higher removal rates of ARGs and ARB were observed in the MBR (from 2.57 to 7.06 log) than in the conventional treatment plants (from 2.37 to 4.56 log).

3.2. Effect of advanced treatments on ARB and gene transfer

Advanced treatments aim at improving the quality of the secondary effluent before of disposal or reuse. Sand filtration, adsorption, membranes and advanced oxidation processes are among the most applied and studied advanced treatment processes/technologies. Unfortunately, despite a lot of available studies regarding the effect of advanced treatment technologies on bacteria inactivation (Gerrity et al., 2011; Madaeni, 1999; Malato et al., 2009), there is a lack of knowledge about their effect on antibiotic resistance (Grabow et al., 1976) as well as on gene transfer (Öncü et al., 2011).

The first study where the effect of advanced treatments on ARB was investigated dates back to the mid of 70s (Grabow et al., 1976). Transferable and non-transferable resistance in coliform bacteria was investigated in different phases (primary, secondary and advanced treatment) of wastewater treatment. The percentage of non transferable resistance to ampicillin, chloramphenicol or streptomycin, but not kanamycin or tetracycline, was found to slightly decrease through the treatment system. On average, the percentage of transferable resistance to one or more drugs in coliforms was reduced by about 50%; the reduction being mainly accomplished by advanced treatment of biofiltration and sand filtration. The authors believed that the fast passage over stony surfaces in biological and sand filters treatment units was unfavourable for conjugation and could damage sex pilli.

Ozonation and TiO_2 heterogeneous photocatalysis were compared with conventional chlorination in terms of effects on DNA structure and integrity (Öncü et al., 2011). Opposite to chlorine, which did not affect plasmid DNA structure at the studied doses, ozone and photocatalytic treatment resulted in conformational changes and the damage increased with increasing oxidant doses. When Fenton and ozone oxidation processes were investigated in the removal of *tetM* gene and its host *E. coli* HB101 from synthetically contaminated cow manure, PCR-based monitoring assays showed that the band intensity of the *tetM* gene gradually decreased by increasing the Fenton reagent and the applied ozone dose (Cengiz et al., 2010). A different approach was used by Paul et al. (2010) to evaluate the effects of photolytic and TiO_2 photocatalytic treatment processes on the antibacterial activity of ciprofloxacin. In particular, quantitative microbiological assays with a reference *E. coli* strain showed that for each mole of ciprofloxacin degraded, the antibacterial potency of irradiated solutions decreased by approximately one "mole" of activity relative to that of the untreated ciprofloxacin solution. The authors inferred that the ciprofloxacin photocatalytic transformation products retain negligible antibacterial activity compared to the parent compound. Moreover, according to their experimental system the lower energy demand (20 J/cm^2) to reduce antibacterial activity by one order of magnitude was achieved by UVA-TiO_2 photocatalysis. Finally, in a recently published work, the efficiency of solar driven photo-Fenton process was investigated in the inactivation of antibiotic resistant enterococci (Michael et al., 2012). All enterococci, including those resistant to ofloxacin and trimethoprim, were completely eliminated at the end of the treatment; comparing the resistance rates for the two antibiotics tested, it was observed that ofloxacin resistance was almost double of that of trimethoprim.

3.3. Effect of disinfection on ARB and gene transfer

The most applied disinfection process in wastewater treatment is chlorination, but UV radiation also finds extended applications. Germicidal effects of chlorine (as chlorine gas or hypochlorites) include the following mechanisms: oxidizing the germ cells, altering cell permeability, altering cell protoplasm, inhibiting enzyme activity and damaging the cell DNA and RNA (USEPA, 1999). Chlorine appears to react strongly with the lipids of the membrane and the membranes that have high lipid concentrations appear to be more susceptible to destruction (USEPA, 1999). The predominant disinfection mechanism depends on the microorganism (the resistance of a particular strain), the wastewater characteristics and chlorine dose (USEPA, 1999). Unfortunately, bacteria injured by disinfection process can survive and re-grow at low chlorine doses (Rizzo et al., 2004; Shrivastava et al., 2004).

UV radiation can also damage DNA, resulting in inhibition of cell replication and, in case of lethal doses, in a loss of reproducibility. The effectiveness of a UV disinfection system depends on the characteristics of the wastewater, the intensity of UV radiation (optimum wavelength to effectively inactivate microorganisms is 250–270 nm), the time the microorganisms are exposed to the radiation and the reactor configuration. In spite of DNA damage due to UV radiation, bacteria may recover replication activity, under visible light, through a process (photoreactivation) catalysed by the DNA repair enzyme photolyase (Oguma et al., 2001). Besides the photoreactivation *recA* gene-mediated dark repair mechanisms do also contribute to the regeneration of injured bacteria including pathogens like enterococci and *P. aeruginosa* (Jungfer et al., 2007). Here, sub-lethal UV doses induce DNA damages that trigger the activation of the *recA* gene expression and other genes involved in bacterial stress responses including DNA repair. It was demonstrated that the induction of DNA repair and the regeneration of bacteria is time and species dependent (Jungfer et al., 2007; Kraft et al., 2011). In a recent study, the inactivation of tetracycline-resistant *E. coli* and

antibiotic-sensitive *E. coli* by UV radiation was investigated to evaluate their tolerance to UV light (Huang et al., in press); but the authors did not find any difference in the inactivation of tetracycline-resistant *E. coli* and antibiotic-sensitive *E. coli* after disinfection treatment. The lack of data regarding the effect of UV-dependent DNA damages on antibiotic resistance, shows that this topic is worthy of investigation.

The first studies on the effect of chlorination on ARB can be traced back to the 70s (Armstrong et al., 1982; Grabow et al., 1976; Murray et al., 1984). A number of bacteria have been shown to be resistant to chlorination process in water (Maillard et al., 1998; Pyle et al., 1994; Ridgway and Olson, 1982). Although it has been demonstrated that the percentage of ampicillin-resistant bacteria in sewage decreased after different doses of chlorination (Grabow et al., 1976), contradictory results were obtained by other authors (Murray et al., 1984). In particular, Murray et al. (1984) isolated 1900 lactose-fermenting bacteria from untreated and treated urban wastewater. Chlorination of inflow resulted in an increase in the proportion of bacteria resistant to ampicillin and cephalothin, the increase being most marked after regrowth occurred following chlorination. Although the chlorination process was found to initially decrease the total number of bacteria in wastewater, it may substantially increase the proportions of ARB. This result is in agreement with a subsequent work showing that the chlorination process can increase the risk of selecting for highly tetracycline-resistant *E. coli* strains (Huang et al., in press). In particular, the inactivation of tetracycline-resistant *E. coli* was found significantly lower than that of antibiotic-sensitive *E. coli* at high chlorine doses (>1.0 mg $Cl_2\,L^{-1}$, 10 min contact time). The opposite result was observed for ampicillin- and trimethoprim-resistant *E. coli* strains (Templeton et al., 2009); according to the authors' conclusions, their results suggest that chlorination process is unlikely to select for ampicillin- or trimethoprim-resistant survivors during water and wastewater treatment. They investigated the effect of free chlorine and ultraviolet (UV intensity 0.247 mW cm^{-2}) disinfection on *E. coli* strains resistant to ampicillin and trimethoprim, in comparison to an antibiotic-susceptible strain of *E. coli* isolated from sewage sludge. Trimethoprim-resistant *E. coli* was found to be slightly more resistant to chlorine than the antibiotic-susceptible isolate and the ampicillin-resistant *E. coli*, under the studied conditions (95% confidence). Moreover, no statistically significant differences between the UV dose–response profiles of the antibiotic-resistant and antibiotic-susceptible *E. coli* strains over the UV dose range tested were observed.

Antibiotic resistant *E. coli* and other coliforms were investigated in an UWTP in Tokyo Metropolitan Prefecture (Iwane et al., 2001). *E. coli* strains, randomly isolated from wastewater samples, were tested for their sensitivity to seven antimicrobial agents in three different UWTP locations: the inflow, before chlorination and after chlorination. Chlorination treatment did not significantly affect the percentage of resistance in *E. coli* to one or more antibiotics (from 14.7 to 14.0%) or specifically to ampicillin (constant at 7.3%) and tetracycline (from 8.0 to 6.7%).

In a study with enterobacteria isolated from raw (189 isolates) and treated (156 isolates) wastewater of an UWTP during 18 months, the susceptibility to antibiotics (amoxicillin, gentamicin, ciprofloxacin, sulfamethoxazole/trimethoprim, tetracycline and cephalothin), disinfectants (hydrogen peroxide, sodium hypochlorite, quaternary ammonium/formaldehyde and iodine), and heavy metals (nickel, cadmium, chromium, mercury and zinc) was investigated (Ferreira da Silva et al., 2007). The authors did not find any significant positive correlation among antibiotic, disinfectant and heavy metal resistance. However, a positive correlation was observed between mercury and tetracycline and sulfamethoxazole/trimethoprim resistance. On other studies, two activated sludge UWTPs were investigated as possible sources of tetracycline resistance via qualitative PCR and qPCR. According to Auerbach et al. (2007), UV disinfection (at a dosage of 30,100 mWs cm^{-2}) did not promote the reduction of the number of detectable *tetR* gene types. Also Munir et al. (2011) investigated the effect of five different UWTPs located in Michigan (U.S.A.) on the occurrence and release of ARGs and ARB into the environment. In particular, the investigators found out that disinfection by chlorination and UV radiation processes did not significantly reduce ARGs and ARB; the statistical *t*-test between concentrations of ARGs in pre- and post disinfected effluent did not show any significant difference between UV and chlorination disinfection processes ($p>0.05$). Moreover, plasmid DNA was treated by chlorine disinfection in laboratory scale tests and the subsequent oxidative damages were analysed in gel electrophoresis by comparing the extent of conformational changes in the DNA structure (Öncü et al., 2011); the authors found out that the chlorination process does not affect plasmid DNA at the studied chlorine doses (0.5–5.0 mg L^{-1}). In another recent study, when 2.0 mg L^{-1} chlorine dose was used to investigate the inactivation of multi-drug resistant *E. coli* strains selected from an UWTP effluent, the number of colonies decreased by 99.999% after 60 min of contact time (Rizzo et al., 2012). However, the minimum inhibitory concentration to the antibiotics amoxicillin, ciprofloxacin and sulfametoxazole was not altered for the surviving cultures. On the other hand, Suquet et al. (2010) reported that CT (disinfectant residual concentration multiplied by the contact time) values higher than ~180 mg L^{-1} min^{-1} were required in 50 mM phosphate buffer pH 7.4, for chlorine to produce detectable genomic fragmentation of *E. coli* DNA. In light of the available data, the effect of chlorine on DNA of bacterial cells may be achieved only for quite high disinfectant dose or CT values compared to those typically used in wastewater disinfection (Dodd, 2012).

4. Concluding remarks

The main conclusions that can be withdrawn based on the scientific literature available on the fate of ARB and ARGs in UWTPs, can be summarized as follows:

- In spite of the lack of information as well as systematic and conclusive studies on the effect of biological process on antibiotic resistance, some studies showed that conventional UWTPs may positively affect ARB spread and selection as well as ARG transfer. Moreover, general trends of antibiotic resistance in relevant human-related environmental bacteria isolated from UWTP have been identified.
- Demonstrating ARG transfer in complex environments remains a difficult task, although indispensable for an adequate risk assessment of resistance spread in UWTPs. In this respect, an adequate tool may involve the use of a specific model-element, allowing the pinpointing of environmental parameters affecting the transfer of specific elements, that can later on be evaluated in real conditions (environmental or in processes) by measuring how these parameters affect the occurrence of ARG or carrier structures such as RIs.
- All known types of antibiotic resistance mechanisms are represented in UWTP, suggesting the relevance of these facilities as reservoirs and environmental suppliers of genetic determinants of resistance.
- Advanced treatment technologies and disinfection processes are regarded as possible tools to control the spread of ARB into the environment because they can effectively decrease the number of ARB, but further studies are necessary to better explain their effect on ARB selection before recommending their use as possible solution to control resistance spread into the environment.

Moreover, according to the reviewed studies, several questions have not yet been addressed. In our opinion, the following are among the most relevant research questions that should be addressed:

- Aiming on the reduction of dissemination of clinically-relevant ARB, culture-based and molecular-based approaches should be defined to survey the effluents from UWTPs for the occurrence of ARB and their genes as well as to track their spread in the environment (e.g., rivers, lakes, soils, sediments).

- Next generation sequencing is a valuable tool to complement conventional PCR and qPCR, but extended genome data from environmental reference bacteria should be implemented in data bank entries.
- Various investigations have been mainly focused on the selection and relative prevalence of ARB and ARG transfer in UWTP irrespective of the biological process, technology and operating parameters. Therefore, this issue is worth of investigation to understand the mechanisms driving the development of ARB and ARGs transfer in order to control/minimize their spread. In this regard, because of the lack of studies, the investigation of the effect of advanced treatment technologies and disinfection on the control of ARB and ARGs transfer is desirable because, irrespective of biological process, they may be operated/focused on ARB inactivation and ARGs transfer control.
- Further and systematic studies on the effect of conventional (e.g., chlorination and UV radiation) and new/alternative disinfection processes on the inactivation of specific ARB as well as the capacity to control resistance spread into the environment are strongly recommended because the few studies available show that, despite an effective decrease of the total number of bacteria, they may simultaneously promote the selection of ARB.
- A public dataset with information such as i) antibiotic resistant bacteria and their phylogenetic lineages; ii) antibiotic resistance genes and respective nucleotide sequences and genetic environment; iii) sampled sites and major characteristics, would represent a valuable tool to a better understanding of antibiotic resistance ecology and control measures.

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References

Alexandrino M, Grohmann E, Szewzyk R, Szewzyk U. Application of culture-independent methods to assess the bacteria removal efficiency of subsurface flow constructed wetlands. Water Sci Technol 2007;56(3):217–22.
Andrews JM. BSAC standardized disc susceptibility testing method (version 8). J Antimicrob Chemother 2009;64:454–89.
APHA. Standard methods for the examination of water and waste water. 21st ed. Washington, DC: American Public Health Association; 2005.
Araújo C, Torres C, Silva N, Carneiro C, Gonçalves A, Radhouani H, et al. Vancomycin-resistant enterococci from Portuguese waste water treatment plants. J Basic Microbiol 2010;50:605–9.
Armstrong JL, Calomaris JJ, Seidler RJ. Selection of antibiotic-resistant standard plate count bacteria during water treatment. Appl Environ Microbiol 1982;44:308–16.
Auerbach EA, Seyfried EE, McMahon KD. Tetracycline resistance genes in activated sludge wastewater treatment plants. Water Res 2007;41:1143–51.
Baquero F, Martínez J-L, Cantón R. Antibiotics and antibiotic resistance in water environments. Curr Opin Biotechnol 2008;19(3):260–5.
Barlow RS, Fegan N, Gobius KS. Integron-containing bacteria in faeces of cattle from different production systems at slaughter. J Appl Microbiol 2009;107:540–5.
Barraud O, Ploy M-C. Actualités sur les intégrons de résistance aux antibiotiques: mises au point. J Anti-infectieux 2011;13:133–44.
Batt AL, Snow DD, Aga DS. Occurrence of sulphonamide antimicrobials in private water wells in Washington County, Idaho, USA. Chemosphere 2006;64:1963–71.
Boczek LA, Rice EW, Johnston B, Johnson JR. Occurrence of antibiotic-resistant uropathogenic *Escherichia coli* clonal group A in waste water effluents. Appl Environ Microbiol 2007;73:4180–4.
Bönemann G, Stiens M, Puhler A, Schluter A. Mobilizable IncQ-related plasmid carrying a new quinolone resistance gene, qnrS2, isolated from the bacterial community of a wastewater treatment plant. Antimicrob Agents Chemother 2006;50:3075–80.
Bonot S. Persistance et dissémination du plasmide pB10, vecteur de gènes de résistance aux antibiotiques, dans des biomasses issues de stations d'épuration d'eaux usées urbaines [dissertation]. Laboratoire de Chimie Physique et Microbiologie pour l'Environnement, UMR 7564 CNRS: Université Henri Poincaré, Nancy-Université; 2010.
Bonot S, Merlin C. Monitoring the dissemination of the broad-host-range plasmid pB10 in sediment microcosms by quantitative PCR. Appl Environ Microbiol 2010;76(1):378–82.
Börjesson S, Melin S, Matussek A, Lindgren P-E. A seasonal study of the mecA gene and *Staphylococcus aureus* including methicillin-resistant *S. aureus* in a municipal wastewater treatment plant. Water Res 2009;43:925–32.
Brown KD, Kulis J, Thomson B, Chapman TH, Mawhinney DB. Occurrence of antibiotics in hospital, residential, and dairy effluent, municipal wastewater, and the Rio Grande in New Mexico. Sci Total Environ 2006;366:772–83.
Cadotte MW, Drake JA, Fukami T. Constructing nature: laboratory models as necessary tools for investigating complex ecological communities. Adv Ecol Res 2005;37:333–53.
Cambray G, Guerout AM, Mazel D. Integrons. Annu Rev Genet 2010;44:141–66.
CA-SFM [internet]. Société Francaise de Microbiologie. [30 August]. Available from: http://www.sfm-microbiologie.org/pages/?all=accueil 2011.
Cengiz M, Uslu MO, Balcioglu I. Treatment of *E. coli* HB101 and the tetM gene by Fenton's reagent and ozone in cow manure. J Environ Manage 2010;91:2590–3.
CLSI [internet]. Clinical and Laboratory Standards Institute. http://www.clsi.org/.
CLSI, Clinical Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing; M100-S17, vol. 27; 2007 (Wayne, PA).
Colomer-Lluch M, Jofre J, Muniesa M. Antibiotic resistance genes in the bacteriophage DNA fraction of environmental samples. PLoS One 2011;6:e17549.
Courvalin P. Predictable and unpredictable evolution of antibiotic resistance. J Intern Med 2008;264(1):4-16.
Czekalski N, Berthold T, Caucci S, Egli A, Burgmann H. Increased levels of multiresistant bacteria and resistance genes after wastewater treatment and their dissemination into Lake Geneva, Switzerland. Front Microbiol 2012;3:106.
Dahlberg C, Bergström M, Andreasen M, Christensen BB, Molin S, Hermansson M. Interspecies bacterial conjugation by plasmids from marine environments visualized by gfp expression. Mol Biol Evol 1998a;15(4):385–90.
Dahlberg C, Bergström M, Hermansson M. In situ detection of high levels of horizontal plasmid transfer in marine bacterial communities. Appl Environ Microbiol 1998b;64(7):2670–5.
Daikos GL, Kosmidis C, Tassios PT, Petrikkos G, Vasilakopoulou A, Psychogiou M, et al. Enterobacteriaceae bloodstream infections: presence of integrons, risk factors, and outcome. Antimicrob Agents Chemother 2007;51:2366–72.
DANMAP [internet]. Danish Integrated Antimicrobial Resistance Monitoring and Research Programme. [30 August]. Available from:http://www.danmap.org/2011.
David MZ, Daum RS. Community-associated methicillin-resistant *Staphylococcus aureus*: epidemiology and clinical consequences of an emerging epidemic. Clin Microbiol Rev 2010;23(3):616–87.
Davies J. Microbes have the last word. A drastic reevaluation of antimicrobial treatment is needed to overcome the threat of antibiotic-resistant bacteria. EMBO Rep 2007;8(7):616–21.
Davies J, Davies D. Origins and evolution of antibiotic resistance. Microbiol Mol Biol Rev 2010;74(3):417–33.
Davies J, Spiegelmann G, Yim G. The world of subinhibitory antibiotic concentration. Curr Opin Microbiol 2006;9:445–53.
De Gelder L, Vandecasteele FP, Brown CJ, Forney LJ, Top EM. Plasmid donor affects host range of promiscuous IncP-1β plasmid pB10 in an activated-sludge microbial community. Appl Environ Microbiol 2005;71(9):5309–17.
Demanèche S, Bertolla F, Buret F, Nalin R, Sailland A, Auriol P, et al. Laboratory-scale evidence for lightning-mediated gene transfer in soil. Appl Environ Microbiol 2001;67(8):3440–4.
Deurenberg RH, Stobberingh EE. The evolution of *Staphylococcus aureus*. Infect Genet Evol 2008;8(6):747–63.
Díaz-Cruz MS, Lopez de Alda MJ, Barcelo D. Environmental behavior and analysis of veterinary and human drugs in soils, sediments and sludge. TrAC Trends Anal Chem 2003;22(6):340–51.
Diehl DL, Lapara TM. Effect of temperature on the fate of genes encoding tetracycline resistance and the integrase of class 1 integrons within anaerobic and aerobic digesters treating municipal wastewater solids. Environ Sci Technol 2010;44:9128–33.
Dobrindt U, Hochhut B, Hentschel U, Hacker J. Genomic islands in pathogenic and environmental microorganisms. Nat Rev Microbiol 2004;2(5):414–24.
Dodd MC. Potential impacts of disinfection processes on elimination and deactivation of antibiotic resistance genes during water and wastewater treatment. J Environ Monit 2012;14:1754–71.
Dröge M, Pühler A, Selbitschka W. Phenotypic and molecular characterization of conjugative antibiotic resistance plasmids isolated from bacterial communities of activated sludge. Mol Gen Genet 2000;263(3):471–82.

EC. 98/83/EC CD of 3 November 1998 on the quality of water intended for human consumption as amended by regulation 1882/2003/EC; 1998.
ECDC, European Centre for Disease Prevention, Control. [21 December]http://ecdc.europa.eu/en/healthtopics/antimicrobial_resistance/Pages/index.aspx2012.
EUCAST, European Committee on Antimicrobial Susceptibility Testing. [30 October] http://www.eucast.org/2011.
Fars S, Oufdou K, Nejmeddine A, Hassani L, Ait MA, Bousselhaj K, et al. Antibiotic resistance and survival of faecal coliforms in activated sludge system in a semi-arid region (Beni Mellal, Morocco). World J Microbiol Biotechnol 2005;21:493–500.
Ferreira da Silva M, Tiago I, Verissimo A, Boaventura RAR, Nunes OC, Manaia CM. Antibiotic resistance of enterococci and related bacteria in an urban waste water treatment plant. FEMS Microbiol Ecol 2006;55:322–9.
Ferreira da Silva M, Vaz-Moreira I, Gonzalez-Pajuelo M, Nunes OC, Manaia CM. Antimicrobial resistance patterns in Enterobacteriaceae isolated from an urban waste water treatment plant. FEMS Microbiol Ecol 2007;60:166–76.
Figueira V, Serra E, Manaia CM. Differential patterns of antimicrobial resistance in population subsets of *Escherichia coli* isolated from waste- and surface waters. Sci Total Environ 2011a;409:1017–23.
Figueira V, Vaz-Moreira I, Silva M, Manaia CM. Diversity and antibiotic resistance of *Aeromonas* spp. in drinking and waste water treatment plants. Water Res 2011b;45:5599–611.
Gajan EB, Abashov R, Aghazadeh M, Eslami H, Oskouei SG, Mohammadnejad D. Vancomycin-resistant *Enterococcus faecalis* from a wastewater treatment plant in Tabriz, Iran. Pak J Biol Sci 2008;11:2443–6.
Galvin S, Boyle F, Hickey P, Vellinga A, Morris D, Cormican M. Enumeration and characterization of antimicrobial-resistant *Escherichia coli* bacteria in effluent from municipal, hospital, and secondary treatment facility sources. Appl Environ Microbiol 2010;76:4772–9.
Gaze WH, Zhang L, Abdouslam NA, Hawkey PM, Calvo-Bado L, Royle J, et al. Impacts of anthropogenic activity on the ecology of class 1 integrons and integron-associated genes in the environment. ISME J 2011;5:1253–61.
Gealt MA, Chai MD, Alpert KB, Boyer JC. Transfer of plasmids pBR322 and pBR325 in wastewater from laboratory strains of *Escherichia coli* to bacteria indigenous to the waste disposal system. Appl Environ Microbiol 1985;49(4):836–41.
Geisenberger O, Ammendola A, Christensen BB, Molin S, Schleifer K-H, Eberl L. Monitoring the conjugal transfer of plasmid RP4 in activated sludge and in situ identification of the transconjugants. FEMS Microbiol Lett 1999;174(1):9-17.
Gerrity D, Gamage S, Holady JC, Mawhinney DB, Quiñones O, Trenholm RA, et al. Pilot-scale evaluation of ozone and biological activated carbon for trace organic contaminant mitigation and disinfection. Water Res 2011;45:2155–65.
Ghosh S, Ramsden SJ, LaPara TM. The role of anaerobic digestion in controlling the release of tetracycline resistance genes and class 1 integrons from municipal wastewater treatment plants. Appl Microbiol Biotechnol 2009;84:791–6.
Gillings MR, Krishnan S, Worden PJ, Hardwick SA. Recovery of diverse genes for class 1 integron-integrases from environmental DNA samples. FEMS Microbiol Lett 2008;287:56–62.
Gibson K, Schwab K, Spencer S, Borchardt M. Measuring and mitigating inhibition during quantitative real time PCR analyses of viral nucleic acid extracts from large volume environmental water samples. Water Res 2012;46:4281–91.
Grabow WOK, van Zyl M, Prozesky OW. Behaviour in conventional sewage purification processes of coliform bacteria with transferable or non-transferable drug-resistance. Water Res 1976;10:717–23.
Guardabassi L, Lo Fo Wong DMA, Dalsgaard A. The effects of tertiary wastewater treatment on the prevalence of antimicrobial resistant bacteria. Water Res 2002;36:1955–64.
Guillaume G, Verbrugge D, Chasseur-Libotte M, Moens W, Collard J. PCR typing of tetracycline resistance determinants (Tet A–E) in *Salmonella enterica* serotype Hadar and in the microbial community of activated sludges from hospital and urban wastewater treatment facilities in Belgium. FEMS Microbiol Ecol 2000;32:77–85.
Guo X, Xia R, Han N, Xu H. Genetic diversity analyses of class 1 integrons and their associated antimicrobial resistance genes in Enterobacteriaceae strains recovered from aquatic habitats in China. Lett Appl Microbiol 2011;52:667–75.
Haagensen JA, Hansen SK, Johansen T, Molin S. In situ detection of horizontal transfer of mobile genetic elements. FEMS Microbiol Ecol 2002;42(2):261–8.
Hall RM, Collis CM. Mobile gene cassettes and integrons: capture and spread of genes by site-specific recombination. Mol Microbiol 1995;15:593–600.
Heuer H, Szczepanowski R, Schneiker S, Puhler A, Top EM, Schluter A. The complete sequences of plasmids pB2 and pB3 provide evidence for a recent ancestor of the IncP-1b group without any accessory genes. Microbiology 2004;150:3591–9.
Heuer H, Schmitt H, Smalla K. Antibiotic resistance gene spread due to manure application on agricultural fields. Curr Opin Microbiol 2011;14:236–43.
Huang J-J, Hua H-Y, Wua Y-H, Wei B, Lu Y. Effect of chlorination and ultraviolet disinfection on tetA-mediated tetracycline resistance of *Escherichia coli*. Chemosphere (in press). http://dx.doi.org/10.1016/j.chemosphere.2012.10.008.
Inoue D, Sei K, Soda S, Ike M, Fujita M. Potential of predominant activated sludge bacteria as recipients in conjugative plasmid transfer. J Biosci Bioeng 2005;100(6):600–5.
Iwane T, Urase T, Yamamoto K. Possible impact of treated wastewater discharge on incidence of antibiotic resistant bacteria in river water. Water Sci Technol 2001;43(2):91–9.
Jungfer C, Schwartz T, Obst U. UV-induced dark repair mechanisms in bacteria associated with drinking water. Water Res 2007;41:188–96.
Kemper N. Veterinary antibiotics in the aquatic and terrestrial environment. Ecol Indic 2008;8:1-13.
Khan IUH, Yadav JS. Real-time PCR assays for genus-specific detection and quantification of culturable and non-culturable mycobacteria and pseudomonads in metalworking fluids. Mol Cell Probes 2004;18:67–73.
Kim S, Jensen JN, Aga DS, Weber AS. Tetracycline as a selector for resistant bacteria in activated sludge. Chemosphere 2007a;66:1643–51.
Kim S, Jensen JN, Aga DS, Weber AS. Fate of tetracycline resistant bacteria as a function of activated sludge process organic loading and growth rate. Water Sci Technol 2007b;55(1–2):291–7.
Kowalchuk GA, de Bruijn FJ, Head IM, Akkermans ADL, van Elsas JD. Molecular Microbial Ecology Manual. Kluwer Academic Publishers; 2004.
Kraft S, Obst U, Schwartz T. Immunological detection of UV induced cyclobutane pyrimidine dimers and (6–4) photoproducts in DNA from reference bacteria and natural aquatic populations. J Microbiol Methods 2011;84(3):435–41.
Kristiansson E, Fick J, Janzon A, Grabic R, Rutgersson C, Weijdegård B, et al. Pyrosequencing of antibiotic-contaminated river sediments reveals high levels of resistance and gene transfer elements. PLoS One 2011;6:e17038.
Kümmerer K. Antibiotics in the aquatic environment—a review—part II. Chemosphere 2009;75:435–41.
Lachmayr KL, Kerkhof LJ, Dirienzo AG, Cavanaugh CM, Ford TE. Quantifying nonspecific TEM beta-lactamase (blaTEM) genes in a waste water stream. Appl Environ Microbiol 2009;75:203–11.
Lal S, Cheema S, Kalia VC. Phylogeny vs genome reshuffling: horizontal gene transfer. Indian J Microbiol 2008;48(2):228–42.
LaPara TM, Burch TR, McNamara PJ, Tan DT, Yan M, Eichmiller JJ. Tertiary-treated municipal wastewater is a significant point source of antibiotic resistance genes into Duluth-Superior Harbor. Environ Sci Technol 2011;45:9543–9.
Liu B, Pop M. ARDB—Antibiotic Resistance Genes Database. Nucleic Acids Res 2009;37:D443–7. [Database issue].
Łuczkiewicz A, Jankowska K, Fudala-Książek S, Olańczuk-Neyman K. Antimicrobial resistance of fecal indicators in municipal wastewater treatment plant. Water Res 2010;44:5089–97.
Luo Y, Mao D, Rysz M, Zhou Q, Zhang H, Xu L, et al. Trends in antibiotic resistance genes occurrence in the Haihe River, China. Environ Sci Technol 2010;44:7220–5.
Lupo A, Coyne S, Berendonk T. Origin and evolution of antibiotic resistance: the common mechanism of emergence and spread in water bodies. Front Microbiol 2012;3:18.
Ma L, Zhang XX, Cheng S, Zhang Z, Shi P, Liu B, et al. Occurrence, abundance and elimination of class 1 integrons in one municipal sewage treatment plant. Ecotoxicology 2011;20:968–73.
Mach PA, Grimes DJ. R-plasmid transfer in a wastewater treatment plant. Appl Environ Microbiol 1982;44(6):1395–403.
Madaeni SS. The application of membrane technology for water disinfection. Water Res 1999;33:301–8.
Maillard JY, Hann AC, Perrin R. Resistance of *Pseudomonas aeruginosa* PAO1 phage F116 to sodium hypochlorite. J Appl Microbiol 1998;85:799–806.
Malato S, Fernández-Ibáñez P, Maldonado MI, Blanco J, Gernjak W. Decontamination and disinfection of water by solar photocatalysis: recent overview and trends. Catal Today 2009;147:1-59.
Manaia CM, Vaz-Moreira I, Nunes OC. Antibiotic resistance in waste- and surface waters and human health implications. In: Barceló D, editor. Emerging organic contaminants and human health. Springer; 2012. p. 173–212.
Marcinek H, Wirth R, Muscholl-Silberhorn A, Gauer M. *Enterococcus faecalis* gene transfer under natural conditions in municipal sewage water treatment plants. Appl Environ Microbiol 1998;64:626–32.
Martin C, Timm J, Rauzier J, Gomez-Lus R, Davies J, Gicquel B. Transposition of an antibiotic resistance element in mycobacteria. Nature 1990;345:739–43.
Martins da Costa P, Vaz-Pires P, Bernardo F. Antimicrobial resistance in *Enterococcus* spp. isolated in inflow, effluent and sludge from municipal sewage water treatment plants. Water Res 2006;40:1735–40.
Mazel D. Integrons: agents of bacterial evolution. Nat Rev Microbiol 2006;4:608–20.
Merlin C, Bonot S, Courtois S, Block JC. Persistence and dissemination of the multiple-antibiotic-resistance plasmid pB10 in the microbial communities of wastewater sludge microcosms. Water Res 2011;45:2897–905.
Mezrioui N, Baleux B. Resistance patterns of *E. coli* strains isolated from domestic sewage before and after treatment in both aerobic lagoon and activated sludge. Water Res 1994;28:2399–406.
Michael I, Hapeshi E, Michael C, Varela AR, Kyriakou S, Manaia CM, et al. Solar photo-Fenton process on the abatement of antibiotics at a pilot scale: degradation kinetics, ecotoxicity and phytotoxicity assessment and removal of antibiotic resistant enterococci. Water Res 2012;46:5621–34.
Miriagou V, Carattoli A, Fanning S. Antimicrobial resistance islands: resistance gene clusters in Salmonella chromosome and plasmids. Microbes Infect 2006;8(7):1923–30.
Moura A, Henriques I, Ribeiro R, Correia A. Prevalence and characterization of integrons from bacteria isolated from a slaughterhouse wastewater treatment plant. J Antimicrob Chemother 2007;60:1243–50.
Moura A, Henriques I, Smalla K, Correia A. Wastewater bacterial communities bring together broad-host range plasmids, integrons and a wide diversity of uncharacterized gene cassettes. Res Microbiol 2010;161:58–66.
Munir M, Wong K, Xagoraraki I. Release of antibiotic resistant bacteria and genes in the effluent and biosolids of five wastewater utilities in Michigan. Water Res 2011;45:681–93.
Murray GE, Tobin RS, Junkins B, Kushner DJ. Effect of chlorination on antibiotic resistance profiles of sewage related bacteria. Appl Environ Microbiol 1984;48:73–7.
Musovic S, Dechesne A, Sørensen J, Smets BF. Novel assay to assess permissiveness of a soil microbial community toward receipt of mobile genetic elements. Appl Environ Microbiol 2010;76(14):4813–8.

Nancharaiah YV, Wattiau P, Wuertz S, Bathe S, Mohan SV, Wilderer PA, et al. Dual labeling of *Pseudomonas putida* with fluorescent proteins for in situ monitoring of conjugal transfer of the TOL plasmid. Appl Environ Microbiol 2003;69(8): 4846–52.
Nandi S, Maurer JJ, Hofacre C, Summers AO. Gram-positive bacteria are a major reservoir of class1 antibiotic resistance integrons in poultry litter. Proc Natl Acad Sci U S A 2004;101:7118–22.
Nesvera J, Hochmannová J, Pátek M. An integron of class 1 is present on the plasmid pCG4 from Gram-positive bacterium *Corynebacterium glutamicum*. FEMS Microbiol Lett 1998;169:391–5.
Novick RP, Schlievert P, Ruzin A. Pathogenicity and resistance islands of staphylococci. Microbes Infect 2001;3(7):585–94.
Novo A, Manaia CM. Factors influencing antibiotic resistance burden in municipal waste water treatment plants. Appl Microbiol Biotechnol 2010;87(3):1157–66.
Novo A, André S, Viana P, Nunes OC, Manaia CM. Antibiotic resistance, antimicrobial residues and bacterial community composition in urban wastewater. Water Res in press. http://dx.doi.org/10.1016/j.watres.2013.01.010.
Oguma K, Katayama H, Mitani H, Morita S, Hirata T, Ohgaki S. Determination of pyrimidine dimers in *Escherichia coli* and *Cryptosporidium parvum* during UV light inactivation, photoreactivation, and dark repair. Appl Microbiol Biotechnol 2001;67:4630–7.
Okoh AI, Igbinosa EO. Antibiotic susceptibility profiles of some Vibrio strains isolated from waste water final effluents in a rural community of the Eastern Cape Province of South Africa. BMC Microbiol 2010;10:143–8.
Oliver JD. The viable but nonculturable state in bacteria. J Microbiol 2005;43:93-100.
Oliver JD. Recent findings on the viable but nonculturable state in pathogenic bacteria. FEMS Microbiol Rev 2010;34:415–25.
Öncü NB, Menceloğlu YZ, Balcıoğlu IA. Comparison of the effectiveness of chlorine, ozone, and photocatalytic disinfection in reducing the risk of antibiotic resistance pollution. J Adv Oxid Technol 2011;14:196–203.
Parsley LC, Consuegra EJ, Kakirde KS, Land AM, Harper WF, Liles MR. Identification of diverse antimicrobial resistance determinants carried on bacterial, plasmid, or viral metagenomes from an activated sludge microbial assemblage. Appl Environ Microbiol 2010;76:3753–7.
Partridge SR. Analysis of antibiotic resistance regions in Gram-negative bacteria. FEMS Microbiol Rev 2011;35(5):820–55.
Partridge SR, Tsafnat G, Coiera E, Iredell JR. Gene cassettes and cassette arrays in mobile resistance integrons. FEMS Microbiol Rev 2009;33:757–84.
Paul T, Dodd MC, Strathmann TJ. Photolytic and photocatalytic decomposition of aqueous ciprofloxacin: transformation products and residual antibacterial activity. Water Res 2010;44:3121–32.
Pontiroli A, Rizzi A, Simonet P, Daffonchio D, Vogel TM, Monier JM. Visual evidence of horizontal gene transfer between plants and bacteria in the phytosphere of transplastomic tobacco. Appl Environ Microbiol 2009;75(10):3314–22.
Pyle BH, Watters SK, McFeters GA. Physiological aspects of disinfection resistance in *Pseudomonas cepacia*. J Appl Bacteriol 1994;76:142–8.
Reinthaler FF, Posch J, Feierl G. Antibiotic resistance of *E. coli* in sewage and sludge. Water Res 2003;37:1685–90.
Ridgway HF, Olson BH. Chlorine resistance patterns of bacteria from two drinking water distribution systems. Appl Environ Microbiol 1982;44:972–87.
Rizzi A, Pontiroli A, Brusetti L, Borin S, Sorlini C, Abruzzese A, et al. Strategy for in situ detection of natural transformation-based horizontal gene transfer events. Appl Environ Microbiol 2008;74(4):1250–4.
Rizzo L, Belgiorno V, Napoli RMA. Regrowth evaluation of coliform bacteria injured by low chlorine doses using selective and nonselective media. J Environ Sci Health A 2004;39(8):2081–92.
Rizzo L, Fiorentino A, Anselmo A. Effect of solar radiation on multidrug resistant *E. coli* strains and antibiotic mixture photodegradation in wastewater polluted stream. Sci Total Environ 2012;427–428:263–8.
Rosewarne CP, Pettigrove V, Stokes HW, Parsons YM. Class 1 integrons in benthic bacterial communities: abundance, association with tn402-like transposition modules and evidence for coselection with heavy-metal resistance. FEMS Microbiol Ecol 2010;72:35–46.
Sabate M, Prats G, Moreno E. Virulence and antimicrobial resistance profiles among *Escherichia coli* strains isolated from human and animal waste water. Res Microbiol 2008;159(4):288–93.
Sanapareddy N, Hamp TJ, Gonzalez LC, Hilger HA, Fodor AA, Clinton SM. Molecular diversity of a North Carolina waste water treatment plant as revealed by pyrosequencing. Appl Environ Microbiol 2009;75:1688–96.
Schlüter A, Heuer H, Szczepanowski R, Forney LJ, Thomas CM, Puhler A, et al. The 64,508 bp IncP-1b antibiotic multiresistance plasmid pB10 isolated from a wastewater treatment plant provides evidence for recombination between members of different branches of the IncP-1b group. Microbiology 2003;149:3139–53.
Schlüter A, Szczepanowski R, Kurz N, Schneiker S, Krahn I, Puhler A. Erythromycin resistance-conferring plasmid pRSB105, isolated from a sewage treatment plant, harbors a new macrolide resistance determinant, an integron-containing Tn402-like element, and a large region of unknown function. Appl Environ Microbiol 2007;73: 1952–60.
Schlüter A, Krause L, Szczepanowski R, Goesmann A, Pühler A. Genetic diversity and composition of a plasmid metagenome from a wastewater treatment plant. J Biotechnol 2008;136(1–2):65–76.
Schwartz T. Strategies to assess and minimize the biological risk of antibiotic resistance in the environment. In: Keen P, Montforts M, editors. Antimicrobial resistance in the environment. John Wiley & Sons; 2012. p. 251–64.
Schwartz T, Kohnen W, Jansen B, Obst O. Detection of antibiotic-resistant bacteria and their resistance genes in wastewater, surface water, and drinking water biofilms. FEMS Microbiol Ecol 2003;43:325–35.
Schwartz T, Volkmann H, Kirchen S, Kohnen K, Schön-Hölz K, Jansen B, et al. Real-time PCR detection of *Pseudomonas aeruginosa* in clinical and municipal wastewater and genotyping of the ciprofloxacin-resistant isolates. FEMS Microbiol Ecol 2006;57: 158–67.
Shrivastava R, Upreti RK, Jain SR, Prasad KN, Seth PK, Chaturvedi UC. Suboptimal chlorine treatment of drinking water leads to selection of multidrug-resistant *Pseudomonas aeruginosa*. Ecotoxicol Environ Saf 2004;58:277–83.
Skurnik D, Le Menach A, Zurakowski D, Mazel D, Courvalin P, Denamur E, et al. Integron-associated antibiotic resistance and phylogenetic grouping of *Escherichia coli* isolates from healthy subjects free of recent antibiotic exposure. Antimicrob Agents Chemother 2005;49:3062–5.
Skurnik D, Ruimy R, Andremont A, Amorin C, Rouquet P, Picard B, et al. Effect of human vicinity on antimicrobial resistance and integrons in animal faecal *Escherichia coli*. J Antimicrob Chemother 2006;57:1215–9.
Smith MS, Yang RK, Knapp CW, Niu Y, Peak N, Hanfelt MM, et al. Quantification of tetracycline resistance genes in feedlot lagoons by real-time PCR. Appl Environ Microbiol 2004;70:7372–7.
Soda S, Otsuki H, Inoue D, Tsutsui H, Sei K, Ike M. Transfer of antibiotic multiresistant plasmid RP4 from *Escherichia coli* to activated sludge bacteria. J Biosci Bioeng 2008;106(3):292–6.
Soge OO, Meschke JS, No DB. Characterization of methicillin-resistant *Staphylococcus aureus* and methicillin-resistant coagulase-negative *Staphylococcus* spp. isolated from US West Coast public marine beaches. J Antimicrob Chemother 2009;64(6): 1148–55.
Sørensen SJ, Oregaard G, De Lipthay J, Kroer N. Plasmid transfer in aquatic environments. In: Kowalchuk GA, de Bruijn FJ, Head IM, Akkermans AD, van Elsas JD, editors. Microbial ecology manual. 2nd ed. the Netherlands: Kluwer Academic Publishers; 2004. p. 1081–108.
Sørensen SJ, Bailey M, Hansen LH, Kroer N, Wuertz S. Studying plasmid horizontal transfer in situ: a critical review. Nat Rev Microbiol 2005;3(9):700–10.
Stalder T, Barraud O, Casellas M, Dagot C, Ploy M-C. Integron involvement in environmental spread of antibiotic resistance. Front Microbiol 2012;3:119.
Stokes HW, Gillings MR. Gene flow, mobile genetic elements and the recruitment of antibiotic resistance genes into Gram-negative pathogens. FEMS Microbiol Rev 2011;35(5):790–819.
Suquet C, Warren JJ, Seth N, Hurst JK. Comparative study of HOCl-inflicted damage to bacterial DNA ex vivo and within cells. Arch Biochem Biophys 2010;493:135–42.
Szczepanowski R, Krahn I, Bohn N, Puhler A, Schluter A. Novel macrolide resistance module carried by the IncP-1β resistance plasmid pRSB111, isolated from a wastewater treatment plant. Antimicrob Agents Chemother 2007;51:673–8.
Szczepanowski R, Bekel T, Goesmann A, Krause L, Krömeke H, Kaiser O, et al. Insight into the plasmid metagenome of wastewater treatment plant bacteria showing reduced susceptibility to antimicrobial drugs analysed by the 454-pyrosequencing technology. J Biotechnol 2008;136(1–2):54–64.
Szczepanowski R, Linke B, Krahn I. Detection of 140 clinically relevant antibiotic-resistance genes in the plasmid metagenome of waste water treatment plant bacteria showing reduced susceptibility to selected antibiotics. Microbiology 2009;155(7):2306–19.
Szewzyk U, Szewzyk R, Manz W, Schleifer KH. Microbiological safety of drinking water. Annu Rev Microbiol 2000;54:81-127.
Templeton MR, Oddy F, Leung WK, Rogers M. Chlorine and UV disinfection of ampicillin-resistant and trimethoprim-resistant *Escherichia coli*. Can J Civ Eng 2009;36: 889–94.
Tennstedt T, Szczepanowski R, Braun S. Occurrence of integron-associated resistance gene cassettes located on antibiotic resistance plasmids isolated from a waste water treatment plant. FEMS Microbiol Ecol 2003;45(3):239–52.
Top EM, Springael D. The role of mobile genetic elements in bacterial adaptation to xenobiotic organic compounds. Curr Opin Biotechnol 2003;14(3):262–9.
Trevors J. Viable but not-culturable (VBNC) bacteria: gene expression in planktonic and biofilm cells. J Microbiol Methods 2011;86:266–73.
USEPA, United States Environmental Protection Agency. Combined sewer overflow technology fact sheet: chlorine disinfection. EPA 832-F-99-034, September http://water.epa.gov/scitech/wastetech/upload/2002_06_28_mtb_chlor.pdf1999.
Valentín-Vargas A, Toro-Labrador G, Massol-Deyá AA. Bacterial community dynamics in full-scale activated sludge bioreactors: operational and ecological factors driving community assembly and performance. PLoS One 2012;7(8):e42524.
Van den Broeck RM, Van Impe JF, Smets IY. Assessment of activated sludge stability in lab-scale experiments. J Biotechnol 2009;141(3–4):147–54.
Volkmann H, Schwartz T, Bischoff P, Kirchen S, Obst U. Detection of clinically relevant antibiotic-resistance genes in municipal wastewater using real-time PCR (TaqMan). J Microbiol Methods 2004;56:277–86.
Volkmann H, Schwartz T, Kirchen S, Carmen SC, Silke K, Obst U. Evaluation of inhibition and cross-reaction effects on real-time PCR applied to the total DNA of wastewater samples for the quantification of bacterial antibiotic resistance genes and taxon-specific targets. Mol Cell Probes 2007;21:125–33.
Watkinson AJ, Micalizzi GB, Graham GM. Antibiotic-resistant *Escherichia coli* in waste waters, surface waters, and oysters from an urban riverine system. Appl Environ Microbiol 2007;73(17):5667–70.
Weyant RS, Edmonds P, Swaminathan B. Effect of ionic and nonionic detergents on the Taq polymerase. Biotechniques 1990;9:308.
WHO. Guidelines for drinking water. 3rd ed. Geneva: World Health Organization; 2008.
Wiegand I, Hilpert K, Hancock RE. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nat Protoc 2008;3(2):163–75.
Wimpeny J, Manz W, Sewzayk U. Heterogeneity in biofilms. FEMS Microbiol Rev 2000;24:661–71.

Wright MS, Baker-Austin C, Lindell AH, Stepanauskas R, Stokes HW, McArthur JV. Influence of industrial contamination on mobile genetic elements: class 1 integron abundance and gene cassette structure in aquatic bacterial communities. ISME J 2008;2:417–28.

Xia R, Guo X, Zhang Y, Xu H. qnrVC-like gene located in a novel complex class 1 integron harboring the ISCR1 element in an *Aeromonas punctata* strain from an aquatic environment in Shandong Province, China. Antimicrob Agents Chemother 2010;54:3471–4.

Xu H, Davies J, Miao V. Molecular characterization of class3 integrons from *Delftia* spp. J Bacteriol 2007a;189:6276–83.

Xu W, Zhang G, Li X, Zou S, Li P, Hu Z, et al. Occurrence and elimination of antibiotics at four sewage treatment plants in the Pearl River Delta (PRD), South China. Water Res 2007b;41:4526–34.

Xu Z, Li L, Shirtliff ME, Peters BM, Peng Y, Alam MJ, et al. First report of class 2 integron in clinical *Enterococcus faecalis* and class 1 integron in *Enterococcus faecium* in South China. Diagn Microbiol Infect Dis 2010;68:315–7.

Zhang XX, Zhang T, Fang H. Antibiotic resistance genes in water environment. Appl Microbiol Biotechnol 2009a;82:397–414.

Zhang XX, Zhang T, Zhang M, Fang HHP, Cheng SP. Characterization and quantification of class 1 integrons and associated gene cassettes in sewage treatment plants. Appl Microbiol Biotechnol 2009b;82:1169–77.

Zhang Y, Marrs CF, Simon C, Xi C. Wastewater treatment contributes to selective increase of antibiotic resistance among *Acinetobacter* spp. Sci Total Environ 2009c;407:3702–6.

Zhang T, Zhang XX, Ye L. Plasmid metagenome reveals high levels of antibiotic resistance genes and mobile genetic elements in activated sludge. PLoS One 2011;6(10):e26041.

Zhang T, Shao MF, Ye L. 454-Pyrosequencing reveals bacterial diversity of activated sludge from 14 sewage treatment plants. ISME J 2012;6:1137–47.