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

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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

Wastewater

Antibiotics

Antibiotics resistant bacteria

Resistance gene transfer

Impact ?

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.

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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., 2009). The increasing interest of the scientific community and international institutions/organizations 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; Boczk 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 Enterococcus 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
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; Lückkiewicz 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.

For example, in the work of Figueira et al. (2011b), characterizing quinolone resistance in Enterococci 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; Lückkiewicz 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), charcterizing quinolone resistance in Aeromonas isolated from water habitats, the gene aacA6-ib-cr was detected mainly in bacteria isolated in the presence of ciprofloxacin.

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.

2.2.1. Antibiotic resistance genes

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 biocones (Lupo et al., 2012; Volkmann et al., 2004). Many studies have demonstrated the presence of clinically-relevant antibiotic resistance genes along the water cycle, 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 mepicillin resistance gene mecA of staphylococci, the β-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 ampC 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.
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 prim-
er and probe design for qPCR (X.X. Zhang et al., 2009a,b). In case of
under-represented ARG in the metagenome of complex microbial com-
munities, 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 anthropo-
genic 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). Further-
more, 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 tar-
get 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 ma-
trices like chemical ingredients, microorganism population composi-
tion 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
centralization 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 sequenc-
ing). It has to be mentioned that metagenome and transcriptome
analyses via next generation sequencing analyses need database
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 compartiments. In contrast to
the HTP approaches, the validity of more conventional techniques
(PCR, qPCR) to assess the fate of genetic markers for taxonomy
analyses via next generation sequencing analyses need databank
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 defini-
tion, 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 atti site are integrated
in or excised from the functional platform by a site-specific recombi-
nation 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; Nesyera et al., 1998; Xu et al., 2010). RIs contain a limited num-
ber of GCs (to date, up to 9) and are located on mobile genetic

### Table 1

<table>
<thead>
<tr>
<th>Wastewater pathways</th>
<th>City A</th>
<th>City B</th>
<th>City C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ampC</td>
<td>vanA</td>
<td>blu\textsubscript{am}</td>
</tr>
<tr>
<td>Housing area</td>
<td>8.9 × 10\textsuperscript{9}</td>
<td>–</td>
<td>3.0 × 10\textsuperscript{3}</td>
</tr>
<tr>
<td>Clinical wastewater</td>
<td>1.5 × 10\textsuperscript{4}</td>
<td>3.3 × 10\textsuperscript{8}</td>
<td>4.9 × 10\textsuperscript{4}</td>
</tr>
<tr>
<td>Inflow UWTP</td>
<td>4.6 × 10\textsuperscript{4}</td>
<td>+*</td>
<td>1.8 × 10\textsuperscript{4}</td>
</tr>
<tr>
<td>Effluent UWTP</td>
<td>1.6 × 10\textsuperscript{8}</td>
<td>–</td>
<td>1.6 × 10\textsuperscript{3}</td>
</tr>
</tbody>
</table>

* Detected, but not quantified.

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
with the non-cultivability of some ARG 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 ARG
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 detec-
tion of possibly rare events/sequences, especially in treatment pro-
cesses that do not favour the acquisition and spread of resistance
genesis in indigenous bacteria.

As discussed above, various approaches have been developed
based on the systematic search and enumeration of either ARG 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 dissemina-
tion should also rely on the detection of the common genetic struc-
tures 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 defini-
tion, an integron is structurally based on a functional platform
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2004; Nesyera et al., 1998; Xu et al., 2010). RIs contain a limited num-
ber of GCs (to date, up to 9) and are located on mobile genetic
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.
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, 1962; 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 pWVO transfers mostly occur at the periphery of the cell aggregate (Haagenes 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; Nancharaih 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; Kümmner, 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; Reinhpthaler 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 meticillin 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; Łuczkiwicz et al., 2010; Martins da Costa et al., 2006). The final effluent of UWTP can discharge about 10^6–10^12 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 cultivable fraction of the bacterial population is being considered, and it can represent only 1% of the total. Indeed, the numerous unculturulable bacteria thriving in wastewaters and related systems (sludge, biofilms) can host a myriad of antibiotic resistance genes (Szczepanowski et al., 2009; XX. 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,
choloramphenicol, fluoroquinolone, macrolides, rifampicin, tetracycline, trimethoprim and sulfonamide as well as efflux pumps) were detected (Szczeniowski 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 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 (Auerebach et al., 2007; Ferreira da Silva et al., 2006; Kim et al., 2007a; Łuczkiwicz 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; Łuczkiwicz 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 conduct 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>
<thead>
<tr>
<th>UWTP&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Biological process</th>
<th>ARB</th>
<th>Resistance to antibiotics&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>UWTP (150,700 inhabitants, 11,000 m&lt;sup&gt;3&lt;/sup&gt;/day) in Beni Mellal (Morocco) receives sewage of essentially domestic origin.</td>
<td>Activated sludge</td>
<td>Faecal coliforms</td>
<td>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 (50.3%), amoxicillin (42.3%), amoxicillin (42.3%) and amoxicillin–clavulanic acid (31.3%).</td>
<td>Fars et al. (2005)</td>
</tr>
<tr>
<td>UWTP (100,000 inhabitants) in northern Portugal, 70% domestic ww and 30% pretreated industrial ww.</td>
<td>Activated sludge</td>
<td>Enterococcus hirae, Enterococcus faecium and Enterococcus faecalis</td>
<td>Tetracycline (31.3%), gentamicin (50.5%), erythromycin (33.2%), ciprofloxacin (9.2%), amoxicillin (0.3%), sulfamethoxazole/trimethoprim (0.1%) and no resistance to vancomycin</td>
<td>Ferreira da Silva et al. (2006)</td>
</tr>
<tr>
<td>14 UWTPs in Portugal</td>
<td>N.A.</td>
<td>Enterococci</td>
<td>983 isolates: multidrug resistance (49.4%), rifampin (51.5%), tetracycline (34.6%), erythromycin (24.8%), nitrofurantoin (21.4%), ciprofloxacin (11.5%), amoxicillin (3.8%), vancomycin (0.6%) and no resistance to vancomycin</td>
<td>Martins da Costa et al. (2006)</td>
</tr>
<tr>
<td>Gdansk–Wschod’ UWTP (700,000 PE) in Northern Poland, mainly domestic ww, only limited industrial (5%) and undisinfected hospital (0.17%) ww</td>
<td>Activated sludge process working in modified UCT (University of Cape Town) type system</td>
<td>Enterococci</td>
<td>153 isolates: amoxicillin (34.5%), ciprofloxacin (6.2%), tetracycline (23%), levofloxacin (15.5%), Trimethoprim/Sulfamethoxazole (14.1%), ciprofloxacin (19.5%),</td>
<td>Łuczkiwicz et al. (2010)</td>
</tr>
<tr>
<td>UWTP (72,000 inhabitants) in Ireland under the effect of ww from four hospitals.</td>
<td>Activated sludge</td>
<td>Escherichia</td>
<td>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%)</td>
<td>Galvin et al. (2010)</td>
</tr>
<tr>
<td>UWTP (100,000 inhabitants) in northern Portugal, 70% domestic ww and 30% pretreated industrial ww.; 400 mg COD/L, 250 mg BOD/L</td>
<td>Activated sludge</td>
<td>Escherichia</td>
<td>Tetracycline (31.2/36.8%), amoxicillin (28/34.8%), sulfamethoxazole/trimethoprim (22.2/22.5%), cephalothin (10.5/20.5%), ciprofloxacin (2.5/9.7%), Tetracycline (0/25%), amoxicillin (20/12.5%), sulfamethoxazole/trimethoprim (0/12.5%), cephalothin (12.3%), ciprofloxacin (0/8.3%), Tetracycline (5/3.1%), amoxicillin (94.7/95.7%), sulfamethoxazole/trimethoprim (8.7/8.7%), cephalothin (5/0.5%), ciprofloxacin (0/4.4%)</td>
<td>Ferreira da Silva et al. (2007)</td>
</tr>
<tr>
<td>2 large-scale Danish UWTPs (240,000 and 500,000 inhabitants)</td>
<td>Activated sludge for N and P removal</td>
<td>Acinetobacter</td>
<td>442 isolates: ampicillin (27/22.3%), gentamicin (7.2/5.8%), tetracycline (11.5/12.9%), multidrug resistance (n.d./0.3%)</td>
<td>Guardabassi et al. (2002)</td>
</tr>
<tr>
<td>UWTP (210,700 PE) in Ann Arbor, Michigan, USA, mostly domestic ww, only limited industrial and untreated hospital ww</td>
<td>Activated sludge for N and P removal</td>
<td>Acinetobacter</td>
<td>366 isolates: trimethoprim (92.2/100%), rifampin (63.1/77.5%), chloramphenicol (25.2/35%), amoxicillin/clavulanic (8.7/20%), sulfamethoxazole (8.7/22.5%), ciprofloxacin (4.9/11.3%)</td>
<td>Y. Zhang et al. (2009)</td>
</tr>
</tbody>
</table>

<sup>a</sup> WW = wastewater,
<sup>b</sup> 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 Łuczkiwicz 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 10^2 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; Łuczkiwicz 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; Łuczkiwicz 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; Łuczkiwicz 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 phylogenetic 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 Nocardiosis* 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; Sanapreddy 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 (Sanapreddy 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.
genes, e.g. vanA, ampC, mecA (Schwartz et al., 2003; Vollmann 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 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.

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

<table>
<thead>
<tr>
<th>Class</th>
<th>Mechanism (Type)*</th>
<th>Examples of genesb</th>
<th>Approachc</th>
<th>Referencead</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aminoglycosides</td>
<td>Modification by adenyllyation (DM)</td>
<td>aad(A1, A2, A13, B)</td>
<td>CD</td>
<td>4; 8</td>
</tr>
<tr>
<td></td>
<td>Modification by phosphorylation (DM)</td>
<td>aph(A, A-3, A-6, 2); str(A, B)</td>
<td>CI</td>
<td>11</td>
</tr>
<tr>
<td>Beta-lactams</td>
<td>Beta-lactam ring cleavage (beta-lactamase production) (DM)</td>
<td>Class A: CTX, GES, NPS,PER, SHV, TEM,TIA, VEB</td>
<td>CI</td>
<td>3; 6; 11; 12</td>
</tr>
<tr>
<td></td>
<td>Class B: IMP, VSM (DM)</td>
<td>CI</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Class C: ampC, CMY (DM)</td>
<td>CI</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Class D: OXA (DM)</td>
<td>CI</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Class D: OXA (DM)</td>
<td>CI</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Penicillin binding protein (TP)</td>
<td>mecA</td>
<td>CI</td>
<td>2; 3; 10</td>
<td></td>
</tr>
<tr>
<td>Glycopeptides</td>
<td>Modified peptidoglycan pentapeptide (TM)</td>
<td>vanA</td>
<td>CD</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Macrolide-efflux protein (DE)</td>
<td>mel</td>
<td>CI</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>Erythromycin inactivation (DM)</td>
<td>ereA2</td>
<td>CI</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>Modification by 23S rRNA methylation (TP)</td>
<td>ermB</td>
<td>CD</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>(TP)</td>
<td>erm(B, F)</td>
<td>CI</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>Macrolide phosphotransferase (DM)</td>
<td>mphi(A, B)</td>
<td>CI</td>
<td>11</td>
</tr>
<tr>
<td>Quinolones</td>
<td>Modification by acetylation (DM)</td>
<td>oaacAg4-ib-cr</td>
<td>CD</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>DNA gyrase protection (TM)</td>
<td>qnr(A3, B1, B2, B4, B5, B2)</td>
<td>CI</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>Sulfonamides Modified dihydropteroate synthase (TM)</td>
<td>sul2</td>
<td>CD</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Tetracyclines</td>
<td>tetrU</td>
<td>CI</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>Tetracycline efflux pump (DE)</td>
<td>tetA</td>
<td>CD</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Ribosomal protection protein (TP)</td>
<td>tetM</td>
<td>CD</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Tetracycline modification (DM)</td>
<td>tetX</td>
<td>CI</td>
<td>7; 11</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>Dihydrofolate reductase (DM)</td>
<td>dfr(A1, A12, 18)</td>
<td>CD</td>
<td>4; 8; 9</td>
</tr>
<tr>
<td></td>
<td>Multidrug resistance efflux pump (DE)</td>
<td>acr(B, D), mex(B, D, F, I, Y)</td>
<td>CI</td>
<td>11</td>
</tr>
</tbody>
</table>

* 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.

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-2.33×10⁶ copies (100 mL⁻¹) and 5.00×10⁻⁵–6.10×10⁵ 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 TiO₂ 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 TiO₂ 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/cm²) to reduce antibacterial activity by one order of magnitude was achieved by UVA-TiO₂ 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. Ger- micial 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 chloride dose (USEPA, 1999). Unfortunately, bacteria injured by disinfection process can survive and re-grow at low chlorine doses (Rizzo et al., 2004; Shivivastava 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. aeruginos (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 cephalexin, 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 Cl₂ 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 (Iwané 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 cephalexin), 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 chlorination 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 sulfamethoxazole 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.
- Demonstrate 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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