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Antibiotic resistance in urban aquatic environments: can it be controlled?

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Abstract

Over the last decade, numerous evidences have contributed to establish a link between the natural and human- impacted environments and the growing public health threat that is the antimicrobial resistance. In the environment, in particular in areas subjected to strong anthropogenic pressures, water plays a major role on the transformation and transport of contaminants including antibiotic residues, antibiotic- resistant bacteria, and antibiotic resistance genes. Therefore, the urban water cycle, comprising water abstraction, disinfection, and distribution for human consumption, and the collection, treatment, and delivery of wastewater to the environment, is a particularly interesting loop to track the fate of antibiotic resistance in the environment and to assess the risks of its trans- mission back to humans. In this article, the relevance of different transepts of the urban water cycle on the potential enrichment and spread of antibiotic resistance is reviewed. According to this analysis, some gaps of knowledge, research needs, and control measures are suggested. The critical rationale behind the measures suggested and the desirable involvement of some key action players is also discussed.

Introduction

The environment is doubly associated with antibiotic resistance: firstly, because the environment is the natural source of antibiotic resistance genes (ARG), including those harbored by commensal and pathogenic bacteria that threat humans and other animals (D'Costa et al. 2011; Vaz-Moreira et al. 2014); and secondly, because the environment is nowadays a major receptor of contaminant antibiotic-resistant bacteria (ARB) and ARG, released from humans and other animals that do not only accumulate in the environment but also spread across different niches (Rizzo et al. 2013b; Vredenburg et al. 2014; Vaz-Moreira et al. 2014). A major driver for the dissemination of antibiotic

resistance is supposed to be the use of antibiotics, which, between the years of 2000 and 2010, increased in 36 %, with major contributions from Brazil, Russia, India, China, and South Africa (Van Boeckel et al. 2014). An impor- tant fraction of these antibiotics is discharged in sewage, together with bacteria of human and animal origin (Michael et al. 2013; Rizzo et al. 2013b). For its part, water is one of the most important habitats and routes of propagation of bacteria, playing a major role in the dissemination of antibiotic resistance between the environment and humans and other animals (Vaz-Moreira et al. 2014). The urban water cycle comprises two major stages, the abstraction of surface or ground water for drinking water purposes, and the collection, transport, and treatment of sewage with its final discharge in the environment. This bipartite structure of the urban water cycle supports the hypothetical definition of potential paths of transmission of antibiotic resistance from the environment to humans and from humans to the environment (Fig. 1). In addition, the urban water cycle creates multiple situations, in which microbial communities can suffer important rearrangements, including the elimination or enhancement of antibiotic resistance (Manaia et al. 2011; Rizzo et al. 2013b; Vaz- Moreira et al. 2014; Fig. 1).

Urban wastewater treatment

Urban wastewater treatment plants receive continuously abundant discharges of ARB&ARG of human and animal origin, antibiotic residues, and other contaminants such as metals, pharmaceutical products, and parabens. These substances, mainly of anthropogenic origin, are mixed with environmental microbiota, suffering, during wastewater treatment, multiple chemical and biological transformations, largely still un- known (Zhang et al. 2012; Michael et al. 2013; Rizzo et al. 2013b). In general, the biological wastewater treatment processes, widely used in urban wastewater treatment plants, offer ideal conditions for bacterial proliferation (Zhang et al. 2012). The high bacterial densities found in the sludge or in biofilters and the large surface area onto which bacteria can adsorb and form biofilm structures, combined with the abundance of nutrients and the presence of different types of selective pressure, are supposed to influence the dynamics of the wastewater microbiome, not only in what concerns microbial community members but also the genetic pool, in which specific genetic elements may present increased fitness (Fondi and Fani 2010; Skippington and Ragan 2011; Zhang et al. 2012). The increased fitness may be due to the fact that it confers an advantage to the host and/or because it is associated with enhanced occurrence of horizontal gene transfer (Fondi and Fani 2010). In this aspect, it is important to mention the well-documented occurrence of antibiotic residues in wastewater (Segura et al. 2009, 2015; Michael et al. 2013). sustaining the hypothesis that, during wastewater treatment, the presence of active antibiotic molecules may impose selective pressure on ARB. Positive correlations between the concentration of antibiotics and rearrangements of the microbial communities, or resistance determinants have been reported (Novo et al. 2013, Varela et al. 2014; Rodriguez-Mozaz et al. 2015). These evidences are supported also by the demonstration of the selective potential of subinhibitory concentrations of antibiotics (Andersson and Hughes 2014). However, skeptical arguments may defend that given the complexity of the wastewater environment, antibiotic residues often at subinhibitory concentrations may have a limited potential to select ARB. In such case, subinhibitory concentrations of antibiotics would not have any selective effect, and it could be argued that acquired antibiotic resistance would not represent any benefit and would tend to be lost during wastewater treatment. Probably, both arguments are valid, and both situations may occur. In- deed, the fate of antibiotic resistance during wastewater treatment and in the environment may depend on the resistance genes and genetic environment, the host of the gene, and the

permissiveness of the receiving environment. Several studies have demonstrated that wastewater treatment using different technologies and complying with the established recommendations regarding treatment efficiency removes, in general, ARB at the same rate as the total bacteria (Novo and Manaia 2010; Rizzo et al. 2013b; Vaz-Moreira et al. 2014). In a study comparing different conventional treatment systems—activated sludge, trickling filter, and submerged aerated filter—it was concluded that the most important factor on antibiotic resistance removal was the capacity of the treatment to remove bacteria, while longer retention times, as those used in la- goons, could be considered a factor favoring antibiotic resistance proliferation (Manaia et al. 2010; Novo and Manaia 2010). In other studies, it was shown that some resistance types, such as quinolone resistance in coliforms or enterococci, tend to become more prevalent in the final effluent, after activated sludge treatment, than in the raw wastewater and this effect could be noticed mainly in the winter (Ferreira da Silva et al. 2006; 2007; Łuczkiewicz et al. 2010; Novo et al. 2013). Although it is nowadays very difficult to identify the best technology to avoid antibiotic resistance selection, an important conclusion can be drawn: Irrespective of any significant variation on the antibiotic resistance prevalence, a well- functioning wastewater treatment plant with secondary treatment will discharge continuously to the environment high doses of ARB&ARG (Vaz-Moreira et al. 2014; Fig. 2a). Probably, this load is much higher than the receiving environment is able to eliminate. The improvement of wastewater treatment processes in order to significantly reduce the loads of ARB&ARG discharged in the environment seems an adequate strategy to mitigate the antibiotic resistance contamination burden (Fig. 3).

Important environmental reservoirs of contaminant antibiotic resistance

For decades, antibiotic resistance has been regarded as a problem restricted to clinical settings. Indeed, it is in the hospital and health care units, where antibiotherapy is intensively used, that the most dramatic selective conditions to ARB are expected (WHO 2014). For this reason, it is arguable that hospital effluents may represent an important environmental reservoir of antibiotic resistance. And, the role of hospital effluents as critical antibiotic resistance reservoirs has been demonstrated in different studies that compared antibiotic resistance prevalence in hospital and municipal wastewater. These studies demonstrated that hospital effluents contain higher prevalence of ARGs (e.g., *tetW*, *blaTEM*, or s*ul1*), including some genetic determinants which occurrence is mainly related with clinical practices, such as the gene *vanA* (Varela et al. 2013, 2014, 2015b; Narciso-da-Rocha et al. 2014; Rodriguez-Mozaz et al. 2015). Hospital effluents can contain higher concentrations of antibiotic residues, metalloids, and metals like arsenic or mercury, and significantly higher abundance of ARB&ARG than municipal effluents (Kümmerer and Henninger 2003; Varela et al. 2014; Rodriguez-Mozaz et al. 2015). Yet, since hospital effluents can be classified as domes- tic effluents, there is no legal recommendation for pretreatment aiming at reducing the microbial loads before its dis- charge in the municipal collectors. This legal gap can be attributed to the fact that hospital effluent discharges represent less than 5 % of the municipal effluents, leading to the readily dilution of antibiotic residues and ARB in the domestic effluents (Varela et al. 2014; Rodriguez-Mozaz et al. 2015). How- ever, this may be a fallacious argument, since ARB&ARG, unlike other environmental contaminants, can proliferate and spread across different niches and hosts, as it has been demonstrated based on the detection of the closely related ARG&ARB in urban water streams, gulls, or hospitalized patients (Varela et al. 2013, 2014, 2015b; Vredenburg et al. 2014). In particular, the effluents from hospital and health care units can represent important reservoirs of ARG associated with bacteria of clinical relevance and with

antibiotics used mainly in hospitals (ECDC 2012). Therefore, hospital effluents can represent a privileged entry portal in the environment of genes particularly widespread in clinical settings, such as *vanA* (vancomycin-resistant enterococci, VRE), *mecA* (methicillin-resistant *Staphylococcus aureus*, MRSA), and *aac(6')- Ib-cr* or *blaCTX-M15* (plasmid-encoded resistance to quinolones and beta-lactams in Gram-negative bacteria) (Novais et al. 2005; Narciso-da-Rocha et al. 2014; Rodriguez-Mozaz et al. 2015; Varela et al. 2015a).

Health care-related effluents are not the only accountable source of antibiotics and antibiotic resistance determinants as environmental contaminants. It has been demonstrated that the largest proportion of the antimicrobial consumption by humans is observed at the community and not in hospitals (ECDC 2012). Moreover, patient's recovery and antibiotherapy are increasingly made at home, and antibiotics are also used in companion and food-production animals (ECDC 2012; EFSA/ECDC, 2015). Directly or indirectly, these sources may contribute to the enhancement of ARB&ARG, part of which will reach the municipal collector. Indeed, some types of antibiotic residues (e.g., tetracyclines or sulfonamides) and some ARG (such as plasmid mediated quinolone resistance genes of the family *qnrS*) can be more frequent in municipal wastewater and biosolids than in hospital effluents (Kaplan et al. 2013; Varela et al. 2014; Rodriguez-Mozaz et al. 2015; Varela et al. 2016).

The widespread distribution of some ARG in unpolluted, although human-impacted, areas makes very difficult the identification of their major reservoirs and routes of dissemination. However, there are evidences of some important nuclei of dissemination of contaminant ARB&ARG. For instance, closely related bacterial lineages of *Escherichia coli* of the multilocus sequence types ST131 or ST10 are found in patients hospitalized and in the environment, in directly or indirectly connected compartments such as wastewater, surface water, or gulls (Varela et al. 2015a, b). This suggests that ubiquitous ARB that can share different environments and hosts will have the potential to contribute to the widespread distribution of ARG. In particular, ARB that can be found either in water environments or in the human microbiome deserves special attention (Vaz-Moreira et al. 2014). The ARB ubiquity explains why nowadays, gene families like *bla- TEM, blaCTX-M*, *blaSHV*, and plasmid-mediated quinolone resistance such as (*aac-Ib-cr* or *qnrS*), *sul 1–3* or *tetX/Q/S/W/O/M*, besides clinically relevant, can be found in surface water, aquaculture environments, or in wildlife (Costa et al. 2006; Simões et al. 2010; Vredenburg et al. 2014; Xiong et al. 2015). If no control measures are taken, in a few years, this same scenario can be observed for other genes such as bla_{NDM-1} (New Delhi metallo-β-lactamase), bla_{VIM} and bla_{KPC} (carbapenemases), or *vanA* (vancomycin resistance operon gene), and then it may be too late to act. Indeed, in some Asiatic regions, the dissemination of the gene bla_{NDM-1} through water, in rivers and drinking water supplies, is reported for several years, and this is mainly attributed to poor sanitation practices (Walsh et al. 2011; Toleman et al. 2015).

Drinking water

One of the reasons why it is important to examine the occurrence of antibiotic resistance along the urban water cycle refers to the risks that the ARB&ARG present in the "unclean" part of cycle can reach the water consumer. This is a legitimate concern given the ubiquity of some bacterial groups, which due to their ecology and physiology can inhabit waste, sur- face, or drinking water and often can also integrate in the human microbiome (Vaz-Moreira et al. 2014). These arguments place drinking water among the potential routes of transmission of antibiotic resistance to humans.

Because the access to safe drinking water is a human right, the Millennium Development Goals included the access to improved sources of drinking water, which since 2010 is accessible to 90 % of the world's population (GEMS-Water 2014; WHO/UNICEF 2015). Unfortunately, the same improvement was not extensive to sanitation, and therefore, the risks of contamination of drinking water may be, at least in some world regions, in- creased (GEMS-Water 2014; UNICEF/WHO 2015). Drinking water can be either abstracted from microbiologically safe water resources or disinfected prior its use, aiming at reducing pathogens to levels below the hazardous limit (WHO 2014). Therefore, it is expected that in drinking water, both the diversity of bacterial species and the possible occurrence of antibiotic resistance will have different characteristics of what can be found in wastewater. Water disinfection is known to cause a dramatic bottleneck on bacterial diversity (Hoefel et al. 2005; Eichler et al. 2006; Vaz-Moreira et al. 2013) and bacteria ubiquitous in aquatic environments, such as members of the genus *Aeromonas*, may be reduced to levels below the limit of detection by culture-dependent methods (Figueira et al. 2011). However, other groups that comprise bacteria with recognized ubiquity and genome plasticity, demonstrated by the acquired capacity to degrade xenobiotics or to resist to some antibiotics, such as members of the genera *Pseudomonas*, *Acinetobacter*, or family *Sphingomonadaceae*, are commonly found in tap water (Vaz-Moreira et al. 2014). In general, it is difficult to demonstrate the origin of bacteria found in tap water, for in- stance if their occurrence results from the survival to the dis- infection process or if they can enter the system along the distribution network (Vaz-Moreira et al. 2011; 2012; Narciso-da-Rocha et al. 2013). Although culture-dependent methods do not suggest that drinking water is an important habitat for bacteria with acquired antibiotic resistance, intrinsic antibiotic resistance is commonly found in tap water in members of groups such as the family *Sphingomonadaceae* or the genera *Pseudomonas* or *Ralstonia* (Vaz-Moreira et al. 2014). Not much is known about the role of the intrinsic antibiotic resistance in the evolution of acquired antibiotic resistance, since the genetic determinants involved cannot, at least easily, be transferred to other bacteria (Fajardo et al. 2008; Alvarez-Ortega et al. 2011). However, even though at the molecular level, intrinsic resistance may be considered to be trapped in the bacterial genome, at the microbial community and population levels, there are no evidences demonstrating that intrinsically, ARB cannot suffer population-based se- lection and that these populations cannot have a role on anti- biotic resistance dissemination. In addition, also some well- known acquired antibiotic resistance determinants, often associated with mobile genetic elements, have been reported in drinking water using culture-independent methods. Genes such as *vanA*, *blaTEM*, *sul1*, or *intI1* (related with vancomycin, beta-lactam and sulfonamides resistance, and class 1 integrons, respectively) have been detected, although at very low abundance, in biofilm or planktonic DNA of tap water samples that fulfilled the quality criteria in different world regions (Schwartz et al. 2003; Xi et al. 2009; our data unpublished). The risks associated with intrinsic or acquired antibiotic resistance in drinking water for the dissemination of antibiotic resistance and its transmission to humans are not known; however, this is an issue that seems to deserve additional research.

Other antibiotic resistance reservoirs associated with the urban water cycle

Important paths of dissemination of antibiotic resistance associated with the urban water cycle are the wastewater reuse, mainly for irrigation purposes, the use of municipal sewage sludge for agriculture soil fertilization and the water use in animal production, in particular fish farming. In all these situations are created opportunities for ARB&ARG to cross the border environment-

humans or animals, with potential risks of contamination of soil, surface, and groundwater or the human food chain. The amount (tons) of antibiotics consumed for food-producing animals is generally higher than for human therapy (EFSA/ECDC 2015; Done et al. 2015). For this rea- son, it is arguable that the selective pressures found in animal production environments are important drivers for antibiotic resistance dissemination (Cabello et al. 2013; Done et al. 2015; Xiong et al. 2015). Although the same active principles can be used in human medicine and in animal production, the pattern of utilization is different (Cabello et al. 2013; WHO 2014; EFSA/ECDC 2015; Done et al. 2015). While human medicine, mainly in what concerns antibiotics administration in health care units, tends to use last-generation antibiotics of classes of fluoroquinolones, cephalosporins, or carbapenems, in animal production, "older" antibiotics such as tetracyclines, sulfonamides, or penicillins are preferred (WHO 2014;

EFSA/ECDC 2015; Done et al. 2015). The use of different antibiotics for animal production and for human medicine may explain the observation of distinct correlations between the bacterial community composition and antibiotic-resistant populations and the concentration in wastewater of tetracyclines, sulfonamides, and penicillins or fluoroquinolones (Novo et al. 2013; Varela et al. 2014). Nevertheless, it is suggested that both sources of contamination with antibiotic residues are probably associated with antibiotic resistance dissemination and bacterial community disturbance. In addition, although effluents of animal production facilities are not demonstrated as important reservoirs of emerging ARG, as those disseminated via hospital wastewater, they are supposedly important suppliers of resistance against sulfonamides, amino- glycosides, tetracycline, or quinolones (Xiong et al. 2015). Moreover, effluents of animal production facilities contain ubiquitous bacteria that may be also found in the human microbiome (Vaz-Moreira et al., 2014; Done et al. 2015). Be- cause ARB&ARG from animal production effluents are able to spread in the environment and enter the human food chain, these facilities should also be placed among the critical control points to mitigate antibiotic resistance propagation (Cabello et al. 2013; Done et al. 2015; Xiong et al. 2015).

The implications of the use of treated wastewater for irrigation purposes in terms of antibiotic resistance dissemination are still unclear due to some contradictory findings. While some studies suggest that ARG in soils do not vary significantly due to irrigation with treated wastewater, others demonstrate that at least some genes tend to accumulate in soils after a continued exposure to wastewater (Negreanu et al. 2012; Wang et al. 2014; Becerra-Castro et al. 2015). Another potential risk for the dissemination of ARB&ARG is the use of sewage sludge for agriculture soil fertilization, which has been suggested as presenting a high potential of spreading through both soils and crops (Rahube et al. 2014). The fate of ARG in soils or their eventual uptake by plants and entry in the human food chain may depend on many different, still unstudied, variables (Becerra-Castro et al. 2015). However, based on what is known today about the evolutionary success of ARB&ARG, it seems advisable to use strict precautionary rules concerning the reuse of wastewater or soil fertilization with municipal sewage sludge.

Factors triggering antibiotic resistance dissemination

Conceptually, one of the most important issues regarding the control of antibiotic resistance dissemination refers to the identification of environmental variables, whose manipulation could attenuate the selective effects that, directly or indirectly, can be exerted upon ARB. Of the multiple environmental variables that are probably influencing the dissemination of ARB or the transfer of ARG, the effect of selective pressures imposed by antibiotics is normally pointed out as the major cause

(Tello et al. 2012; Perry and Wright 2014). Other environmental contaminants, such as heavy metals, have also been reported among the list of possible selectors of ARB&ARG (Graham et al. 2011; Seiler and Berendonk 2012). Nevertheless, the occurrence of environmental contaminants does not exhaust all the possible causes of antibiotic resistance enhancement. Different types of stress, such as nutrients scarcity, presence of reactive oxygen species, different types of cellular damage, or high-temperature shocks, are capable of activating cellular responses that will hamper antimicrobial activity (Poole 2012). A good evidence of the direct influence of the SOS response in the capture and spread of antibiotic resistance genes was the demonstration of the role of regulator protein LexA on the control of integron integrase gene expression and, therefore, on the regulation of gene-cassette recombination (Guerin et al. 2009). This kind of evidence suggests how different environmental factors may contribute for ARB or ARG selection in the environment. The control of this still poorly understood that network of factors that can influence antibiotic resistance proliferation would probably contribute to mitigate its dissemination from relevant hotspots (Guerin et al. 2009). The consistent observation of significant positive correlations between antibiotic consumption and antibiotic resistance prevalence has consolidated the perception of a possible cause-effect relationship between both (Bell et al. 2014). Since domestic wastewater contains antibiotic residues, it is arguable that such residues can impose selective effects on ARB, although, as discussed above, the low concentrations found in wastewater can make this issue controversial. Some recent reports have assessed possible correlations be- tween antibiotics and metals concentration and the microbiota in wastewater or in freshwater samples (Graham et al. 2011; Huerta et al. 2013; Novo et al. 2013; Narciso-da-Rocha et al. 2014; Varela et al. 2014; Rodriguez-Mozaz et al. 2015). As hypothesized by the authors, these studies showed significant positive correlations between the concentration of antibiotic residues or metals and ARB&ARG or specific bacterial com- munity members in aquatic environments. However, hardly, these findings can be interpreted as straightforward evidences of cause-effect relationships. In particular, the complexity of waste- or fresh-water composition in terms of nutrients, particles and microbiota, may make difficult a reliable prediction of the bioavailability of antibiotic residues or of their effects on target bacteria. In spite of these shortcomings, in wastewater, it was possible to demonstrate that the concentration of ciprofloxacin, tetracycline, sulfamethoxazole, or arsenic could be correlated with the prevalence of ARB, the abundance of the genes *blaTEM*, *vanA*, or *intI1* (related with beta-lactam and vancomycin resistance and class 1 integrons, respectively) or the increase of populations of *Bacteroidetes*, *Clostridia*, or *Proteobacteria* of the classes *Gamma* or *Epsilon* (Novo et al. 2013; Narciso-da- Rocha et al. 2014; Varela et al. 2014). Also in surface water and sediments, similar correlations were observed, suggesting that in the environment downstream the wastewater treatment plant, selective pressures may still be exerting some effects. Graham et al. (2011) observed significant correlations between the abundance of tetracyclines and beta-lactams ARG in river sediments and the concentration of Cu or ampicillin. Similarly, in a lacus- trine system, it was demonstrated a correlation between the presence of macrolides-related ARG, antibiotic pollution, and some bacterial community members of the phyla *Actinobacteria* and *Firmicutes* (Huerta et al. 2013). The experimental approach used in these studies does not distinguish if we are in the presence of a simple co-occurrence of antibiotic residues and specific bacterial populations or genes, or if, by the contrary, significant positive correlations are due to cause-effect relationships. Yet, those studies suggest that antibiotic resistance selection is associated with noticeable rearrangements of the resistance gene pools and bacterial communities, demonstrating that antibiotic resistance dissemination is probably a multifactorial process. It is, thus, suggested that a better under- standing of antibiotic resistance evolution and of its control may be only possible based on a multifactorial assessment of the factors triggering antibiotic resistance selection and on the permissiveness of a given environment to the

penetrance of ARG. In other words, antibiotic resistance evolution and dissemination can only be understood based on the simultaneous assessment of the selective pressures, the ARG, and its genetic environment and the microbial community.

Measures contributing to prevent antibiotic resistance dissemination

Nowadays, scientific evidences and technological and societal developments can be put together in order to control antibiotic resistance dissemination. Three major strands of intervention can be proposed: methods harmonization and data sharing, improved frameworks for reliable risk assessment and prevention and mitigation interventions at critical control points (Table 1).

Methods harmonization and data sharing

The screening of environmental samples for ARB or ARG has been supported by either culture-dependent or culture- independent methods (Fig. 4). The first involves the isolation of bacteria and antibiotic susceptibility testing, using methods and classification criteria originally proposed for clinical bacteria (e.g., European Committee on Antimicrobial Susceptibility Testing, EUCAST or the Clinical and Laboratory Standards Institute, CLSI). The selection of bacteria tolerant to antibiotics supplemented in the culture media has also been used (Watkinson et al. 2007; Novo and Manaia 2010; Manaia et al. 2011; Novo et al. 2013). These methods have provided a good overview of the resistance occurrence in some bacterial groups of human-commensal and environmental bacteria. However, culture-dependent methods fail to detect bacteria that due to the absence of nutritional requirements, possible cell injuries or latency state, or slow growth, are transiently or permanently in a non-culturable state. Therefore, culture- independent methods are important complements to assess the occurrence of antibiotic resistance in the environment. Culture-independent approaches, mainly real-time quantitative PCR (qPCR), have gained popularity (Auerbach et al. 2007; Zhang et al. 2009; LaPara et al. 2011; Laht et al. 2014; Narciso-da-Rocha et al. 2014; Rodriguez-Mozaz et al. 2015). These methods, although offering a good overview of the occurrence of antibiotic resistance in different types of environment (Vaz-Moreira et al. 2014; Fig. 2), hardly can be used to compare world regions, assess timescale trends, or evaluate the effects of control measures, since most of these data is obtained under non-harmonized conditions. The harmonization of methods, successfully established for clinical data (e.g., EUCAST, CLSI), is also possible for environmental samples, mainly in laboratories where routine monitoring is already implemented, as for example in waste and drinking water treatment facilities. Indeed, the analyses of water micro- biological quality are fairly harmonized worldwide (ISO 9308−1:2014; Council Directive 98/83/EC 1998; WHO 2004), and it would be feasible to extend these procedures to assess also the antibiotic resistance status. Also, the protocols for qPCR would benefit from some harmonization, if world- wide and timescale comparisons are aimed. Indeed, the data available nowadays hardly can be compared, being difficult to assess geographical patterns or the efficiency of the disinfection methods used. This is evident from the analysis of Fig. 2b, in which it is represented that the load of selected ARG occurring in 100 mL of final effluent produced with different treatment processes, in distinct world regions, and with data obtained with different qPCR protocols is presented. The in- vestment on antibiotic resistance monitoring would not be a stray effort, since it could be framed in a broader context of ongoing problems associated with water quality, related not only with pollution but also with climate variability, among other, and that require the action of regulatory authorities at local, national, and global scales (GEMS-Water

2014). In re- search laboratories, high-throughput qPCR and metagenomics analyses of ARG are increasingly the state of the art (Nesme et al. 2014; Port et al. 2014; Li et al. 2015). and these will be important contributions to understand antibiotic resistance evolution. However, a thorough understanding of antibiotic resistance evolution can only be achieved in a global context, in which the microbial ecology, the level, and typology of environmental anthropogenic impacts or the geographical distribution must be taken into account. Therefore, data on anti- biotic resistance occurrence should be combined with data on other microbiological and chemical and physical parameters, in order to allow systematic analyses on the major drivers for antibiotic resistance evolution and dissemination in the environment. The establishment and maintenance of public data-bases with the aforementioned information would be an important contribution to assess the antibiotic resistance status and to evaluate the efficiency of control measures.

Improved frameworks for reliable risk assessment

Still a major gap for the control of antibiotic resistance in the environment regards risk assessment. On one hand, it is missing a framework to assess the risk of antibiotic resistance emergence and dissemination in the environment. In particular are missing adequate tools for the identification of environmental stressors that can trigger antibiotic resistance selection or propagation and to ascertain the maximal dose of an ARG that will be able to auto-proliferate under a given conditions set. On the other hand, although it is very well-known that ARB&ARG are able to spread in the environment, mainly due to human contamination, the risks of transmission back to humans hardly can be estimated. For both approaches of risk assessment evaluation, underpinning measures rely on the establishment of global data- bases. The risk associated with an ARG can be estimated based on the likelihood of being transferred among bacteria and of integrating the genome of a primary or opportunistic pathogenic bacteria for humans (Martínez et al. 2015). This implies that for each ARG, it would be important to explore preferential bacterial hosts and propagation pathways, including in what concerns the interaction between the environmental and human microbiome resistome. In parallel, the proposal of models capable of predicting antibiotic resistance fate, fitness, and major constraints under different environmental conditions is also important to prevent and control antibiotic dissemination (Nguyen et al. 2014; Amos et al. 2015). In both cases, such developments are strongly associated with the existence of reliable and systematically acquired data of ARB&ARG in different environmental compartments and its interface with humans.

Prevention and mitigation interventions at critical control points

While the current state of the knowledge evidences the need to improve environmental monitoring and surveillance in the interface environment-humans, the information available shows that, in general, receptors of human and food- production animal excreta are major reservoirs and suppliers of antibiotic residues, ARB&ARG to the environment. In particular, sewage treatment plants are important connectors in the spread of antibiotic resistance to natural waterways, groundwater, soil, wildlife, and eventually the food chain (Berendonk et al. 2015). The quality of wastewater treatment worldwide can easily be correlated with the degree of contamination of water bodies with pharmaceutical products, including antibiotics (Segura et al. 2015). This observation, combined with the fact that antibiotic consumption is raising, warns for the need not only to encourage the appropriate use of antibiotics but also to develop measures that can prevent the

environmental contamination with ARB&ARG (Van Boeckel et al. 2014; Berendonk et al. 2015). These concerns and measures must be implemented internationally, since the past has already shown that ARB&ARG do not respect geographical boundaries (e.g., Walsh et al. 2011).

Whilst appropriate sanitation is not available in some world regions, tertiary treatment and advanced wastewater treatment are increasingly being implemented in some high-income regions (GEMS-Water 2014; Segura et al. 2015). However, in most world regions, conventional wastewater treatment is the state of the art. Conventional wastewater treatment includes well-established physical and biological methodologies, constituting the primary and secondary treatments, respectively. These processes, which were mainly developed to reduce the organic load of the raw wastewater, are able to remove the microbial load up to 2–3 log-units (EPA 1986; Norton-Brandão et al. 2013). However, conventionally treated waste- water still contains high microbial loads, with cultivable heterotrophic bacteria reaching 10^5–10^7 colony-forming units (CFU)/100 mL (Novo and Manaia 2010; Novo et al. 2013; Rizzo et al. 2013b). Part of these bacteria holds acquired antibiotic resistance to one or more classes of antimicrobial agents (Rizzo et al. 2013b; Vaz-Moreira et al. 2014). Depending on the antibiotic, in enteric bacteria, percentages of anti- biotic resistance varying from 2 % (e.g., to ciprofloxacin) up to 70 % (e.g., to sulfamethoxazole) have been described (Rizzo et al. 2013b; Novo et al. 2013). As a consequence, it can be estimated that conventionally treated wastewater discharged by a well-functioning treatment plant may supply to the environment more than a teradose of culturable ARB (10^{12}) per day (Vaz-Moreira et al. 2014). Such high loads are confirmed by multiple studies on the quantification of ARG in conventionally treated wastewater (Fig. 2) being estimated that more than 10^{14} to 10^{18} copies of genes encoding for tetracycline or beta-lactam resistance are released per day for the surrounding environment.

Advanced wastewater treatment and the control of antibiotic resistance

In different world regions, the recognition of the adverse impacts of high microbial loads resultant from conventionally treated wastewater has been leading to recommendations on the implementation of a disinfection step before the discharge to receiving waters (e.g., EPA 1986; EPA Victoria 2002). Chlorination as well as UV- and O3-based processes (the latter are included in the group of advanced oxidation processes [AOPs]) is amongst the most common technologies currently applied in wastewater treatment for disinfection purposes worldwide. In general, to date, there have been few and conflicting reports regarding the efficiency of disinfection processes in removing ARB from wastewater. Although it has been demonstrated that ARB&ARG can be reduced in waste- water after disinfection (Dodd 2012). recent studies suggest that much more investigation is still needed. Chlorination process was found to initially decrease the total number of bacteria in wastewater; nonetheless, many studies indicated that chlorine might substantially increase the proportions of ARB (Rizzo et al. 2013a). Conner-Kerr et al. (1998) reported the effective inactivation of a methicillin-resistant strain of *Staph- ylococcus aureus* and vancomycin-resistant *Enterococcusfaecalis* following UV disinfection at fluence of 77 mJ cm^{-2}.

Also, UV disinfection process resulted in a total reduction of the proportion of *E. coli*, which was resistant toward amoxicillin, ciprofloxacin, and sulfamethoxazole after 60 min of irradiation (1.25×10^4 μW s^{-1} cm^{-2}) (Rizzo et al. 2013a). On the other hand, UV process did not contribute to a significant reduction of tetracycline- and sulfonamide-resistant *E. coli* (Munir et al. 2011).

Ozonation led to a reduction of total and antibiotic-resistant *E. coli*, enterococci, and staphylococci be- tween 0.8 and 1.1 log-units compared to the respective concentrations of the effluent (Lüddeke et al. 2015). Other AOPs, e.g., UV/H_2O_2-mediated oxidation, photo-Fenton, TiO_2 photocatalysis, or activated-persulfate oxidation, have also been demonstrated to be effective in removing ARB&ARG from conventionally treated wastewater (Cengiz et al. 2010; Michael et al. 2012; Michael-Kordatou et al. 2015; Ferro et al. 2015). However, it is still an open question whether the operational conditions of AOPs may select bacteria with higher capacity to resist oxidative stress conditions (e.g., enhanced defense mechanisms, horizontal gene transfer, or mutation rates). Currently, there is a big gap in knowledge with regard to the design/operating parameters that may influence the mechanisms through which ARB&ARG are removed, sur- vive, multiply, or evolve during the application of advanced treatment. For example, limited information is currently avail- able in the scientific literature regarding the effect of UV fluence on the ARB removal. Guo et al. (2013a, b) reported the high efficiency of UV treatment in removing erythromycin-resistant heterotrophic bacteria at a UV fluence of 5 mJ cm^{-2} (with the corresponding log reduction being 1.4± 0.1), while when the fluence increased to 20 and 50 mJ cm^{-2}, the counts of total ARB were below 1 CFU mL^{-1}. Ozonation resulted in oxidative damage on the plasmid DNA of multidrug-resistant *E. coli* and the damage increased with increasing oxidant doses (Öncü et al. 2011). Also, it was recently reported that the pretreatment type of wastewater (e.g., various filtering materials) seems to significantly affect the resistance level of the treated effluents following ozonation (Lüddeke et al. 2015). For sure, a deeper insight into the mechanistic aspects would help to improve the capacity of advanced treatment processes in removing ARB. Moreover, even if ARB are removed, intact remnants of DNA present in cell debris can hypothetically promote ARG dissemination (Dodd 2012). Also, one critical issue governing the application of disinfection (UV- and O_3-based processes)/AOPs technologies is the formation of "new" compounds resulting from the oxidation of the antibiotics present in wastewater, which may possess their own antimicrobial activity contributing thus to the overall evolution of antibiotic resistance in bacterial strains of the treated wastewater samples. In alternative, membrane filtration technologies, due to the benefits they may have when compared with these well- established methods, in particular concerning the high efficiency on the removal of bacteria, metals, and some organic compounds, may be preferred. However, the high investment and operational costs may be a limitation (Norton-Brandão et al. 2013; Michael-Kordatou et al. 2015). In summary, it can be said that the selection of the method should be done on a case-by-case basis considering the overall characteristics of the wastewater treatment plant, namely the size, the operating conditions, the level of disinfection to be reached, and the environmental impact of the process (EPA 1986; EPA Victoria 2002). To achieve the goals manifested in international guidelines (e.g., WHO or EU) regarding antibiotic resistance and wastewater discharge and/or reuse, it is imperative that efforts be made by the wastewater treatment facilities to invest on advanced treatment technologies to remove/minimize ARB&ARG from wastewater. However, there is no universal solution, since each of the disinfection technologies available has advantages and disadvantages (Table 2), being the capital and operation costs important aspects to consider when the choice for a given method is required.

At the moment, one of the possible interventions of the scientific community might be the demonstration of how wastewater treatment processes could be improved. For in- stance, it is possible to estimate the levels of ARB discharged in the environment for which the probability of proliferation or dissemination can be extremely low. Some simple calculations demonstrate how

advanced wastewater treatment can prevent the environmental contamination with ARB&ARG. In Fig. 3 are illustrated different hypothetic scenarios of the impact of antibiotic resistance dissemination via treated waste- water, demonstrating the potential benefits of applying advanced wastewater treatment. In comparison with a secondary treatment, an advanced treatment process will contribute to reduce the burden of the discharge of ARB, even if it implies an increase of 10 times in the prevalence of antibiotic resistance, in treatments achieving bacterial removal rates of 2 log- units, or of 100 times in the case of bacterial removal rates of 4 log-units (Fig. 3). But, it is important to note that the improvement of wastewater treatment processes is an issue that cannot be oversimplified or be a subject of universal solutions. For instance, the influence of the environmental temperature on the fitness of bacteria of human and animal origin in the environment seems to be crucial to assess the burden of antibiotic resistance dissemination by wastewater treatment plants, with higher temperatures favoring the bacterial proliferation of mesophilic bacteria (Fig. 3). In addition, besides cost-effectiveness, also the properties of the receiving environment and other conditions must be considered and need to be carefully assessed. It seems clear that advanced treatment methods should be optimized for each region taking into account different environmental variables, especially the abundance and prevailing types of environmental bacteria, climate conditions, in particular temperature, and average rainfall or chemical composition, either natural or due to anthropogenic impacts. Thus, comprehensive research strategy based on both advanced engineering and microbiological tools is without doubt needed to identify the most critical operating, chemical, and microbiological parameters that are needed to improve disinfection and advanced treatment processes with regard to antibiotic resistance control.

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Ethical statement

The content of this article is the authors' responsibility and neither the financing entities nor any person acting on their behalf is responsible for the use, which might be made of the information contained in it.

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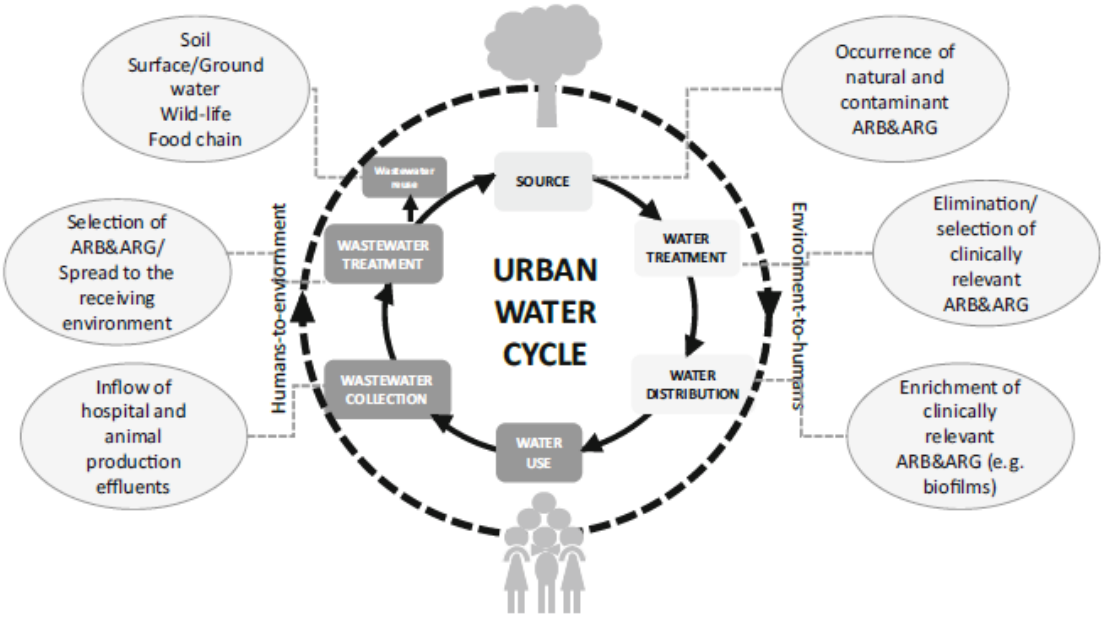

Fig. 1 Schematic representation of the urban water cycle and of sites or processes potentially critical for antibiotic resistance selection, spread, and control

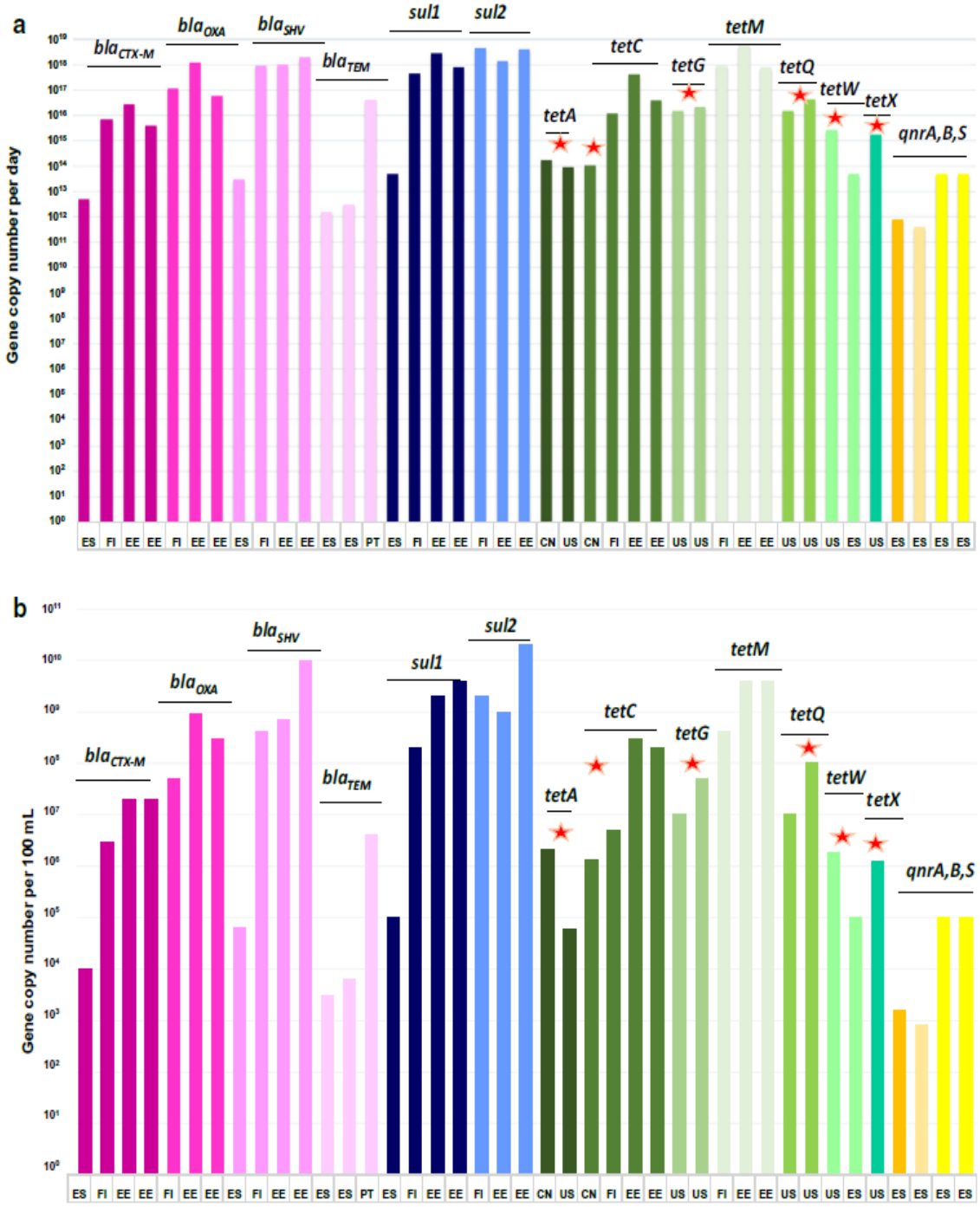

Fig. 2 Estimated abundance of antibiotic resistance genes, based on the same data set, discharged per day (a) or per 100 mL (b) of treated effluents of municipal wastewater treatment plants in different world regions (Spain (*ES*)—Rodriguez-Mozaz et al. 2015; Finland (*FI*)—Laht et al. 2014; Estonia (*EE*)—Laht et al. 2014; Portugal (*PT*)—Narciso-da- Rocha et al. 2014; China (*CN*)—Zhang et al. 2009; USA (*US*)— Auerbach et al. 2007; LaPara et al. 2011). Water disinfection by chlorination or UV is indicated by the *red star*

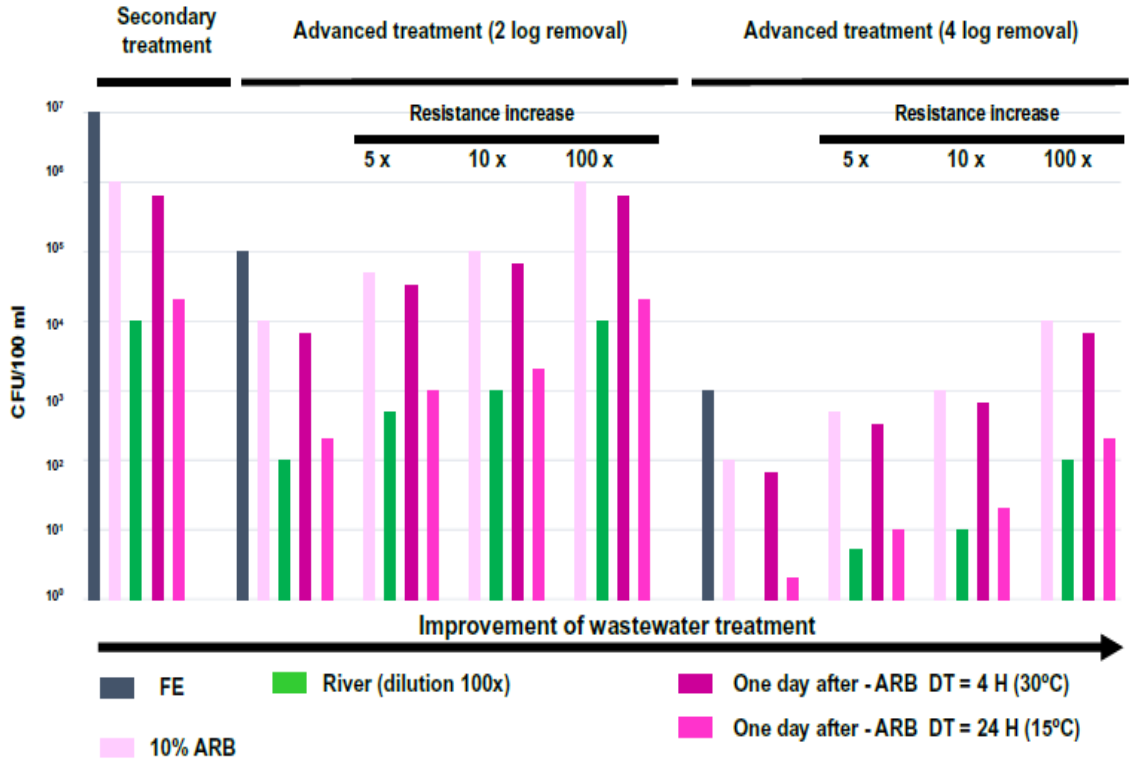

Fig. 3 Different scenarios of the impact of antibiotic resistance dissemination via treated wastewater (final effluent, *FE*) discharges, considering different variables: (a) type of treatment—no advanced treatment (secondary treatment) or advanced treatment with 2 log-units removal or 4 log-units removal); (b) potential increase of antibiotic resistance prevalence promoted by the wastewater advanced treatment—of 5-, 10-, or 100-fold; and (c) effect of environmental temperature on the fitness of the ARB of animal and human origin— duplication time (*DT*) of4 h at 30 °C or of 24h at 15 °C

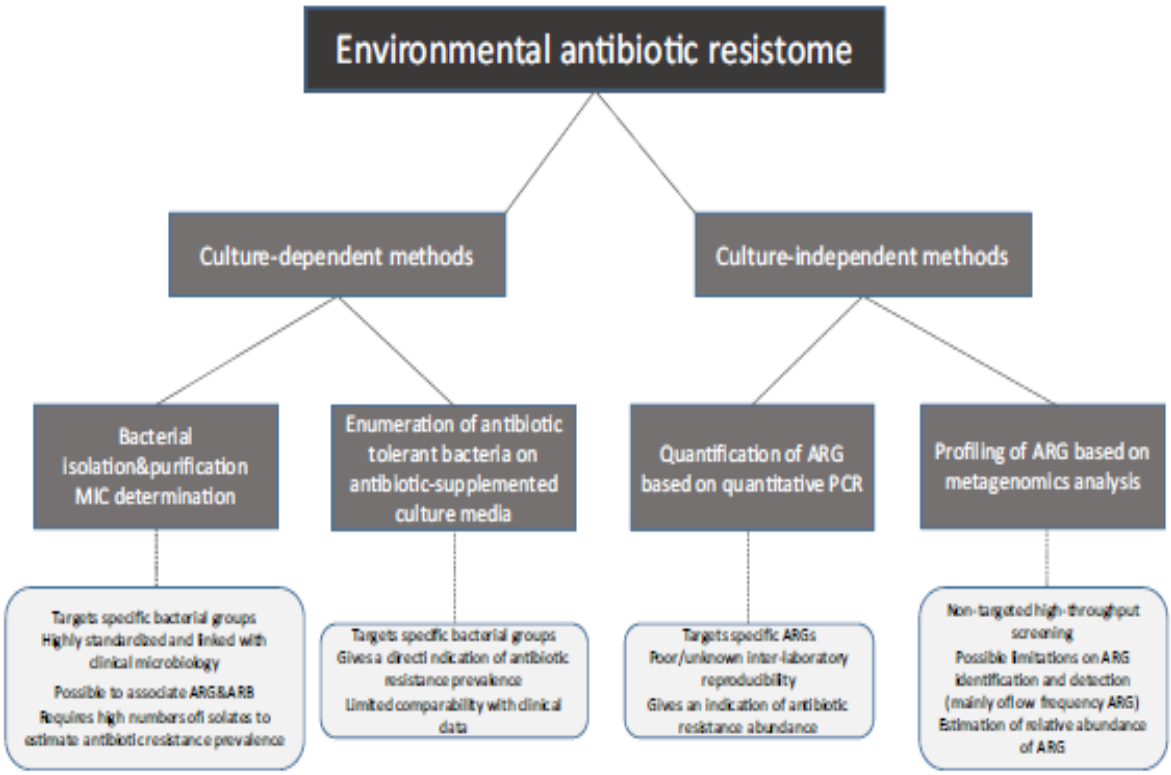

Fig. 4 Overview of some commonly used methodologies to assess the quantity and/or diversity of ARB or ARG in the environment

Table 1 Antibiotic resistance and the environment: major current limitations and useful key actions of the scientific community and policy-making entities

Limitations	Key actions
Methods harmonization and data availability	
Current situation: Different sampling and analyses methods limit a coherent overview of geographical distribution, temporal evolution, and definition of critical control points of antibiotic resistance Limitations: Risk assessment of spreading in the environment and transmission to humans Evaluation of temporal evolution and efficiency of possible control measures Establishment of legal recommendations (e.g., maximum admissible AR levels in effluents discharged into municipal collectors or of effluents discharged in the environment)	Actions: Establishment of guidelines for antibiotic resistance measurement concerning sampling procedures and sample analyses (e.g., target genes, bacteria, and analytical procedures) Sample characterization in terms of organic matter, key environmental contaminants, and other factors (e.g., C- and N-pool content, antibiotic residues, metals, oxic/anoxic conditions) Creation of global public databases, curated, and maintained by public entities Key players: the scientific community, routine monitoring laboratories, public health, and environmental protection entities
Risk assessment	
Current situation: The factors triggering antibiotic resistance emergence and spread are not known (e.g., effects of subinhibitory concentrations of antibiotics in the environment, effects of other stressors, the role of the microbial community impacted by antibiotic resistance discharges) The fate of antibiotic resistance in the environment cannot be predicted (e.g., after its discharge from a wastewater treatment plant into a river, irrigated soil, or surrounding wildlife) The risk of transmission of antibiotic resistance from different environmental sources to humans cannot be quantified	Actions: Multifactorial studies (environmental variables known to influence microbial activities, including antibiotic residues, microbial community, gene pool) Development of molecular epidemiology and modeling tools to assess antibiotic resistance persistence, ubiquity, and preferential routes of dissemination Coordination between human and environmental human microbiome data in the assessment of the risks of transmission of antibiotic resistance to humans, taking into account a rationale ranking of risk in resistomes Key players: the scientific community
Define and act at critical control points	
Current situation: There are multiple evidences of important reservoirs of antibiotic resistance in the environment (e.g., hospital and health care effluents, municipal effluents, animal production). The intervention in these critical control points would diminish the risks of antibiotic dissemination	Actions: Definition of legal maximum admissible levels of key antibiotic resistant bacteria or genes in wastewaters, prior to their discharge in the receiving environment Development of cost-effective, environmentally sustainable, and technically feasible processes of advanced treatment for wastewater (Table 2) Key players: the scientific community, wastewater treatment companies, public health, and environmental protection entities

Table 2 Some characteristics of currently used wastewater disinfection processes

	Chlorination	Ozonation	UV irradiation	Membrane filtration
Cell damage mechanism	Oxidation	Oxidation	DNA damage	Physical retention
Residuals	Yes, toxic to aquatic life[a]	Short lived	No	No
Formation of toxic by-products	Yes	Yes	Yes	No
Increase in the level of total dissolved solids	Yes	No	No	No
Resistant microorganisms	Yes	Unknown	Yes	No
pH dependence	Yes	Yes	No	No
Suspended solid dependence	Yes	Yes	Yes	Yes
Dissolved organic matter dependence	Low	High	Moderate	Moderate
Contact time	Long (min)	Moderate (min)	Short (s)	NA
Capital costs[b]	Low/medium	High	Low/high	High
Operation costs[b]	Medium	Medium	Medium	High
Energy consumption	Low	High	High	High
Possible disadvantages	Formation of TPs[c]	Formation of TPs[c]	Formation of TPs[c]	Biofouling; high microbial load of retentate

NA not applicable

[a] Requires a dechlorination step

[b] Dependent on size of the WWTP

[c] Formation of transformation products of antibiotics (TPs) that may still possess antibacterial properties