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REVIEW

Contribution of the Antropically – Impacted aquatic Ecosystems to the Resistance Reservior

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Abstract. Aquatic ecosystems are reservoirs of antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs), having a major role in their occurrence, accumulation and dissemination. The purpose of this review is to highlight the influence of wastewater treatment plants (WWTP) effluents, manure and biosolids use in agriculture as well as of aquaculture upon the development of antibiotic resistance (AR). Research indicates the need to streamline treatment strategies in order to minimize the risk of AR spread in the aquatic environment through wastewater.

Keywords: *Antibiotic resistance, antibiotic-resistant bacteria, antibiotic resistance genes, wastewater treatment plants*.

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Introduction

Throughout the world, in recent decades, after the *golden age* of antibiotics, their excessive consumption, both in human therapy and in animal husbandry, has led to the development and enrichment of different media in antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs) (Doma et al., 2015).

The World Health Organization (WHO) classified antibiotic resistance (AR) as one of the three most important threats to public health (Chen et al., 2017; Alexiu, 2019), causing hundreds of thousands of deaths annually (WHO, 2019). The European Commissioner for Health and Food Safety, Vytenis Andriukaitis, claims that in the EU, around 25,000 deaths a year are caused by rising AR. He states that AR "*is a global problem that needs a global solution. Unfortunately, the accumulation of factors - especially the excessive and inappropriate use of antibiotics and poor infection control practices - has progressively turned AR into a massive threat to humanity. The increase in AR and the lack of preventive measures can lead to a return to the pre-antibiotic era, when people died from*

common infections and minor injuries. This would have major consequences for human health and the economy, not only in Europe but also around the world" (Vytenis, 2017).

After the introduction of a new antibiotic in the clinic, the development of resistance is inevitable. The rate of emergence of bacterial strains resistant to new drugs is of the order of 1%, but after 8-12 years of intensive use of antibiotics (in human clinic and animal husbandry), multidrug-resistant bacterial strains have become very common (Mihăescu et al., 2007). Many of the bacterial pathogens, which have caused epidemics among the human population, have evolved into AR forms versus several classes of antibiotics (MDR = multidrug resistance) (Alekshun and Levy, 2007; Moisoiu et al., 2016). The term "*superbugs*" defines microorganisms which, as a result of multiple mutations, cause increased morbidity and mortality and cause a high level of AR to the classes of antibiotics specifically recommended for their treatment. In some cases, super-resistant strains also have increased virulence and increased transmissibility, suggesting that AR may even be considered a virulence factor (Moisoiu et al., 2016).

Currently, there is a danger of continuous development of AR in pathogenic bacteria, a fact of particular concern for the species from the "ESKAPE group" (Pendleton et al., 2013; Peneş et al., 2017). In 2019, the emergence of an extensively drug-resistant (XDR) strain of *Klebsiella pneumoniae* (XDR), including carbapenems and colistin, has been reported (https://www.ecdc.europa.eu/sites/default/files/documents/Communicable-disease-threats-report-5-Oct-2019-rs.pdf), in Germany, Slovenia, Spain, Iran, Turkey, United Kingdom, Greece, Switzerland, France (Robert Koch Institute. Datenbank Resistenzübersicht 2018. RKI; 2019, https://ars.rki.de/Content/Database/ResistanceOverview.aspx).

If initially, the research has focused on the AR acquired in the hospital environment, subsequently, a major role in the development and dissemination of AR was attributed to the natural environment (Gillings et al., 2013). Antibiotics and ARGs existing in the natural environment may in part originate from anthropogenic contamination (Gillings et al., 2013; Anthony et al., 2017). The development of ARBs and their distribution in microbial populations throughout the biosphere are the result of many years of relentless selection pressure since the introduction of antibiotics (Barber and Rozwadowska-Dowzenko, 1948; Crofton and Mitchison, 1948; Watanabe, 1963; Levy, 1982; Davies, 1995; Chopra and Roberts, 2001; WHO, 2014).

The volume of antibiotics used in the outpatient setting is much higher than in hospitalized patients, up to 30% of all outpatient antibiotic prescriptions being considered unnecessary and up to 50%, inadequate for the pathological condition (Schmidt et al., 2018). In agriculture, antibiotics are also used in excess, not only to fight infections, but also to stimulate the growth of some animals (birds, pigs and cattle) (Mecena, 2017). The pharmaceutical industry in

conjunction with the increased use of antibiotics in human and veterinary medicine are closely linked to the increasing prevalence of ARB, favoring their release along with human and animal waste in the environment, and especially in aquatic ecosystems such as industrial, community, clinical and agricultural wastewater (Li et al., 2010; Rizzo et al., 2013; Devarajan et al., 2015; Barancheshme and Munir, 2017; Anthony et al., 2017). In most cases, the concentration of ARB in the environment correlates with that of ARGs (Kristiansson et al., 2011; Anthony et al., 2017).

The aquatic environments are important in the circulation and storage of antibiotics and are therefore considered true hotspots for the development and dissemination of ARGs (Baquero et al., 2008; Berglund et al., 2014; Ma et al., 2017). Rizzo and co-workers described wastewater treatment plants (WWTPs) as critical points for the occurrence of AR (Anthony et al., 2017). Because WWTP collects wastewater from domestic sources, respectively from hospitals or the food industry, where the consumption of antibiotics is quite high, it was found that, although water is subjected to treatment and disinfection, ARB and ARG are not completely eliminated. In other words, these water sources have a major impact on ARG transfer and dispersion, representing an immediate concern for researchers (Rizzo et al., 2013; Rodriguez-Mozaz et al., 2015; Xu et al., 2015; Paul et al. colab., 2018).

Main categories of chemicals that act as selective pressure factors for AR in the environment

The literature highlights three categories of chemicals with antimicrobial action: antimicrobials (antibiotics, antifungals, antivirals and antiparasitics), heavy metals and biocides (disinfectants and surfactants) (Singer et al., 2016).

Antibiotics

Antibiotics are heterogeneous chemicals produced by microorganisms (actinomycetes, Gram-positive bacilli and microscopic filamentous fungi) through biosynthetic processes, in order to kill or inhibit the growth of other species of microorganisms (Doma et al., 2015). Semi-synthetic or artificially synthesized substances are currently included in the category of antibiotics (Mihăescu et al., 2007).

Before the 1930s, mortality due to infections was huge, this period being described as the "*dark age*" or pre-antibiotic period. The first active antibiotic, benzylpenicillin, was discovered in 1928 by Alexander Fleming (Fleming, 1929), introduced into infection therapy only in 1942, when pharmacologist Howard Florey and biochemist Ernst Boris Chain purified and widely produced penicillin, the miracle drug of the twentieth century.The resounding success of human subjects has triggered numerous researches completed with the appearance of numerous antibiotics. After the 1930s, the "*era of antibiotics*" began (Jesman et

al., 2011; Preoţescu et al., 2014), with the glory years 1940-1960, in which most of the antibiotics used today were discovered. In 1940, Chaine and Florey were able to isolate crystallized penicillin in pure form, only after studying its qualities, it was used for therapeutic purposes. The resounding success of human subjects triggered numerous researches completed with the appearance of numerous antibiotics, so in 1944 streptomycin was discovered, in 1947, chloramphenicol, in 1948, chlorotetracycline, in 1958 semisynthetic penicillins, in 1960 cephalosporins, in 1980 fluoroquinolones etc. (Giguere, 2006; Doma et al., 2015).

In the years 1980-1990, genetic studies were performed on the bacterial genome and, implicitly, the targets on which antibiotics act on the bacterial cell, as well as ARG (Giguere, 2006; Doma et al., 2015). In developed countries, the average life expectancy has increased by about twenty years due to the discovery of antibiotics, besides active immunization through vaccination (Doma et al., 2015). Antibiotics are also frequently used in the veterinary sector (e.g., penicillins, tetracyclines, macrolides, aminoglycosides) (Giguere, 2006; Doma et al., 2015). On the other hand, antibiotics pose a major risk to human health because they are considered the main selective factors responsible for the occurrence and spread of AR both in the clinic and in natural bacterial communities (Bayarski, 2006; Sengupta et al., 2013; Cristea et al., 2015).

Improper management of AR has exacerbated the situation, so many bacterial conditions (eg. infections with *Escherichia coli* O157: H7, opportunistic Enterobacterales such as *Klebsiella* sp., methicillin-resistant *Staphylococcus aureus*, or with vancomycins-resistant enterococci, Lyme disease, toxic shock syndrome, destructive skin streptococcal infections) need totally new antibiotics (Keyes et al., 2003; Giguere, 2006; Wulf et al., 2007; Aarestrup et al., 2008; Doma et al., 2015).

In human common infections with *Campylobacter* and *E. coli*, resistance to fluoroquinolones occurs due to the use of fluoroquinolones in animal feed, and through meat there is subsequent transmission of ARB to humans (Doma et al., 2015). Increased frequency of quinolone resistance has been also demonstrated for other enteric pathogens, such as *Salmonella enteritidis* and *Campylobacter spp.*, while *Salmonella typhimurium* manifests multiple resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracycline (Hendriksen et al. 2004, Doma et al. , 2015).

In soil and water, low concentrations of antibiotics can probably allow the selection of ARB, thus enriching the collection of ARG in the environmental *resistome* (Doma et al., 2015). Due to the permanent release of microbes from sediments into the water, the constructed wetland deposits are major sources of ARGs. The operational and environmental factors of constructed wetlands greatly influence the types and concentrations of ARGs, more than the source of pollutants. Household sewerage is the main source of ARGs in the constructed

wetland (Barancheshme and Munir, 2017). Fang et al. highlighted the presence of ARB, ARGs [*sul* (1), *sul* (3), *tet* (A), *tet* (C), *tet* (E) and *qnr* (S)] and the presence of contaminants, such as antibiotics and metals (Fang et al., 2017). In one study, Chen et al. detected the presence of *sul* (1), *sul* (2), *sul* (3), *tet* (G), *tet* (M), *tet* (O), *tet* (X), *erm cml* (A) and *flo* (R) resistance genes in six constructed wetlands mesocosms (Chen et al., 2016).

Studies have shown that wastewater and animal waste contain a large number of resistant bacteria and, although they are subjected to various treatment processes in WWTP, the bacteria can reach the receiving waters (Rizzo et al., 2013; Czekalski et al., 2014). At least locally, the continuous discharge of these contaminants causes an increase in the level of natural resistance. Under these conditions, the probability of ARGs reaching back to human and animal microbiota or even pathogens is increasing (Cantas et al., 2013; Czekalski et al., 2014).

Antibiotics and their metabolites are eliminated in the urine and feces of patients, reaching hospitals effluents. Similarly, people who are treated at home with different classes of antibiotics, eventually release antibiotics and ARB in the wastewater treatment system. Subsequently, antibiotics end up in the sludge of the stations and, through it, in the field, in the form of fertilizer, or, directly in the receiving surface waters (Berglund, 2015).

Wetlands are exposed to antibiotic contamination through the wastewater to be treated. In animal and poultry farms, antibiotics are used both as growth promoters and for therapeutic purposes. These aspects determine the expansion, through animal excrement, of antibiotics and metabolites, in fields and in groundwater. If antibiotics are used on fish farms, they get directly into the aquatic environment. Whenever antibiotics are spread, ARB is likely to follow the same dispersal pathways. Thus, there are environments that have a mixture of antibiotics, ARB and bacterial mcirobiota, which can also aquire ARGs turning them into resistance hotspots that favor the proliferation of ARGs and the creation of new resistant strains, by HGT (Berglund, 2015).

The selective forces involved in maintaining ARGs include not only the direct pressure of antibiotic, but also the co-selection exerted by other substances present, such as metals and biocides (Baker-Austin et al., 2006; Wales and Davies, 2015). Resistance determinants for some of these compounds can be co-located on the same mobile genetic elements as ARG (Pal et al., 2015). Similarly, exposure to other stressors other than antibiotics may select genes encoding efflux pumps, which in turn may generate bacteria less sensitive to antibiotics.

Heavy metals

Anthropogenic contamination of the environment with heavy metals is a major problem in the AR control, as it contributes to the selection and co-selection of ARB and ARG. Co-selection is due to the coupling of resistance mechanisms

to antibiotics and heavy metals, by locating genes that confer resistance to solvents, biocides and heavy metals on the same mobile genetic elements or by selecting ARGs encoding cross-resistance to these classes of substances (Seiler and Berendonk, 2012; Pal et al., 2015; Johnson et al., 2016; Curiao et al., 2016; Singer et al., 2016).

For centuries, metals, in the form of metal salts, such as copper (Cu), magnesium (Mg), mercury (Hg), tellurium (Te), arsenic (As) and gold (Au) have been used as antibacterial agents, an example being the copper (Cu) and silver (Ag) vessels used to maintain the quality of stored drinking water (Kayne, 1935; Alexander, 2009; Pal et al., 2014). Currently, Cu and Ag are widely used in household products (Russel, 2003; Pal et al., 2014). Titanium (Ti), Cu and Ag are widely used as nanoparticles in food, textiles, household, industrial and hospital products (Selck et al., 2016).

Globally, agricultural practices contribute to environmental pollution by introducing heavy metals such as lead (Pb), Cu, zinc (Zn), cadmium (Cd) and As in feed additives, nutritional supplements, biocides used to disinfect stables, fertilizers, pesticides and fungicides (Pal et al., 2014; Zhu et al., 2017). The main source of contamination with heavy metals, such as Cu and Zn, of arable land is agriculture, and 30% of the contribution of Cd in the soil comes from inorganic fertilizers (Seiler and Berendonk, 2012). Heavy metals, along with antibiotics used in agriculture both as growth promoters and in the treatment and limitation of the spread of infections in animals (Burridge et al., 2010) reach soil enriched with manure and can play an important role in the evolution of ARB (Seiler and Berendonk, 2012). At the level of WWTPs, major urban contributions of heavy metals come from households, sewage, car washes, atmospheric deposition, traffic-related emissions, gasoline and oil leaks (Karvelas et al., 2003; Ardestani et al., 2015). Although urban wastewater is treated by various processes, detectable concentrations of antimicrobial compounds have been found in the receiving media (Rodriguez-Mozaz et al., 2015); thus, in the sediment samples of several rivers, high concentrations of antibiotics, ARB and ARG were found. A study conducted at Arizona State University by researchers at the Institute of Biodesign that targeted 11 countries inspected the use of 47 antibiotics in various aquacultures (salmon, catfish, trout, tilapia, swai, shrimp) and found traces of five antibiotics (Done and Halden, 2015; Barancheshme and Munir, 2017).

The presence of heavy metals in sewage treatment plant influents has been shown to disrupt biological processes used in the wastewater treatment, such as nitrification (Braam and Klapwijk, 1981), denitrification (Waara, 1992) and removal of organics (Ajmal et al., 1983).

Bacteria have developed tolerance mechanisms to avoid the toxicity of heavy metals, by: i) binding and sequestration of toxic metals (Silver and Phung, 1996), to reduce the concentration of free toxic ions in the cytoplasm. Biosorption

of toxic metals occurs at the level of cell membranes, cell walls and extracellular polymeric substance (EPS) of biofilms (Teitzel and Parsek, 2003; Harrison et al., 2007); ii) detoxifiation by reducing intracellular ions (Nies, 1999), under the action of enzymes, such as Hg-reductase, encoded by the *merA* gene. *MerA* protein reduces Hg^{2+} to the less toxic Hg^{0} (Schiering et al., 1991). Hg^0 will then diffuse out of the cell due to its low evaporation point (Nies, 1999); iii) extrusion of toxic ions by efflux systems (Nies, 1999).

Previous studies have shown that bacteria that carry metal resistance genes carry ARG more frequently, and these genes can be found in the structure of plasmids (Pal et al., 2015; Di Cesare et al., 2016). A study of 4 582 plasmids showed that in only 5% of cases i the same plasmid carried both ARG and metal resistance genes (Pal et al., 2015).

Biocides

They are disinfectants commonly used in hospitals, veterinary medicine, in the cosmetics industry, household cleaners, preservatives, on farms and in a number of industrial processes, including for field control (Kahrilas et al., 2015; Singer et al., 2016). Between 1992 and 2007, there was a 40% increase in the use of biocides globally (Sattar et al., 2007). In Europe, the marketing, use and disposal of biocidal products are regulated by Regulation (EU) 528/2012 on biocidal products (European Commission, 2012).

The most commonly used biocides are: ethanol, formaldehyde, chlorhexidine, triclosan and quaternary ammonium compounds (QACs - alkyldimethylbenzyl ammonium chloride (ADBAC), stearalkonium chloride, isothiazole-benzyl chloride), cetylpyridinium chloride, alkylaminoalkyl glycine and didecidimethylammonium chloride (DDAC) (Buffet-Bataillon et al., 2012; Singer et al., 2016). Triclosan and other biocidal substances, such as chlorhexidine and QAC, but also their sub-lethal concentrations have been shown to contribute to the selection of AR of clinical importance (Webber et al., 2015; Buffet-Bataillon et al., 2016). One study found that for six different biocides, the minimum inhibitory concentration (MIC) was between 0.4 and> 1000 mg / l against 16 different clinical bacterial strains of *P. aeruginosa*, *E. coli*, *S. aureus* and *Citrobacter* sp. (Webber et al., 2015).

Biocides and antibiotics have a common path in the environment and reach, in particular, the WWTPs, which may increase the load of biocides released into the environment, and consequently increase the likelihood of selecting AR (Chapman, 2003; Baker-Austin et al., 2006; Singer et al., 2016).

The use of biocides and heavy metals as antibacterials appears to promote multidrug resistance (Baker-Austin et al., 2006; Pal et al., 2015; Wales and Davies, 2015; Pal et al., 2017).

The scientific literature mentions the presence in soil and water of pathogenic bacteria belonging to the genera *Escherichia*, *Pseudomonas*,

Salmonella, *Shigella*, *Aeromonas*, *Klebsiella* (Stokes and Gillings, 2011), harobring plasmids that carry resistance determinants to at least one heavy metal (Cu, Ni, Pb, Cd, Co, Hg, Zn) and to antimicrobial substances from different groups (tetracyclines, quinolones, aminoglycosides, sulfonamides, β-lactams and chemotherapeutics) (Seiler and Berendonk, 2012; Cantas et al., 2013). These soil bacteria form a reservoir of resistance determinants that can be transferred to the microbial community, including pathogenic bacteria.

Dissemination of ARB in the environment

The dispersal pathways of ARB through the environment have not been fully investigated, but the main route of exposure of humans to resistant pathogens is contact with other people, either in clinics or in the community, directly or indirectly, through aerosols and food prepared by contaminated persons (Livermore, 2000).

In addition to interhuman transmission, the environment plays an important role in the spread of AR (Allen et al., 2010; Finley et al., 2013; Pruden et al., 2013; Levin, Baquero and Johnsen, 2014; Huijbers et al., 2015). Research aimed at identifying the sources and associated health risks has generated some knowledge about the persistence and reinfection potential of human intestinal bacteria released into the environment, for example, in untreated wastewater (Harwood et al., 2014). In this context, sewers, sewage treatment plants, aquatic environments, travel, but also aerosols, dust and food, are important vectors that allow the transmission of bacteria between hosts, through the environment (Fernando, Collignon and Bell, 2010; Molton et al., 2013; Rolain, 2013; Pruden, 2014; Barberan´ et al., 2015; Bengtsson-Palme et al., 2015; McEachran et al., 2015; Pal et al., 2016; Bengtsson -Palme, 2017).

Rizzo and co-workers described wastewater treatment plants as critical points for the occurrence of AR (Anthony et al., 2017). Because WWTP collects wastewater from domestic sources, respectively from hospitals or the food industry, where the consumption of antibiotics is quite high, it was found that, although water is subjected to treatment and disinfection processes, ARB and ARG are not completely eliminated. In other words, these water sources have a major impact on ARG transfer and dispersion, representing an immediate concern for researchers (Rizzo et al., 2013; Rodriguez-Mozaz et al., 2015; Xu et al .; 2015; Paul et al., 2018). WWTPs provide opportunities for interaction for a number of different bacterial species and provide sufficient conditions for resistance selection (Rizzo et al., 2013). One of the environmental factors influencing AR transmission is the total organic carbon concentration (TOC) that can affect selection for certain genes [*tet* (A), *erm* (B) and *qnr* (S)]. High concentrations of TOC in the effluent of a WWTP can alter the extent and dissemination of ARG in

receiving aquatic environments (Di Cesare et al., 2016; Barancheshme and Munir, 2017).

WWTPs generally discharge their effluent, often harboring ARG (Bengtsson-Palme et al., 2016) into aquatic environments, and contaminated water is often used to irrigate agricultural land, for recreational swimming, and not least as drinking water, after additional treatment. Surface water (which is not treated) is used for animal watering and, through them, ARBs are subsequently spread to humans. Untreated wastewater released into aquatic environments poses a considerably higher risk than sewage treatment plant effluents in ARB release, as the relative abundance of most ARGs is reduced in effluents and the total bacterial abundance is also low (Bengtsson-Palme et al., 2016; Karkman et al., 2016).

In order to limit the spread of bacteria (resistant or not) associated with humans, it is necessary to understand the barriers to their dispersion in the environment. Contrary to the bacteria in the clinical environment, in the case of community-transmitted bacteria, relevant barriers to their dispersion have been identified. Bacteria have very different survival requirements outside their preferred habitat, which also indicates specific dispersal barriers. For example, many intestinal bacteria are anaerobic, and therefore their ability to disperse outside the human body is limited. The survival of associated human bacteria (resistant or not) is essential in their dissemination through the environment, and the presence of ARG is essential for the survival of species that use only the environment as a dispersion matrix, in the presence of low concentrations of antibiotics. For bacteria that use the environment as an alternative habitat - or even their main habitat - and therefore can easily grow, exposure to antibiotics contributes to the selection of ARG during dissemination. This category includes opportunistic and emerging pathogens such as *P. putida*, *Stenotrophomonas maltophilia* and *B. cereus* (Berg, Eberl and Hartmann, 2005). Therefore, the factors influencing the selection of resistant strains during dispersal are specific to both the antibiotic and the bacterial species.

Another important factor in dispersal processes is the ability to form latent inactive stages, such as highly resistant spores formed by some pathogenic bacteria, including *B. anthracis* (Leggett et al., 2012). Stages of inactivity could facilitate the survival of bacteria in the dispersion matrix, but they return to the vegetative state when they reach a suitable host (Lennon and Jones 2011; Shade et al., 2012).

Wind and water movement are under-investigated physical factors that contribute to the dissemination of bacteria, including BRA, over long distances (Allen et al., 2010). Many bacteria have been isolated from the air, including species of the genera *Micrococcus*, *Staphylococcus*, *Bacillus*, and *Aeromonas* (Gorny and ´Dutkiewicz, 2002; Tsai and Macher, 2005; McEachran et al., 2015; Pal et al., 2016; Gat et al., 2017). However, due to the lack of nutrients in the air,

the ability to transfer ARG becomes an almost negligible factor compared to survival and persistence in the air. However, horizontal transfer of ARGs between aerosol bacteria and dust particles remains a possibility, with a lower probability of persistence of transferred genes in the recipient genome, unless they are subsequently exposed to antibiotics in a growth-stimulating environment.

Wild birds and animals also contribute to the spread of ARG over long distances (Baquero, Martinez and Canton, 2008; Bonnedahl et al., 2009; Stedt et al., 2015).

Global food trade is another way to spread resistant pathogenic bacteria worldwide, an example being the 2011 German outbreak of Shiga toxin-producing *E. coli* (O104: H4) (Buchholz et al., 2011; Rasko et al., 2011). The pharmaceutical industries in conjunction with the increased use of antibiotics in human and veterinary medicine are closely linked to the increasing prevalence of ARB, favoring their release with human and animal waste (Li et al., 2010; Anthony et al., 2017).

Most existing ARGs, including those not yet described, are most likely to be present in environmental bacteria (Allen et al., 2010). Bacteria that are not normally associated with the human microbiome can interact with other organisms, for example, wild animals, and can be transmitted to humans through the intake of raw food or contaminated drinking water (Allen et al. 2010; De Boeck et al. 2012 ; Ghaly et al. 2017). In addition, ARGs identified in soil bacteria have also been identified in clinical pathogens (Sommer et al., 2009; Frosberg et al., 2012). There are other directions through which human bacteria can interfere with those associated with animals and the environment, a key point in these contexts being the length of the dispersal path (Baquero, Alvarez-Ortega and Martinez, 2009). A pathogen (or commensal agent) that acquires a new resistance factor from an environmental bacterium, but is eradicated before returning to a human host, never causes clinical resistance problems, becoming real threats to human health only those that, finally, they are transmitted effectively to humans. Transfer of resistance factors from human pathogens to environmental bacteria is also possible, allowing human bacteria to use bacterial populations in the environment as reservoirs of resistance genes, which are later recruited into the associated human resistance (Baquero, Martinez and Canton, 2008).

Despite efforts to clearly identify horizontal ARG transfer events from environmental strains to pathogenic strains, only a few examples have been documented, namely aminoglycoside-modifying enzymes (AMEs), resistance to vancomycin in clinical isolates of *E. faecium* in Europe was subsequently identified as being associated with actinomycetes producing glycopeptide antibiotics in soil (Leclercq et al., 1988; Marshall et al., 1997, 1998), but also to

bacteria that do not produce antibiotics from the genera *Paenibacillus* and *Rhodococcus* in the soil.

Recently, it has been shown that several ARGs (genes that confer resistance to β-lactams, aminoglycosides, tetracyclines, sulfonamides and phenicols) have been transferred between environmental bacteria and clinical pathogens (Forsberg et al., 2012).

Research shows the transfer of ARGs between various organisms such as *Clostridium perfringens*, *S. pneumoniae*, *E. faecalis* and *Bacteroides* strains (Shoemaker et al., 2001). Opportunistic soil pathogens, such as *Burkholderia cepacia*, *Ochrobactrum intermedium* and *S. maltophilia* (Berg, Eberl and Hartmann, 2005; Johnning et al., 2013), may be intermediate organisms that can act as receptors for resistance genes from associated bacteria. human and which could further transfer those genes or may even infect humans.

Strategies to reduce the emergence and spread of resistance in the aquatic environment

It is ideal for the spread of ARG to human pathogens to be attenuated before they reach the human microbiota. Thus, an important component in the assessment and management of AR risks should be the detection of ARGs before they spread to clinical isolates (Bengtsson-Palme and Larsson, 2015).

In order to establish appropriate AR mitigation strategies, the environments with the highest risks must first be identified. Some researchers claim that the most severe risk scenarios are constructed based on previously known ARGs reported to be located in the structure of mobile genetic elements present in human pathogens (Martinez, Coque and Baquero, 2015). ARGs from mobile genetic elements spread easily with human feces, are detected in the external environment, and may be a parameter of human fecal contamination (Pruden et al., 2006, 2013, 2014; Pruden, Bengtsson- Palme, Larsson and Kristiansson, 2017). The main risks associated with the dissemination of AR are: mobilization and permanent establishment of new ARGs, recruitment of ARGs through HGTs, dissemination through the environment to the human population of ARBs (pathogens or not) (Bengtsson-Palme et al., 2017).

Areas with a high risk of AR occurrence and mobilization are those in which antibiotic concentrations are higher than the established minimum selective concentrations (MSCs), or even MIC values for many bacteria. This category includes the human and animal microbiota during antibiotic treatment, antibiotic-assisted intensive aquaculture (Cabello, 2006), as well as environments polluted with high levels of antibiotics from industrial sources (Larsson, 2014). Although not sufficiently explored, the conditions faced by bacteria in WWTPs from antibiotic production are probably very selective, so that the bacteria present in such environments have a very limited diversity (Marathe et al., 2016). ARG

mobilization and transfer increase during antibiotic exposure (Hocquet et al., 2012), occurring even at sub-inhibitory antibiotic concentrations (Jutkina et al., 2016, 2017).

Wastewater may also contain sufficient concentrations of toxic substances to induce HGT. For the transfer of resistance to human pathogens, the abundance of pathogenic bacteria that can act as receptors is crucial. In this sense, the human microbiome could play a role in this process, and human microbiota can act as reservoirs of intermediate resistance (Sommer, Curch and Dantas, 2010; Forslund et al., 2013). Important steps to mitigate the development of environmental AR are: limiting discharges of pharmaceutical waste from antibiotic production and reducing unnecessary use of antibiotics in humans, animals and aquaculture (Pruden et al., 2013; Bengtsson-Palme and Larsson 2016a, b; Review on antimicrobial resistance, 2016). Furthermore, identifying and limiting the dispersal pathways of ARBs to the human microbiome should be a priority.

Because sewage treatment plants, especially those that receive wastewater from hospitals, are considered hot spots for MDR bacteria (Anthony et al., 2017), one of the most important concerns is the microbiological safety of recovered wastewater. Municipal wastewater is a favorable environment for both survival and bacterial resistance transfer, due to mixtures of bacteria, abundant nutrients and antimicrobial agents (Yuan et al., 2015). In wastewater treatment plants, critical control points have a special importance in limiting the spread of resistance (Berendonk et al., 2015), for example, disinfection of treated effluent could be an effective means of controlling the spread of ARB.

From a technical point of view, recycled water is considered municipal wastewater, subject to various treatment processes and can only be used if it meets specific water quality criteria. The US Environmental Protection Agency (USEPA) has argued the importance of subjecting urban wastewater to secondary and / or tertiary treatment to significantly reduce organic and inorganic constituents. This reduction is evaluated according to biochemical and chemical oxygen consumption (Hong et al., 2013).

The treatment used should reduce faecal coliforms to less than 200 faecal coliforms / 100 ml in the recovered water, before the water can be used for irrigation of any food crop, orchard or vineyard (Hong et al., 2013).

Activated carbon, filtration, ozonation, photonic nanotechnologies are technologies used to remove ARB and ARG from wastewater (Anthony et al., 2017). Numerous studies aimed at evaluating the efficacy of different treatments applied to wastewater for the elimination of ARB and ARG suggest that the efficacy of elimination depends on the physicochemical properties of antibiotics and the treatments applied, which should lead not only to inactivation of pathogens but also to the destruction of ARG (Rizzo et al., 2013; Flecher, 2015; Barancheshme and Munir, 2017).

Anaerobic and / or aerobic treatment

Through aerobic treatment processes, which are performed in the presence of microorganisms and oxygen, organic contaminants are transformed into carbon dioxide, water and biomass. Through anaerobic treatment processes, which take place in the absence of oxygen and microorganisms, organic contaminants are transformed into methane gas, carbon dioxide and biomass (Barancheshme and Munir, 2017). A study conducted at a WWTP that assessed the variation of five ARGs (*tet* (G), *tet* (W), *tet* (X), *sul* (1) and *intl* (1)) showed that anaerobic treatments and anoxic effectively remove ARG, because in anaerobiosis, microorganisms have low bioactivity and ARG transfer is inhibited (Du et al., 2015). In the wastewater treatment process, a significant positive correlation is observed between the reduction of *tet* (W), *intl* (1) and *sul* (1) and the reduction of 16S DNA (Du et al., 2015). If anaerobic and aerobic treatments are applied successively, biological treatment methods successfully eliminate antibiotics, ARB and ARG. The efficiency of aerobic treatments is lower if they are not followed by anaerobic ones (Barancheshme and Munir, 2017). Better efficiency of ARG removal is obtained when biological treatment is used in combination with filtration-based technologies (e.g., ultrafiltration, nanofiltration and reverse osmosis), such as biological membrane reactor (BMR) facilities.

Constructed wetlands

They are small semi-aquatic ecosystems that favor the multiplication of different microbial communities and the achievement of various physico-chemical reactions (Barancheshme and Munir, 2017). These anthropogenic ecosystems represent an attractive approach for the treatment of municipal, industrial and agricultural wastewater, in terms of their simplicity, low costs and, especially, the elimination effect of ARG (Fang et al., 2017). Water volume, plant species and types of flows (surface flow, horizontal and vertical zoning) are characteristics of the constructed wetland on which the efficiency of ARB and ARG removal depends (Barancheshme and Munir, 2017). In the case of constructed wetlands, a significant role in reducing the load of nutrients, antibiotics and ARG has the biodegradation, but also other processes, such as adsorption at the substrate and plant absorption (Chen et al., 2016). The constructed wetland showed elimination rates of 77.8% (in the winter season) and 59.5% (in the summer season) of the total of 14 ARGs analyzed (Fang et al., 2017). The removal of pollutants, especially antibiotics is achieved by the presence of plants, proving more effective in the case of submerged constructed wetlands, as opposed to wetlands built in surface flow (Chen et al., 2016).

Disinfection

It is frequently applied in wastewater treatment plants for the destruction of pathogenic microorganisms and viruses (Barancheshme and Munir, 2017). The disinfection processes used in wastewater treatment are chlorination, ozonation,

UV radiation, Fenton reaction and photocatalytic systems, among them, chlorination being the most frequently applied due to its availability and efficiency.

Many heterotrophic MDR bacteria are found in the effluents of drinking water treatment plants, so effective disinfection is a vital process for the removal of various antibiotics (eg., cephalexin, ciprofloxacin, chloramphenicol, erythromycin, gentamicin, rifampicin, sulfadiazine, and tetracycline), ARB and ARG (Barancheshme and Munir, 2017).

Some studies have compared two or more disinfection processes in terms of their effectiveness and mechanism. In this regard, the results of a recent study carried out in a municipal sewage treatment plant that used three different disinfection methods (chlorination, single UV irradiation and sequential UV / chlorination) to control ARG (*sul* (1), *tet* (X), *tet* (G), *intl* (1) and rRNA 16S) highlighted a positive relationship between ARG elimination and disinfection parameters (Sharma et al., 2016). Thus, the combination of sequential UV irradiation / chlorination had the maximum efficiency of ARG removal, as opposed to single UV irradiation, which proved to be less effective. Another study conducted on a municipal wastewater treatment effluent applied three different disinfection methods (chlorination, UV irradiation and ozonation) to investigate the removal of *sul* (1), *tet* (G) and *intl* (1). The obtained results support the increased efficiency of chlorination for ARG elimination, compared to the other methods used (Zhuang et al., 2015).

Although most researchers argue the effectiveness of chlorination in reducing the number of ARBs, however, some studies show contradictory results, according to which chlorination would contribute to ARG enrichment due to co-resistance or cross-resistance to disinfectants and antibiotics (Ma et al., 2017). For example, chloramphenicol, trimethoprim, and cephalothin-resistant bacteria are reported to be chlorine tolerant, reducing chlorination efficiency (Yuan et al., 2015). Also, another study by Iwane and colleagues claims that chlorination did not significantly alter the percentage of ampicillin and tetracycline resistance in *E. coli* (Iwane et al., 2001), a conclusion also confirmed by Grabow and his collaborators, according to which the percentage of ampicillin-resistant bacteria in wastewater changed slightly or not at all, after chlorination. The study by Luo and colleagues in New Delhi shows that, using chlorination as a method of water treatment, the NDM-1 gene remains at significant levels in effluents (Luo et al., 2013). Yuan et al. they also support the idea that ARG transfer to ampicillin and tetracycline in wastewater cannot be inhibited by chlorination, due to the low doses of chlorine used (Yuan et al., 2015).

Bacterial cells are inactivated by chlorine due to the strong oxidizing effect of hypochlorous acid (HClO). On the other hand, there is a possibility that ARG will not be destroyed, but will persist as free DNA. Consequently, a

decrease in ARG concentration after chlorination may be indicated by the absence of free DNA (Yuan et al., 2015).

Antimicrobial nanotechnologies

Aruguete and collaborators studied the antimicrobial potential of nanomaterials, their ability to limit the dissemination of MDR pathogens (Aruguete et al., 2013). In their experiments they used a combination of functional nanomaterials with antibiotic molecules, and the results highlight the antimicrobial capacity of nanomaterials through the toxic effects manifested against MDR bacteria, such as *P. aeruginosa* (Aruguete et al., 2013; Sharma et al., 2016; Barancheshme and Munir, 2017). On the other hand, nanomaterials can lead to the development of antibiotic resistance (Aruguete et al., 2013). In addition, some nanomaterials have demonstrated toxic effects on fauna, flora and humans (cytotoxicity, tissue ulceration, etc.) (Srivastava et al., 2015).

Coagulation

In WWTPs, coagulation is used as a tertiary treatment process, with good results for improving water quality and removing contaminants (Barancheshme and Munir, 2017). In recent years, the effectiveness of this method for removing ARG from treated wastewater has been investigated (Li et al., 2010). Inorganic coagulant, ferric chloride ($FeCl_3$) and inorganic polymeric coagulant, poly-ferric chloride (PFC) have been shown to be active in removing ARG *sul*, *tet* and *intl* from the effluent of treatment plants (Li et al., 2010). Zirconium coagulant has led to an improvement in water quality and a low content of dissolved organic carbon residue (Jarvis et al., 2012). Phosphorus contamination can also be eliminated by coagulation. The presence of residual phosphorus in the effluent of wastewater treatment plants can cause eutrophication and damage to the environment (Tran et al., 2012).

All wastewater treatment strategies aimed at reducing ARB and ARG have certain limitations, requiring further research in this area to increase the effectiveness of existing strategies or to develop new innovative approaches (Barancheshme and Munir, 2017).

The mechanisms by which biological processes influence ARB development and ARG selection / transfer are insufficiently known. Future studies that aim to implement wastewater treatment technologies capable of ensuring the production of effluents with an acceptable level of ARB, should consider the following aspects: risk assessment to allow accurate estimates of the maximum allowable abundance of ARB in effluents from wastewater treatment plants that would not pose a risk to human health and the environment; understanding the factors and mechanisms that lead to the maintenance and selection of AR in wastewater habitats (Rizzo et al., 2013).

Conclusions

A major threat to global health in the 21st century is AR. The driving force for increasing AR is the extensive use of antibiotics in the clinical setting, therefore, this phenomenon is strongly associated with hospitals and other clinical settings. The high spread of bacterial resistance in the community and in the environment is the result of the increased use of antimicrobial drugs for human and veterinary infections. ARBs and ARGs are frequently reported in different environments (sewerage, treated drinking water, river, soil and air). There is a growing awareness that AR in the environment is favored by WWTP effluents, the use of manure and the use of biosolids in agriculture and aquaculture. The ARB can be introduced into the human food chain and in clinical settings are often associated with gene transfer in both directions, perpetuating the AR cycle.

Therefore, further research is needed in this area to innovate treatment approaches and strategies, increasing the effectiveness of reducing the spread of AR in the aquatic environment. Concentrated efforts of scientists, regulatory authorities, industry, doctors, funders, etc are needed to built multidisciplinary approaches and strategies.

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