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Review

Antibiotic resistance from, and to the environment

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Abstract: Antibiotic resistance currently causes hundreds of thousands of deaths worldwide; it is a major and growing public health threat globally. The origins of many resistance genes in pathogenic bacteria can be traced down to the environment; while a staggering number of resistant bacteria and resistance genes, selected for by human activities, are released into the environment. The nature, quantities and fates of this bidirectional flux of organisms and genes are mostly unknown. In order to understand the evolution of resistance within clinical settings, and the impact of the release of resistant bacteria into the environment, it is crucial to assess these questions and to assemble an integrated view of the problem. This review aims at providing an update on related issues previously discussed elsewhere, and to contribute to the comprehensive understanding of the environment as a source, receptacle and reservoir of antibiotic resistance.

Keywords: antibiotic resistance; wastewater; soil bacteria; antibiotic usage; horizontal gene transfer

1. Introduction

Bacterial resistance to antibiotics was, before the advent of COVID-19, one of the most pressing public health issues worldwide. Even during the first year of COVID-19, the recognized death toll worldwide (about 1,800,000, according to the Oxford Martin School, ourworldindata.org) was "merely" 2.5-fold the estimated number of deaths that, year by year, have been attributed to antibiotic resistance (approximately 700,000, according to O'Neil [1]). Moreover, while it is likely that the mortality of COVID-19 would eventually diminish, after the application of vaccines already available, antibiotic resistance is predicted to keep growing, and to claim the lives of 10 million people each year, by 2050 [1]. Three years after the publication of the report containing the figures

above, and of a high level meeting of the United Nations General Assembly [2], no significant advance was achieved to try and harness the increasing trend of antibiotic resistance [3].

For many people, being a health crisis means that antibiotic resistance is only to be found in the clinical setting; for others, although aware of the presence of resistance in the environment, this is not more than a biological curiosity, with little clinical repercussion. Of course, in the short term and for the infected patient, it is much more of a threat to find an antibiotic resistance phenotype in a bacterial pathogen than it would be to detect a resistance gene in an innocuous, environmental microorganism. However, these harmless bacteria are acting as reservoirs and "incubators" of sorts, that collect, mix and release resistance determinants along with mobility and perhaps even virulence genes which, sooner or later, make their way back to human populations. To have an accurate idea of the presence and extent of antibiotic resistance in the environment serves at least two main purposes: (a) to measure the resistance that is being selected by, and released into the environment by human-related activities, ranging from clinical and agricultural usage, to wastewater treatment and non-antibiotic biocides; and (b) to timely detect resistance determinants that are yet to cause clinical problems, but that are likely to arrive to the clinical setting, reducing the efficacy of our already limited antimicrobial arsenal. Many of the issues analyzed below have been previously reviewed (e.g., [4]); this paper will update aspects pertaining to antibiotic resistance in the environment.

There are two different ways to address the issue of antibiotic resistance in the environment. On the one hand, it is possible to analyze the presence of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARG) in the environment, much before antibiotics were discovered by humans and released massively into the environment. Under this scope, the public health crisis posed by antibiotic resistance might very well be the consequence of clinically-relevant bacteria acquiring such ancient resistance determinants, under the selective pressure of human-made antibiotics. On the other hand, the release of ARB and ARG into the environment, that have been selected for by the clinical and agricultural use of antibiotics, can be seen as a form of anthropogenic environmental pollution. To think of the release of organisms, or even genes, as "environmental pollution", might sound exaggerated or paradoxical; but ARB/ARG are just that. While not "human-made" in the sense of actual manufacture, as are chemical pollutants, or noise and light; ARB/ARG have been vigorously selected by the reckless abuse of antibiotics, and are being released in massive amounts. As both scopes are true, it is necessary to address them simultaneously, and to understand their interactions and intricacies to have a full picture of the nature and impact of the presence of resistance in the environment. This is a dynamic, bidirectional process, that varies widely from one location to another, because of the differences in antibiotic usage, wastewater management, and the presence of non-antibiotic selective pressures, among many other things. Most examples and study cases come from developed countries, although it is likely the non-developed countries the ones that contribute the most to this problem. Hence, while there is plenty of information available, surely there are many "unknown unknowns" (Donald Rumsfeld *dictum*) on this subject.

2. What is "resistance"?

Before actually addressing the presence of ARB/ARG in the environment, it would be necessary to define "resistance". The first relevant issue around resistance is to distinguish intrinsic resistance

from acquired resistance: the former can be considered as an inherent ability of certain bacterial species to withstand the effect of certain antibiotics (e.g., anaerobic bacteria are intrinsically resistant to aminoglycoside antibiotics, because those drugs require the presence of an active respiratory chain to get into the bacterial cell; enterococci are intrinsically resistant to cephalosporins, because they lack the particular kind of penicillin-binding proteins to which cephalosporins bind). Acquired resistance, on the other hand, is a trait that is not inherent to the species, but was gained, either by mutation or horizontal gene transfer. As intrinsic resistance defines the spectrum of each antibiotic, it has always been a limitation of such antibiotic; acquired resistance is the one that poses a serious public health threat.

Being essentially a clinical problem —resistance hinders the ability of antibiotic treatments to cure infections, it is defined in clinical terms. Hence, an organism is deemed "resistant" if such acquired trait results in the increase in the minimal inhibitory concentration (MIC) of an antibiotic, high enough to cause the therapeutic failure of such antibiotic if it is used to treat an infection caused by the organism. This simple definition introduces a first problem, as the concentration of an antibiotic varies from one site in the body to another, making then possible, for instance, to treat an infection caused by the same germ in the urinary tract, because the antibiotic reaches high urinary concentrations; but not on the skin, because the same antibiotic fails to reach inhibitory concentrations in such tissue. Therefore, under the previous definition, the same germ is "susceptible" if it causes a urinary infection, but "resistant" if it causes a skin infection. Nevertheless, there are recognized "breakpoints", i.e., MIC values below which an organism is deemed "susceptible", and above which it is deemed "resistant". This oversimplification allows for the epidemiological studies to calculate rates of resistance, regardless of the body site each organism is isolated from. (There is a remaining issue about who and how those "breakpoints" are established; there are several organizations tasked with this, in different countries or regions, such as the CLSI in the US, and EUCAST in the EU. Due to slight variations, the same organism can be "susceptible" to an antibiotic in the US, and "resistant" in Europe, or vice versa.) As a natural consequence, an ARG is the one that encodes the resistance phenotype as described above. Clear cut examples could be the genes encoding beta-lactamases, which can cause a hundred- or thousand-fold increase in the MIC of beta-lactam antibiotics. There are, however, some cases where this definition is not clearly followed; for instance, *qnr* genes, that are plasmid-borne genes mediating "resistance" to quinolone antibiotics, can cause an increase in the ciprofloxacin MIC, from 0.06 μg/mL to 0.5 μg/mL, but still below the typical breakpoint for resistance, which is 4 μg/mL. While the role of *qnr* genes in clinical failure, as well as in the gradual increase to full-resistance, is well documented, genes such as these are more in a "grey zone" in terms of definition.

This issue gets much more complicated when ascertaining resistance in the environment. As many environmental bacteria are mostly innocuous, what antibiotics should be tested, to begin with? The ability to withstand clinically-achievable concentrations of antibiotics seems irrelevant for non-pathogenic bacteria. The line between intrinsic and acquired resistance is also blurred, as the susceptibility of environmental bacteria to antibiotics has only recently being explored in numbers large enough to distinguish between either kind of resistance. All these issues pose technical problems when trying to assess the existence of resistance in the environment, both the one predating the human use of antibiotics, and the one caused by it. To decide which antibiotics to test, and what

MIC breakpoints are relevant, is still a matter of discussion. With metagenomic assessments getting in vogue, the definition of ARG is crucial, as databases currently used list many non-transmissible, "housekeeping" genes, that when mutated or overexpressed lead to a resistance phenotype, but pose little to no danger in clinical terms. These "resistance" genes, very much akin to intrinsic resistance, are causing serious misinterpretations of metagenomic data of all sorts. There is a pressing need for these kinds of studies to evolve beyond the mere detection of "resistance" genes with the sole purpose of publishing a paper. Again, all of these have been previously reviewed and discussed [4]; however, it is important to remind the reader of a relevant paper on the issue [5].

3. Resistance in the environment

It is important to set aside two main categories of ARG/ARB that have been found in the environment: (a) those that predate the discovery and usage of antibiotics by humans, which could be taken literally as detected before the 1940's, but that most often refers to those that have existed for thousands or millions of years; and (b) those that have been selected in human-made environments (i.e., clinical and agricultural settings) and then released into the environment, to be detected in soils, water bodies and wildlife. As will be discussed below, the former have been, and continue to be the source of many ARG of clinical relevance, once they get out of their ancient reservoir and into pathogenic bacteria; while the latter can serve as indicators of the wide reach of this kind of "biological pollution" and of undetected or unknown selective pressures that keep or even enriches their presence in supposedly antibiotic-free environments. The release of "human made" ARG/ARB into the environment is also a major cause of concern, as these organisms often also carry determinants that confer resistance to non-antibiotic agents, as well as mobile genetic elements and even virulence traits that could potentially mobilize and rearrange once in the open environment. A graphic summary of the sections below is in Figure 1.

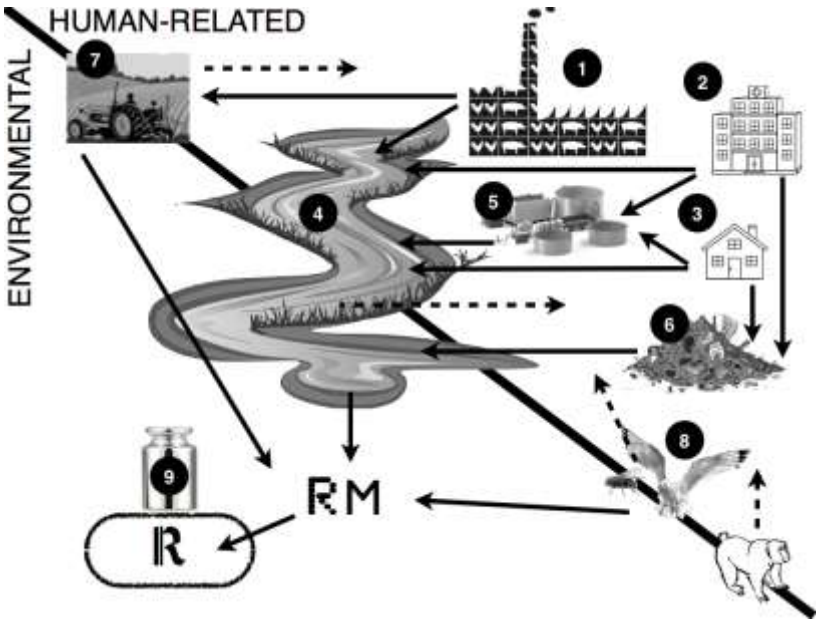

Figure 1. Resistance in the environment. Bacterial resistance to antibiotics became a problem due to the human massive production and use of antibiotics; this is represented

in the top-right side of the figure (human-related), mainly including the use of antibiotics in industrial farming (1) and other agricultural purposes; and the clinical use, both in hospitals (2) and outpatients (3). The main interface between urban or otherwise "human-related" environments, and open, "human-free" environments (bottom left side), are water bodies (4), that receive ARB/ARG in the discharge of wastewater from farms, hospitals and houses (either directly, or after treatment, in wastewater treatment plants (5)), and from leaching and runoff from garbage dumpsters (6). Also, manure from antibiotic-fed animals is applied directly to soils (7). Other "vectors" of ARB/ARG are flying animals (8). All these contribute to a pool of resistance genes, an "R" in dot-matrix font to represent its human-related origin; these genes are often linked to mobile elements, the "M", which facilitate their transfer to other microorganisms. On the other hand, environmental bacteria (bottom left) also carry ancient resistance genes (an "R" in old-fashion font), some that have already made their way to human pathogens, and many that do not. These environmental organisms are receiving loads of "new" resistance genes, along with mobile elements from human-made environments, and are under the selective pressure (9) of antibiotics, other antibacterial xenobiotics (e.g., disinfectants, non-antibiotic drugs with antimicrobial properties) and toxic metal ions that are also released by humans into the environment. The result of gene rearrangements, lateral transfer, and new combinations of old and new resistance genes, return to the human environment (dashed arrows) by the same water bodies, along with vegetables grown in fertilized soils, carried by flying animals, and perhaps even by the hunting and handling of wildlife.

3.1. Resistance already in the environment

As Julian Davies reported nearly fifty years ago [6], some ARG can be traced down to the organisms that produce antibiotics, most of them soil bacteria. Considering that nearly all antibiotics currently in clinical use derive from naturally occurring molecules (i.e., aminoglycosides, beta-lactams, chloramphenicol, fosfomycin, glycopeptides, lipopeptides, macrolides, rifamycins, tetracyclines, in alphabetical order), mechanisms that enable producing organisms to survive their own metabolites have existed for millennia. Some such mechanisms have made their way to clinically relevant bacterial species (e.g., [7,8]), through horizontal gene transfer, that then thrive under the selective pressure of ubiquitous antibiotic presence. However, many other ARG found in pathogenic bacteria do not seem related to those found in the respective antibiotic-producing organism. This apparent disconnection could be caused by "sampling bias", i.e., ARG do come from antibiotic-producing bacteria, just different from those used industrially to manufacture antibiotics. In any case, an analysis of nearly 500 strains of soil *Streptomyces*, reports the presence of many "resistance" traits [9]; although many of them are the result of a poor methodology (e.g., arbitrary breakpoints for assessing resistance, inclusion of phenotypes most likely to be intrinsic resistance), it is clear that there is a large gene pool of resistance determinants in the environment.

The sole notion that antibiotics have been in the environment for millions of years should led to the inevitable conclusion that ARG are also ancient, in antibiotic-producing organisms, and/or in

organisms naturally exposed to such antibiotics. Nevertheless, the fact that antibiotic resistance is ancient has been recently "re-discovered" by papers in top journals (e.g., [10]). Despite the known shortcomings of metagenomic approaches, discussed briefly above, these studies provide evidence supporting the existence of ARG much before of the release of industrial antibiotics into the environment. Another interesting finding is that many such "resistance" traits have never been reported in clinically relevant bacteria: perhaps this is a mere consequence of molecular analyses being performed only in a minority of clinical isolates; perhaps it is only a matter of time for such traits to travel into pathogenic bacteria; or perhaps HGT is not so powerful after all, and known examples of ARG originating in the environment are more the exception than a rule. Mobility elements in environmental bacteria are fewer, and far away from putative ARG, than in human pathogens [11]; this could point to a very limited mobilization of ARG from the environment, but also to a potential increase should the prevalence of mobility elements also increases in the environment.

In addition to older cases of ARG found in pathogenic bacteria that can be traced down to ancient genes, discussed above, there are a few examples of recently discovered determinants in clinical isolates that clearly have environmental origin. These cases could illustrate the dynamic nature of the ongoing evolution of resistance; but can also be simply the consequence of the evolution of the molecular methodologies we use to study it. In other words, they could be genes recently introduced into the resistome of human pathogens; or just recently discovered genes that have been there for a while. Perhaps the better example is the extended-spectrum beta-lactamase (ESBL) CTX-M. The genes encoding this enzyme originated from *Kluyvera* spp., a genus of soil bacteria; this kind of ESBL emerged globally in clinical isolates during the 1990's, to become the prevalent enzyme in enteric bacteria in many parts of the globe [7]. While the $bla_{\text{CTX-M}}$ gene is chromosomal in *Kluyvera* spp., it is almost always plasmid-borne in enterics, and such plasmids often also carry other *Kluyvera*'s neighboring chromosomal genes [12]. This is an interesting example of an ancient resistance gene, of (yet) unknown function in the environment, from the chromosome of an organism that does not produce any antibiotic, jumping into a protagonic resistance role in human pathogens. Another example is the *qnr* group of plasmid-borne genes, mediating a low-level resistance to fluoroquinolones: these came from the chromosome of *Shewanella algae*, a water bacterium, and were first detected in the clinical environment by the late 1990's. This ARG has the additional puzzling feature that it provides protection against a group of synthetic antibiotics, which precludes the notion that it plays a role in the protection against, or in the synthesis of a naturally occurring molecule. Although some other natural compounds are, as quinolones, topoisomerase inhibitors, hence such ancient gene could have had a protective role against them [13], this is far from clear. Getting back to *Shewanella*, it has been recently proposed that one of the plasmid-carried genes mediating polymyxin resistance, *mcr-4*, also originated from the chromosome of this water bacterium [14]. In the end, and considering the short time since metagenomic studies arrived into resistance research, it is perhaps only a matter of time to find many other examples of ARG of environmental origin causing the clinical failure of antibiotics. After all, only about $1x10^{-21}$% of the total DNA on Earth has been sequenced [15].

3.2. Resistance being released into the environment

ARB/ARG selected for by the intensive use of antibiotics by human activities, mainly clinical and agricultural use, are released into the environment in a number of ways. The two most abundant sources of ARB/ARG related to human activities are wastewater and manure; however, resistance gets into the environment in many other ways, ranging from lixiviates and runoff from garbage and other wastes, to airborne carriage by flying animals, such as birds and even flies. Considering that clinical antibiotic usage, in countries where there is some measurement of it, typically ranges around 10–20 daily defined doses per thousand inhabitants per day (DDD/TID) [16], something between 75 and 150 million people each day is receiving an antibiotic. Considering that the average weight of human feces is about 120 g/day, and that half of that weight is bacteria, medicated people would be releasing 4.5–9 million kg of antibiotic-exposed bacteria into the environment daily (something in the order of 10^{21}–10^{22} bacterial cells). Only a fraction of those organisms would actually be ARB, and many are killed shortly after abandoning their host, or during wastewater treatment; however, plenty of ARG are still released daily into the environment. Furthermore, most humans still live either, in rural settings or in cities without proper sewage and/or wastewater treatment plants. On the other hand, manure from antibiotic-fed animals are directly applied to soils; just the US, taken as an example, fertilize with such manure a surface equal to the whole of Portugal [17]. Environmental contamination with ARB/ARG is both, intensive and extensive.

The nature of these ARB/ARG released into the environment includes essentially the whole spectrum of clinically relevant organisms and genes, perhaps with the exception of those to fragile and/or released in minute quantities (e.g., multi-resistant *Neisseria gonorrhoeae*). The most critical resistances, such as metallo-beta-lactamase-mediated (e.g., the New Delhi Metallo-beta-lactamase, NDM) resistance to carbapenems [18], or the plasmid-mediated resistance to polymyxins [19], have been found in the environment. Other, more abundant ARB/ARG, such as ESBL-producing enteric bacteria, are much more commonly reported in different kinds of environmental samples. In the urban settings, both the hospital and domestic sewage contribute to the ARB/ARG: selection and release in hospitals is much more intense, as is the use of antibiotics, making for identifiable "hotspots"; but the amount of antibiotics used by outpatients is much larger, leading to an overall larger contribution. For instance, the prevalence of resistance in *Escherichia coli* isolated from the hospital and municipal sewage in Gothenburg, Sweden, was always higher in the former, but the differences were rather modest: resistance rates hospital/municipal were, for example, 19/10% for amoxicillin-clavulanate, 5/1% for ceftazidime, 12/5% for ciprofloxacin, 5/0.4% for tobramycin and 20/11% for trimethoprim-sulfamethoxazole. While 5.5% of the 721 hospital isolates produced ESBLs, only 1.8% of the 531 municipal isolates did [20]. In addition to the obvious elimination of resistant organisms in the feces and other bodily fluids of antibiotic-treated patients, clinical settings have an overall different microbiota than other built environments: a diminished microbial diversity, a shift towards gram-negatives, and an increased diversity in ARG [21]. In the end, the metagenomic analysis of ARG in sewage correlates with factors affecting the local prevalence of resistance in clinical isolates, and could be used as indicator of such prevalences worldwide [22].

Developing countries also tend to have more crowded cities, with deficient urban infrastructure (i.e., sewage, waste collection and manage, wastewater treatment) that make for further sources of

environmental pollution by ARB/ARG. A couple of examples from Mexico, a middle-income developing country, with diminishing use of antibiotics but high resistance rates [23], could illustrate the point: wastewater running in open canals, and open-air dumpsters of bio-hazardous hospital waste (Figure 2), both represent a clear source of environmental pollution with ARB/ARG. Cities in poor countries tend to concentrate risk factors for exposure to pathogenic bacteria and their byproducts (e.g., pro-inflammatory lipopolysaccharides), as well as antibiotic resistance [24]. They also commonly have air pollution problems, due to poor regulation and/or enforcement of industrial contaminants, higher prevalence of old motor vehicles lacking maintenance, etc. Bacteria exposed chronically to these forms of atmospheric pollution have a number of physiological changes, some encoded by adaptive mutations [25], that could provide protection against antibiotics. Finally, it is important to realize that globalization applies also to microbes: ARB/ARG can cross frontiers, within people or animals, and also in ships [26] and airplanes [27]. Therefore, the consequences of poor regulation and managing of environmental pollution in developing countries will affect the whole globe.

Figure 2. Examples of unchecked release of ARB/ARG into the environment of developing countries**.** Top: an open sewage running through an urban setting, Cartagena canal, in Ecatepec, State of Mexico; all sorts of solid garbage, animal carcasses and occasionally dead people are also dumped into this canal. Bottom: the backyard of a company that collect bio-hazardous waste from hospitals and other healthcare facilities, in Toluca, State of Mexico; this garbage is supposed to be incinerated, but the overload

caused by the COVID-19 crisis made them to store the waste in an open yard for many days.

3.3. Wastewater treatment plants: a peculiar pre-release niche

While most of the ARB/ARG selected by human activities that are released into the environment make their way without further changes, those few that get trapped by wastewater treatment plants (WWTP) are particularly worrisome. WWTP are a "luxury item" mostly found in developed countries: 90% of WW is treated before release in the US and Canada, 66% in Europe, but only 14% in Latin America and less than 1% in Africa. WWTP were designed to reduce both, chemical pollutants and organic load, including bacteria; however, those processes were not instrumented having antibiotics and antibiotic resistance in mind. And while treatments do reduce significantly the bacterial load of WW prior to its release into the environment, those microorganisms that survive are a major cause of concern. Within WWTP bacteria from different sources (e.g., hospital- and community-acquired pathogens, along with commensal and saprophytic bacteria) are put together, concentrated in semisolid networks that enhance the cell-to-cell contact enabling horizontal gene transfer, all while being subjected to diverse chemical stressors (e.g., antibiotics and other drugs, disinfectants and other toxic xenobiotics, heavy-metals) that select for, and/or induce the transfer, rearrangement or expression of ARG. Viable bacteria that escape the treatment, and perhaps even free DNA containing ARG, could potentially be much more dangerous than the ones that enter the process. A couple of examples illustrate the results of WWT: in terms of antibiotic resistance prevalence, resistance to individual antibiotics went, from 9, 25 and 63% towards amoxicillin-clavulanate, chloramphenicol and rifampin, respectively, in *Acinetobacter* spp. isolates from raw sewage; to 38, 69 and 84% post-treatment; multi-resistance went from 33 to 72% [28]. Measuring ARG copies per milliliter, *sul* genes encoding resistance to sulfonamide antibiotics were reduced from 10^6–10^7 copies/mL in hospital effluent, to 10^5–10^6 copies /mL in WWTP effluent; but the ratio *sul1*/16S-rRNA increased from 3 to 8%, and resistant isolates to 8 or more antibiotics went from 30 to 60% [29]. The extensive nature of gene exchange and rearrangement of ARG within WWTP, especially those linked to IncP-1 plasmids, was analyzed in a landmark paper [30]. Among the things that can be assembled in these plants is an IncF, 120-kb conjugative plasmid found in one such site: resistance to ampicillin, chloramphenicol, erythromycin, kanamycin, neomycin, streptomycin, sulfonamides, trimethoprim, tetracycline and mercury, scattered between remnants of transposons Tn*21*, Tn*10*, Tn*1* and Tn*402*, and insertion sequences IS*26* and IS*6100*, and a class 1 integron [31].

In addition to the mere confluence of ARB, ARG, and selective/inducing agents in the WW, the likelihood of the chemical or physical processes used to reduce the bacterial load in WWTPs ending up enriching the content of ARB has been explored recently. For instance, ESBL-carrying *E. coli* isolated from WWTP effluent seem to be more resistant to UV-exposure than their non-resistant counterparts [32]. Results of "standard" treatments tend to be contradictory, as they are all but "standard"; hence some authors report ultrafiltration being more efficient than ozone treatment for the removal of pathogenic bacteria and their ARG from wastewater [33], while others report that ozone treatment is just as effective [34]. The whole issue has been recently reviewed [35], including

the efficiencies of filtration, ozonization, UV-irradiation and chlorination. As the release of ARB/ARG in treated wastewater is increasingly concerning, efforts towards optimizing such treatments, aiming specifically at the removal of resistance, are now in course. However, it may take several years before an enhanced method is universally agreed upon, and many more to have it deployed in existing WWTPs. Meanwhile, it may be worth considering the risk/benefit balance of the treatment of wastewater.

4. Persistence of ARB/ARG within the environment

Once released into the environment, ARB/ARG from human-related origin can follow many paths: from being rapidly killed, which is likely for many enterics not capable of surviving in open environments; to being acquired and become transient or permanent members of the microbiota of wildlife. If they survive long enough to interact with the surrounding microbiota, either soil/water or animal, the potential for gene rearrangement and exchange seems limitless. Mobile genetic elements, often carried by ARB coming from human-related environments (e.g., class 1 integrons are much more common in *E. coli* isolates from humans and urban environments [36]; and the presence of such elements in isolates from human sources have been increasing steadily during the "antibiotic era" [37]), can transfer themselves into environmental ARB, fostering the intra-cellular mobilization of ARG between chromosomes and plasmids. New "compilations" of ARG, genes mediating resistance to other biocides, virulence genes and/or mobile elements, can arise from these interactions, all happening under the selective pressure of a number of environmental pollutants released along ARB/ARG (see below). This was perhaps the path followed by $bla_{\text{CTX-M}}$, *qnr* and *mcr-4* genes, described above: all chromosomal genes of environmental bacteria ending up in conjugative plasmids of human pathogens.

Genes can be mobilized intra- and inter-cellularly. Intra-cellular mobilization typically includes the movement of genes from chromosomes to plasmids and vice versa; this could be accomplished by transposons and integrons, that mediate several variations of "non-homologous" recombination. Plasmids are known to act as "collections" of transposons, often gathering several of these mobile elements. Integrons, on the other hand, "collect" gene cassettes, which frequently contain ARG. Inter-cellular mobilization, or horizontal gene transfer, is typically grouped into three categories: acquisition and expression of free DNA (transformation), cell-to-cell transfer of DNA (conjugation), and viral-mediated DNA transfer (transduction). Overall mechanisms have been extensively reviewed before (e.g., [38,39]). The only known mechanism of fully self-encoded mobilization is conjugation: conjugative elements, plasmids or integrated conjugative elements (ICEs) carry the genes that mediate the whole process. The release of transposons, integrons and conjugative plasmids, frequently carried by ARB from human-related sources, enable the gene rearrangements and mobilization discussed earlier. Along with these elements, transduction also plays a significant role; phages are perhaps the most abundant organisms on Earth, and a calculated $1x10^{23}$ phage infections occur per second [15], making this rather "accidental" mobilization actually inevitable. Transformation is supposedly limited by the short lifespan of DNA in open environments, the small number of (known) bacterial species capable of achieving transformation competence, and the need for a minimum sequence homology both, for DNA uptake, and for recombination leading to

incorporation. Nevertheless, transformation is recognized as a means for transfer of ARG between clinically relevant bacteria, such as pneumococci; it is certainly possible for it to also play a role in the spread of ARG in some environments, soils in particular. This has been proven possible around the *aadA* gene conferring streptomycin resistance used in the construction of transgenic tobacco plants [40,41]. Considering that a significant amount of extracellular DNA can be recovered from soils [42], the likelihood of transformation as means of lateral transfer of ARG is certainly there.

ARB/ARG have been found in open, "human free" environments, wherever they have been searched for: water bodies, soils and wildlife [4]. Of course, the closest they are to human influence, the higher the prevalence and diversity of resistance traits. Culture-based methods have been rapidly displaced by metagenomic technologies that appear to be much more powerful. This is a mistake, as culture-based assessments can provide much more information on the actual origin and risk of detected ARG [43]. For instance, the detection of an ARG in a metagenomic analysis of remote environments could fail to assess if such a gene is indigenous, or was introduced from human-related sources. The risk posed by such an ARG can therefore be missed or overstated. Culture-based and metagenomic approaches are complementary.

5. Antibiotics and other biocides in the environment

Although the aim of this review is not to analyze the environmental pollution by antibiotics and other biocides, it is necessary to, at least tangentially, include some remarks. Antibiotics dispensed to people and animals are excreted in active form in about 70% of the total administered amount (this, of course, is a coarse estimate, that varies widely between antibiotics and animal species receiving them). These antibiotics are also discharged into wastewater, manure and garbage, along with ARB; some of them are rapidly decomposed, such as beta-lactams, while others can survive for many years in waters and soils. Some modeling predict that, should reduce prescribing of antibiotics be the only way to reduce antibiotic pollution of waters, it would be necessary to reduce in 77% the prescription of macrolides, and in 85% the prescription of fluoroquinolones, to reach non-selective concentrations in wastewater [44]. Pharmaceutical companies, particularly those in developing countries, release massive amounts of antibiotics in untreated wastewater, sometimes reaching concentrations similar to those found in the blood of treated patients. Antibiotics are released directly into the aquatic environment by aquaculture; although some of these practices are done in confined tanks, other are done in cages or nets within natural water bodies. About 10,000 tons of antibiotics are used for aquaculture worldwide [45]. Non-antibiotic drugs, that are also released into the environment via wastewater, can also exert effects upon pathogenic and environmental bacteria [46]; while this has been extensively reviewed before, a couple of recently reported examples can illustrate the issue: (a) ticagrelor, an "antiplatelet" drug, has measurable antibacterial activity [47]; and (b) carbamazepine, an anticonvulsant, promotes horizontal gene transfer [48]. While drug pollution of water bodies is a growing cause of concern, almost nothing is known about the effects of such pollution upon bacterial communities.

In addition to drugs, antibiotics or not, a large number of other chemical agents can affect microbial physiology in ways that are relevant to the issue at hand. Disinfectants are a clear example: quaternary ammonium compounds do select for the presence of class 1 integrons, that often carry the

qac genes encoding low-level resistance to such agents; bacteria isolated from environments contaminated with these disinfectants carry integrons more often than those from disinfectant-free environments [49]. Triclosan, on the other hand, while not actually selecting for resistance, can induce antibiotic tolerance [50], which in turn can facilitate the acquisition of canonic resistance. Other biocides, such as herbicides, can also affect bacterial susceptibility towards antibiotics: paraquat act as a redox-cycling compound, inducing oxidative stress responses that also protect against antibiotics [51]; glyphosate and dicamba modify the response to antibiotics in *E. coli* and *Salmonella*, increasing susceptibility to some, decreasing it to others [52]. Metal ions have bactericidal properties, and there are many genes that confer resistance towards those agents, some linked to ARG. Mercury-resistance genes *mer* are well-known examples, but also cadmium/zinc resistance genes have been found linked to macrolide and aminoglycoside resistance genes. Bacteria carrying both ARG and metal-resistance genes are common among clinical isolates, less in isolates from humans and domestic animals, and very rare among environmental isolates; plasmids carrying both kinds of determinants are also more likely to be conjugative [53]. Interestingly, while there are reports of metal ions co-selecting and inducing horizontal gene transfer [54], zinc and copper specifically inhibit the expression of conjugative genes, hence diminishing such lateral transfer [55]. Again, while this review does not focus on antibiotic pollution, it is a crucial piece of the puzzle to understand the consequences of the release of ARB/ARG into the environment.

6. Risks of environmental pollution by ARB/ARG

While writing a news piece about an article on the detection of ARB in the feces of wildlife of a Mexican forest [56], one of the external scientists contacted for his opinion stated that "antibiotic resistance is everywhere and most people that make a big deal out of finding it don't understand the bacteriology". This is, of course, partially true: as has been reviewed above, resistance, in general, is as ancient as antibiotics themselves, so it is somehow to be expected to find ARB in the microbiota of non-domesticated animals. However, to find ESBL-producing enteric bacteria among free-living jaguars, for instance, is peculiar for two main reasons: (a) some ESBL-encoding genes, although ancient, did not originate in Enterobacteriaceae (see above), therefore their presence in this bacterial group is at least indicative of horizontal transfer of resistance genes within the environment and in the apparent absence of selective pressure; and (b) if the gene or the organism was acquired from human-related sources, it would be a remarkable feat for a not-so-common trait among community-acquired pathogens to find its way to an animal living some kilometers away from the nearest human settlement and, again, in the absence of known selective pressures. Nevertheless, this or any other finding related to ARG/ARB in the environment could seem like an interesting subject for the microbiological, molecular biological, or environmental sciences, but with scarce clinical impact, if any. After all, if such a resistant organism is to cause an infection in a wild animal, it is very unlikely that the fate of the infection or the infected would be any worse because of the resistance trait. The risk for a human to get infected by a resistant pathogen coming from the environment is a real possibility, but apparently limited to some groups at risk, from people living in the vicinity of wastewater canals, via aerosols and flies, or through contaminated wounds; to fishermen and agricultural workers exposed to polluted environments; to perhaps even people that came in

proximity to wildlife carrying such resistant bacteria. Among the recognized pathogens that spread from the environment or wildlife, most are viruses and only a few are bacteria (e.g., *Borrelia burgdorferi*, *Yersinia pestis*, *Mycobacterium bovis*). Deforestation, habitat fragmentation, and climate change are among the factors that tend to increase the risk of such transmission to occur [57]. However, those bacterial pathogens seldom carry resistance genes. In any case, the risk would be much less than that of acquiring an infection by a multi-resistant microorganism within a hospital or other healthcare facility, where resistance is much more common. But that is not the whole picture.

The immediate effects of ARB/ARG release into human-related environments would not be discussed here. Obviously, vegetables grown in manured soils, and fish grown in antibiotic-laden waters carry ARB that can directly cause infections, or at least contribute ARG to the microbiota of exposed people. Globalization makes it possible for such foodstuff to travel across the world, affecting people even in countries where the use of antibiotics is better regulated. Birds, flies, cockroaches and rats can carry ARB from farms or landfills, contaminating environments not supposed to have such organisms. People living close by, or even within heavily polluted environments, such as in the vicinity of irrigation canals carrying raw WW, are more likely to be affected by the increased presence of ARG. However, in the end, this would all be part of a human-related environment: the damage would be contained within.

The changes induced both, by the release of massive amounts of human commensal and pathogenic bacteria, often loaded with ARG, and of selective chemical agents, into the environment, ought to change the microbiota at the receiving end of the process. In soils, where naturally occurring antibiotics play signaling roles rather than "chemical warfare" [58], the introduction of organisms capable of destroying such signaling molecules can potentially disrupt delicate ecosystems. The simultaneous presence of ARG in conjugative plasmids, and of minute concentrations of antibiotics known to induce conjugative transfer [59], could enhance the chances of ARG being acquired by indigenous microbiota. Changes in soil microbiota could affect geochemical processes as important as nitrogen fixation [60]. Environments receiving even minute concentrations of antibiotics (below those considered as "predicted no effect concentrations", PNEC, a figure established based on effect of metazoan species) could have their antibiotic-susceptible microbiota reduced and substituted by ARB from human-related origins; this could in turn affect the ecosystem "services" these microbial communities provide (e.g., nutrient cycling, metabolism and degradation of organic and inorganic compounds). There is also, of course, the possibility of such indigenous microbiota to acquire those ARG introduced into the environment, creating unknown new risks [61]. Even saprophytic bacteria, such as Pseudomonads, that have been considered as limited participants in the "gene internet" deployed by plasmid-mediated horizontal gene transfer, do carry a lineage of mobilizable plasmids bearing, among other things, IMP, BIM and VIM metallo-beta-lactamases [62]. The transfer of the gene encoding another of these enzymes, NDM-1, from enteric bacteria into *P. aeruginosa* and *Acinetobacter baumannii* occurs readily in biofilms [63], a common way of life of bacteria in the environment.

The effects of changes in the microbiota of wildlife could be even more dangerous. Commensal or pathogenic bacteria in humans are more likely to survive within another mammal than in the open environment, and also more likely to find phylogenetically related organisms in the indigenous microbiota of such animals, with which gene exchange may be more successful [11]. Considering the

extensive physiological, metabolic and even behavioral impact of changes in the human microbiota, there is every reason to suspect the same in wild animals. A recent report of microbiota changes affecting learning processes in mice [64] illustrates this. Animals exposed to ARB from human-related environments can suffer such changes. And while the impact of the acquisition of antibiotic resistance may have little direct effect upon the affected animal's health –after all, wild animals are not likely to receive antibiotic treatments, the linkage of some of these resistance determinants with virulence genes could potentially cause new or more severe infections in wildlife.

In the end, for many people the most concerning possibility is that, as did happen with CTX-M beta-lactamase and other resistance traits that originated in the environment, other unknown or perhaps even yet inexistent threats do reach us in the future, in the form of untreatable infections. This can happen in a wide variety of ways: (a) dangerous ARG have been detected even in tap water (e.g., [65]), indicating that ARB/ARG dumped in water bodies, and perhaps even ancient, naturally occurring ones, can reach us through water faucets; (b) handling and eating wildlife can be a risk of exposure to resistant pathogens; this might sound as limited scenarios, of "sport" hunters (who likely deserve the risk) or remote communities, but even free-living marine fish carry ARG, mostly localized in mobile elements [66]; (c) flying animals can carry ARB and cross the faint line between human-related and "human-free" environments; while the main risks these animals are associated with is the carriage of ARB/ARG from, for instance, countries that keep using antibiotics agriculturally to those that have banned these practices [67], they could also bring ARB/ARG "concocted" in the open environment to our doors.

7. Concluding remarks

Once the COVID-19 crisis is over, we would come out from the rubble to find antibiotic resistance still waiting for us, perhaps even at an increased pace. While the evidence of the accelerating emergence of new resistance is non-conclusive [68], there are many reasons to think it may be so. The presence of resistance in the environment may very well be an important factor contributing to this phenomenon. With pharmaceutical companies still demanding "incentives" to return to research and development of new antibiotics [69], it is crucial to assess the presence of resistance in the environment, and to stop releasing more into it.

Conflict of interest

The author declares no conflicts of interest in this paper.

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