Chapter 1

Antibiotic Resistance in Aquatic Environments of Rio de Janeiro, Brazil

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1. Introduction

Aquatic environments possess ecological and economical relevance. Unfortunately, these sites are subjected to high levels of anthropogenic impact worldwide [1]. Urban, industrial and farming activities are responsible for the discharge of pollutants. Fertilizers, sludge, organic compounds, heavy metals and all sources of wastewater are released into water bodies often without appropriate treatment. A few possible outcomes from these contaminations are: eutrophication, hypoxia, toxicity, bioaccumulation, and dissemination of pathogens [2]. These pollutants can spread across long distances. Thus, sparsely populated areas where human activities are less intense are also affected by those disturbances [1].

Environmental contamination affects microbial communities in a myriad of ways. Source and amount of pollutants together with ecosystem dynamics modulate the responses of microorganisms to anthropogenic impacts [3]. Microbe communities react with drastic changes in ecosystem functioning, species composition and abundance [2,4]. Several consequences may arise from aquatic pollution. The connection between these impacts and potentially pathogenic bacteria is of particular relevance for human welfare [2].

High amounts of organic matter deposited in water bodies leads to nutrient enrichment. This promotes growth of heterotrophic bacteria, which include various opportunistic pathogens such as *Shigella sp.*, *Salmonella enterica* and *Vibrio cholerae*. These are the etiological agents of waterborne diseases, like cholera and diarrhea, which affect millions of people worldwide. Those illnesses are most frequent in developing countries, where access to treated water and sanitation is limited [5]. Also, pathogenic bacteria originated from human feces are released directly into the environment through wastewater discharges, compromising water quality [2].

Since the discovery of inhibitory properties of antibiotics they have been widely used for treating bacterial infections. Approximately 250 of these molecules are currently in use [6]. These drugs are natural or synthetic substances capable of killing bacteria or retarding their growth. However, the effectiveness of treatments based on antibiotic therapy has been reduced over time, as a result of the spread of antibiotic resistant bacteria [7].

The selective pressure imposed by the use of antibiotics (ABs) results in death of susceptible bacteria while favors resistant strains. Bacteria may be inherently tolerant to these drugs or incorporate resistance genes by assimilating exogenous DNA. Extensive genetic exchange occurs in the environment, where opportunistic pathogens (commonly found in free-living communities) may become resistant upon acquiring resistance mechanisms.

The increasing spread of antibiotic resistance (AR) among environmental bacteria has led some authors to consider antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARG) as emerging pollutants [6,8,9]. These entities have a special property when compared to other contaminants: their ability to amplify and spread, persisting in the environment [9]. Since human populations are dependent on aquatic environments, the propagation of ARB and ARG within these sites represents a serious threat to public health [10].

However, antibiotic resistance is not just a medical issue but also an ecological matter. To understand the process by which resistance propagates, it is necessary to consider not only hospital settings, but also the ecology and evolution of resistant microorganisms [11]. In this chapter, we discuss how ARG and ARB arise and disseminate in aquatic environments, with special attention to how pollution affects this phenomenon. We describe the origins of antibiotic resistance genes and their interactions with environmental and pathogenic bacteria. In addition, we present 3 case studies regarding resistance in pristine and impacted aquatic environments from Rio de Janeiro. Finally, we describe prospects for reversing, or at least to mitigate the dissemination of antibiotic resistance among environmental bacteria. Through the text, the terms 'microbial communities', 'microbes' and 'microorganisms' are used to refer exclusively to members of the Bacteria domain.

2. Antibiotics and resistant bacteria

In the mid-1900s several new drugs originated from environmental microbes were discovered. During this time, most antibiotics currently in use were first characterized. This was started by the discovery of substances originated from free-living organisms that were able to inhibit

microbial growth, such as penicillin [12]. Antibiotics belong to several classes of molecules that act impairing key microbiological processes like metabolic pathways and protein synthesis [13,14] (Table 1). As a result, high enough levels of these drugs lead to death or inhibition of growth.

Antibiotics are mainly used for treating human infections. Although, the use of these drugs is not restricted to medical purposes. These substances are extensively applied in animal husbandry for veterinary objectives and also as growth promoters. ABs are also used in agriculture and aquaculture [23] for both prophylaxis and to fight bacterial contaminations [6]. World consumption of antibiotics has been estimated at up to 200.000 tons per year [24].

Although widely in use, the efficiency of antibiotics has decreased over time [5]. This is a result of the spreading of ARB; organisms capable of continuously growing in toxic concentrations of these drugs. Millions of individuals have their health compromised by infections caused by resistant microorganisms, making resistance a global concern [13]. The problem is so great that resistance now covers all known classes of antibiotics [25]. For some pathogens there is virtually no drug available for their treatment [14,26]. In addition, some bacterial diseases previously deemed treatable (e.g. gonorrhea and typhoid fever) or considered under control (e.g. tuberculosis) are once again a menace due to the emergence of resistant strains [5,9].

Class	Antimicrobial agent	Mechanism of action	Resistance mechanism
Aminoglycosides	Gentamicin, Kanamycin, Streptomycin	Inhibition of protein synthesis [15-17]	Efflux, enzymatic inactivation, mutated target [15,17]
Amphenicols	Chloramphenicol	Inhibition of protein synthesis [14]	Efflux [14,21]
Macrolides	Clarithromycin, Erythromycin	Inhibition of protein synthesis [14,18]	Efflux, mutated target [18]
Tetracyclines	Tetracycline, Doxycycline	Inhibition of protein synthesis [15,18]	Efflux [15,18]
Beta-Lactams*	Penicillin, Aztreonam, Cefotaxime ,	Inhibition of cell-wall synthesis [15,18]	Enzymatic inactivation, mutated target [15,18]
Glycopeptides	Vancomycin, Bleomycin	Inhibition of cell-wall synthesis [15,18]	Cell wall modification, efflux [15,18]
Quinolones	Nalidixic Acid, Ciprofloxacin	Inhibition of nucleic acids synthesis [15,18]	Efflux, mutated target [15,18]
Sulfonamides	Sulfamethoxazole	Inhibition of folate synthesis pathway [14,16]	Alternative enzymes, mutated target [14,16]
Lipopeptides	Daptomycin	Cell membrane depolarization [14,19]	Cell membrane modification, mutations [17,19]
Amino-acid derivates	Polymyxin B	Cell membrane permeabilization [14,20]	Cell membrane modification [20,22]

*Divided into four subclasses: Penicillins, Cephalosporins, Carbapenems and Monobactans

Table 1. Antibiotic classes: action and resistance mechanisms

Microorganisms resistant to at least three classes of ABs are considered multidrug-resistant. It is estimated that 400,000 cases of infections caused by multi-resistant bacteria occurred in Europe only during 2007, which can be connected to 25,000 deaths [27]. World Health Organization estimates that multidrug-resistant tuberculosis causes at least 150,000 deaths every year [28].

Multidrug-resistant pathogens are associated with increased morbidity and mortality, since they are much less susceptible to antibiotic therapy, our main weapon against bacterial infections [28]. Diseases caused by these microbes are more expensive to treat, because they usually require longer treatments, more clinical trials and a larger number of drugs. This is of special relevance in developing countries where little budget is directed for the acquisition of more efficient and expensive pharmaceuticals [5,13,26]

The concept of antibiotic susceptibility is dose dependent. At sufficiently high concentrations all organisms are likely to be susceptible. However, these doses are often so high that they are toxic to humans. While some strains tolerate concentrations barely above the clinical levels, others are able to survive at concentrations up to fifty times higher [29].

Several resistance mechanisms are well documented (Table 1). These adaptations may confer tolerance to a single drug or several of the same class. Common resistance strategies include: i) enzymatic inactivation, use of proteins capable of hampering antibiotic activity. ii) Mutations in target sites, expression of mutated proteins with reduced affinity for antibiotics. iii) Efflux pumps, inter-membrane proteins capable of removing antibiotics from cytoplasm. Intrinsic resistance occurs in the absence of target sites or due to inability of antibiotics to cross cell walls or membranes [8,12,30].

3. The resistome

Antibiotic resistance is not restricted to pathogenic bacteria. Resistance is widespread among environmental microbes. Natural ecosystems are hotspots of resistance mechanisms, due to the large genetic diversity found among free-living microorganisms. The set of genes involved in microbial AR has been named the Resistome (Figure 1). This concept includes not only genes that encode real resistance determinants but also precursor genes that can evolve into such traits [12]. Soil and aquatic bacteria often present resistance determinants, even in pristine sites, like deep terrestrial subsurface and Antarctic waters [31,32]. Also, soil communities harbor bacteria capable of metabolizing antibiotics and subsisting on them as a sole carbon source [29].

Several microbes are known antibiotic producers. These organisms are sources of resistance genes. They control the toxic properties of these substances using self-preservation mechanisms. It is unlikely that these producers can generate inhibitory concentrations of antibiotics among free-living microbial communities [33]. Therefore, their use of ABs is probably distinct from its commonly known bactericidal purposes. In the environment, antibiotics play a different role. They function as signaling molecules used for inter-microbial communication, mediating community homeostasis [11,30,32]. At sub-inhibitory concentrations ABs are

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capable of inducing significant changes in gene expression patterns [33]. These substances can affect biofilm formation and cytotoxicity; therefore, at non-toxic levels, ABs act as signals with regulatory effects, in opposite to the bactericidal properties for which they are applied in medicine [34]

Antibiotics and resistance mechanisms were already present in Earth's microbiome long before humans started making use of these substances [31]. Environmental bacteria harboring resistance mechanisms against β-lactams, Vancomycin and Tetracycline may be more than 30.000 years old [35]. Evidence suggests that some natural ABs appeared on the planet over 100 million years ago. Beta-lactamases, enzymes that inactivate β-lactams are reportedly 2 billion years old [12].

ARG probably evolved from genetic units with physiological functions not related to resistance, such as detoxification, secretion and signaling pathways [36,37]. These genes encoded precursor proteins that, given the proper selective pressure can evolve into real AR features (Figure 1). Therefore, all bacterial genomes are likely to harbor resistome genes [12].

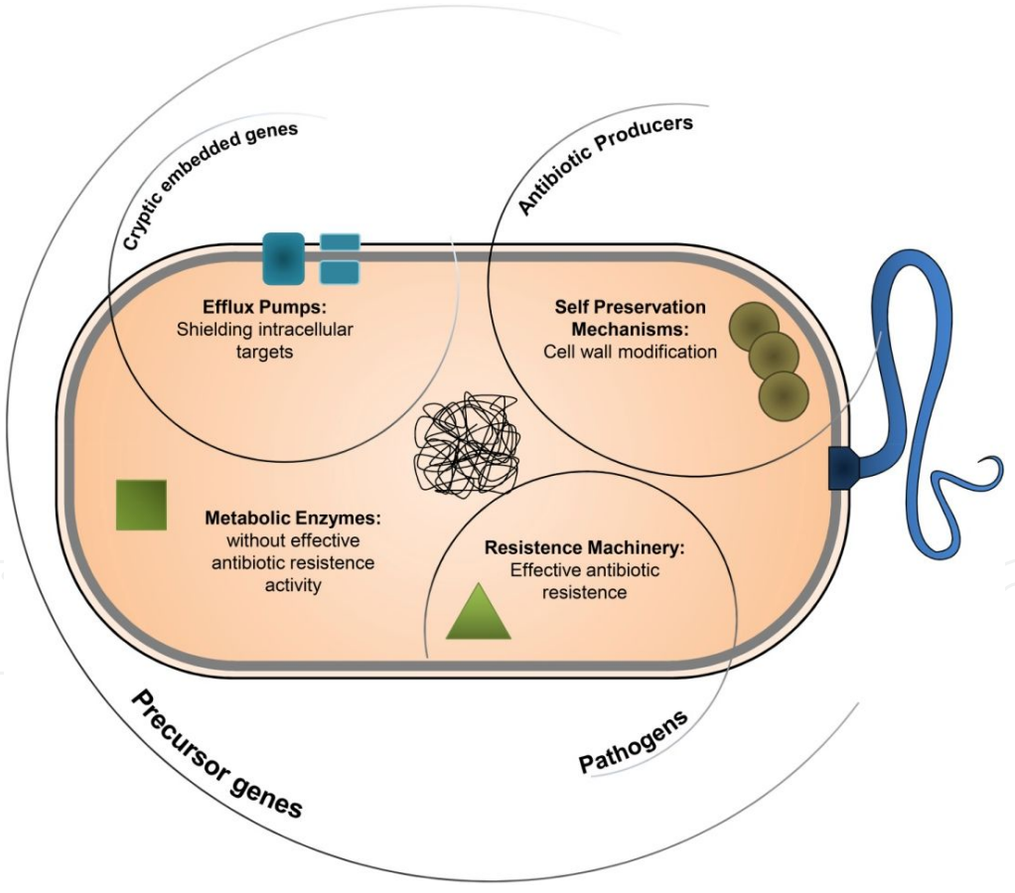

Figure 1. Functional mechanisms of the microbial resistome.

Resistance genes are plentiful and highly disseminated among environmental bacteria, which appear to be predominantly multi-resistant, often tolerating extremely high doses of ABs [25,

29,31]. The complete genome of *E. coli* encodes nearly 600 proteins responsible for the outflow of small molecules, many of which can be associated with elimination of toxic compounds, which makes them possible mechanisms of innate resistance [12].

Studies have shed light on the evolution of ARB, suggesting that resistance traits can be associated with reduced fitness, which is a measure of how adapted an organism is to a particular environment. It is measured by comparing bacterial growth rates against control strains. Higher growth rates are interpreted as higher fitness and vice versa. Resistant bacteria have higher fitness than susceptible strains in the presence of antibiotics. By contrast, in the absence of such substances resistant bacteria tend to grow slower and are overcome by their susceptible counterparts. Therefore, when ABs are absent, resistance genes would be disadvantageous and tend to disappear from microbial populations.

Although, the lower relative fitness associated with AR is not absolute. Compensatory mutations are capable of reversing costs that arise from harboring resistance genes. Absence of fitness costs favors the persistence of ARB and ARG on microbial populations for extended periods after removal of antibiotics. In addition, AR traits may carry very little to no fitness costs, and in certain situations even increase it [7,12,32].

4. Evolution and dissemination of antibiotic resistance: The role of pollution

Bacteria have very short life cycles compared to eukaryotes. This trait allows for the rapid emergence of new adaptations. In short periods of time, originally susceptible bacteria may become resistant through mutations or by acquiring resistance genes. When antibiotics are present, resistance traits tend to rapidly spread among microbial populations [38]. These characteristics of bacterial evolution contribute to a rapid development of multi-resistant pathogenic strains [10]. Consequently, once a new antibiotic is put into use, it does not take long until it is challenged by novel resistant microorganisms [25].

Dissemination of resistance is intensified as human populations grow and increase the use of antibiotics. The more these drugs are consumed, more intense is the selective pressure they impose on bacterial communities. As a result, resistance mutations are evolutionary successful and therefore tend to progressively increase their abundance among bacterial populations. This phenomenon takes place wherever bacterial communities are subjected to antibiotics as a relevant selective pressure, such as in hospitals.

When a multicellular organism is submitted to antibiotic therapy, drastic shifts occur in its symbiotic community. During this process, the richness and diversity of human-associated microbiota decreases [39]. Eventually, low abundance taxa may be eliminated [40]. Although, resilience of the overall community allows diversity to be restored over time [39]. Another consequence is the positive selection of ARB, which show higher abundance following antibiotic treatment [40]. ARG and ARB present in the human microbiota reach aquatic environments through wastewater discharges. Moreover, environmental bacteria are con-

stantly inoculated into the human organism, through direct contact with these sites or indirectly through food and water.

Water bodies are sites of genetic exchange where environmental bacteria interact with microbes originated from humans and other animals, exchanging genes through horizontal gene transfer (HGT). Opportunistic pathogens often have large and versatile genomes, prone to sharing genetic material. This trait helps these organisms to colonize a more diverse set of environments. As consequence, aquatic ecosystems may become a threat to human wellbeing when they are affected by pollution carrying resistant bacteria [36].

Genetic transfer can be performed between pathogenic and environmental bacteria and even between very phylogenetically distant organisms such as species of Gram-positive and Gram-negative bacteria [41,42]. When ARG are not encoded in the bacterial chromosome but in mobile genetic elements (such as plasmids, transposons or integrons) the ability of these entities to be transferred is enhanced. [37,43,44]. As a result, antibiotic resistance may persist on environmental communities for long periods after the first contact with AR determinants [32] (Figure 2). Furthermore, phages are likely to play an important role in the spread of resistance. These viruses are abundant in aquatic ecosystems, and have been shown to carry ARG, both in pristine and polluted aquatic habitats [45].

Although environmental and pathogenic bacteria harbor ARG, the regulation of these genetic units is distinct between them. Pathogens usually carry these genes on mobile genetic elements and express them constitutively. Meanwhile, AR in free-living bacteria is usually chromosomally encoded and expression is initiated following exposure to antibiotics [12]. Since pathogenic organisms are much more likely to be subjected to toxic levels of ABs the continuous expression of ARG makes them permanently prepared to respond against these drugs. On the other hand, environmental bacteria are not constantly in contact with antibiotics; therefore repressing their resistance genes is probably advantageous.

The role of pollution in the rise and spread of resistant bacteria is not fully understood. Nevertheless, a growing body of evidence suggests that pollution promotes propagation of ARB within aquatic environments (Figure 2). In these sites, wastewater discharges from domestic sources affect the diversity of resistant bacteria [46-48]. Those impacts also shape the genetic pool of water bodies by increasing abundance of antibiotic resistance genes within these habitats [49]. As previously stated, in clinical situations, resistant microbes are the most successful and hence increase their numbers. Thus, hospital effluents have been shown to be rich in resistance genes [50] and resistant bacteria [51].

Farms make use of high doses of antibiotics as growth promoters. Animals submitted to these substances represent another source of resistant bacteria [9]. Multicellular organisms exchange members of their associated microbiota with other organisms and with their surroundings. Due to that, animals can spread ARB among other animals, humans and throughout the environment [30].

Antibiotic resistance is commonly encoded on the same mobile genetic elements that carry heavy metal resistance genes. Those genes are positively selected in presence of heavy metals. Along with them, ARG are co-selected. This occurs even in the absence of a selective pressure

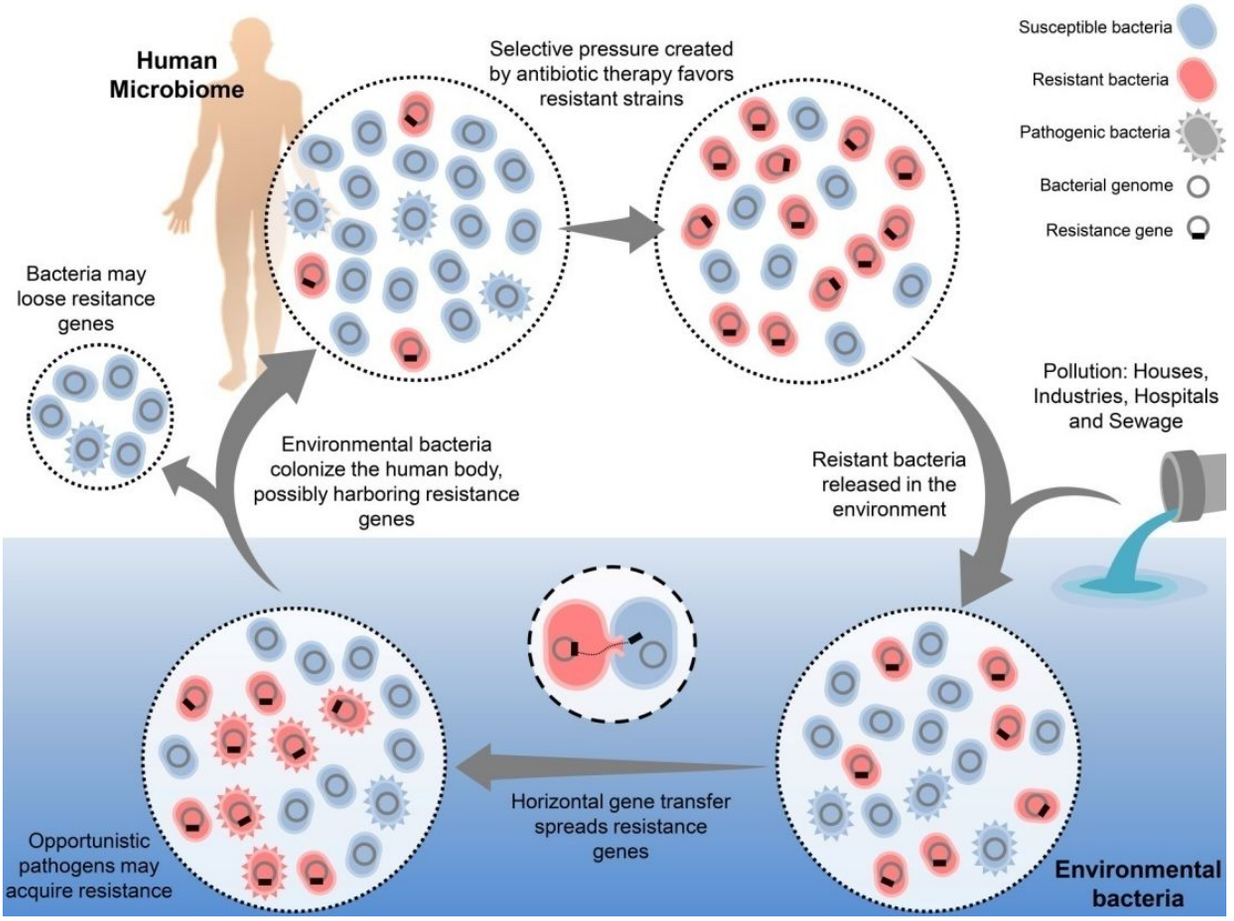

Figure 2. Schematic representation of the interactions between pollution, resistant bacteria and aquatic environments.

imposed by antibiotics [52,53]. Unlike organic matter, metals are not subjected to degradation. They persist in the environment for long periods, possibly promoting co-selection of antibiotic resistance genes [52-54].

Additionally, natural forces like wind and water disseminate microorganisms across long distances, along with AR determinants encoded in their genomes.

5. Case studies: Interactions between water pollution and antibiotic resistant bacteria in Rio de Janeiro, Brazil

5.1. Diversity of antibiotic resistant bacteria in aquatic environments of Rio de Janeiro

Rio de Janeiro city is situated around Guanabara Bay, a brackish environment submitted to intense pollution. Untreated sewage from domestic, clinical, industrial and agricultural origins is constantly released into the bay's waters [3]. Mixing of marine, freshwater and wastewater grants this habitat high microbial diversity [4]. Our group has investigated microbial ecology

in the bay. We have worked to elucidate the impacts that anthropogenic activities exert on bacterial communities in this site.

Currently, we are exploring the connections between wastewater discharges and the diversity of ARB within the bay and nearby aquatic environments. For that purpose, we analyzed the diversity of cultivable bacteria resistant to ampicillin in water bodies situated in the state of Rio de Janeiro.

We assessed the diversity of ampicillin resistant bacteria from three impacted sites located in Rio de Janeiro city (BT, GB and CC). These communities were compared against bacterial diversity of three aquatic environments from Ilha Grande (PR, PB and MS), a pristine island located in a preserved area of the Atlantic rainforest (Figure 3). Ampicillin is a widely prescribed beta-Lactam antibiotic of the penicillins sub-class. This substance is also used in laboratory practices, in which bacteria receive ampicillin resistance genes through molecular cloning.

Inoculants for mixed bacterial cultures were obtained from each of the six sampling sites. Culturing was performed on four antibiotic concentrations. Bacteria were grown in clinical resistance and super-resistance (50 times higher [29]) concentrations of ampicillin. Super-resistance cultures showing growth were inoculated on culture media supplemented with antibiotic concentrations 600 times higher than clinical levels. Bacteria were also cultivated on ampicillin-free media for control. All cultures were grown in Luria-Bertani liquid media for 24 hours at 37°C followed by DNA extraction for construction of 16S rRNA gene libraries.

Communities from pristine environments of Ilha Grande only showed growth in antibiotic-free cultures. Although, it is likely that ARB are present in these sites but could not be detected by our culturing methodology. All cultures from impacted environments of Rio de Janeiro city produced growth in all ampicillin concentrations. Vibrionales order was abundant in all gene libraries regardless of source of inoculants or antibiotic concentration. Diversity of resistant bacteria from impacted sites (BT, GB and CC) included members of orders Enterobacteriales, Firmicutes and Bacteroidales. These organisms are abundant in the intestinal tract of vertebrates, and their presence is an evidence of sewage contamination. Gene libraries analysis indicated that resistance is less disseminated among pristine and low impact environments (unpublished).

Bacteria from Guanabara Bay were isolated on solid agar plates containing ampicillin through functional selection. A total of nine isolates were obtained from these plates but only 5 could be identified by 16S rRNA gene sequencing. Three isolates were classified as multi-resistant due to their tolerance to ampicillin, tetracycline and kanamycin. One of these isolates was identified as *Acinetobacter calcoaceticus* and the remaining two as *Klebsiella pneumoniae*. We obtained a high molecular weight plasmid from *K. pneumoniae* that granted ampicillin resistance upon electroporation into competent DH10B *E. coli* (unpublished).

Unpolluted sites (MS, PB and PR) showed lower diversity and relative abundance of resistant bacteria. Impacted sites (BT, GB and CC) were rich in ARB, many of which identified as fecal bacteria or as opportunistic pathogens. Potentially pathogenic bacteria showing ampicillin resistance were detected in gene libraries of all polluted environments. These results suggest that the degree of dissemination of AR depends on the levels of anthropogenic impact to which

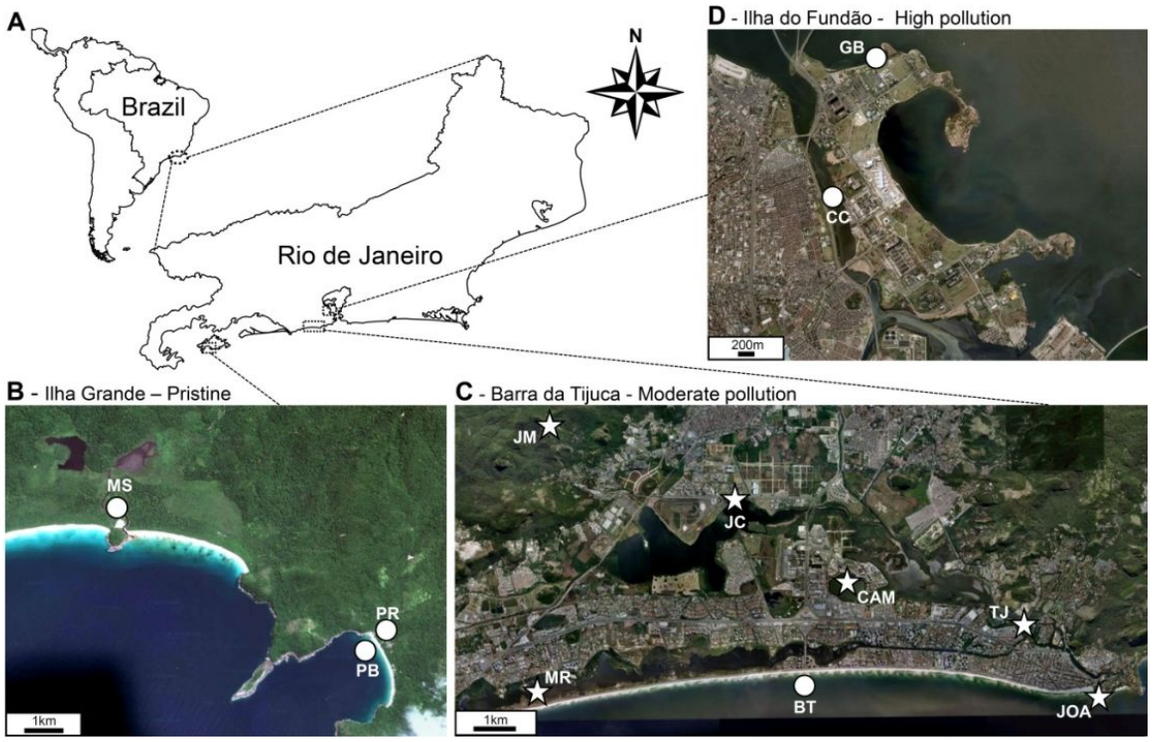

Figure 3. Map of the sampling sites located in the state of Rio de Janeiro. Case study 1 - Samples represented by circles: Mangrove system (MS), Paranaioca River (PR) and Parnaioca Beach (PB), Barra da Tijuca (BT), Guanabara Bay (GB) and Cunha Channel (CC). Case Study 2 - Samples represented by stars: Engenho Novo (JM), Jacarepaguá lagoon (JC), Camorim lagoon (CAM), Tijuca lagoon (TJ), Marapendi lagoon (MR) and Joá channel (JOA). Satellite images obtained from Google Maps.

each sampling site is submitted. Therefore, pollution and spread of antibiotic resistance within these environments are associated.

5.2. Impacts on water resources in the Jacarepaguá lagoon system revealed by polyphasic approach

Jacarepaguá lagoon system is located within the metropolitan area of Rio de Janeiro. Covering an area of 280 Km^2, the lagoon system consists of four major lakes: Jacarepaguá, Camorim, Tijuca and Marapendi. Several small rivers flow into those lakes, among which is the pristine stream of Engenho Novo (JM station). Joá Channel connects the Atlantic Ocean to the lagoon system, making the lakes brackish environments (Figure 3C). All lagoons are subjected to some degree of metropolitan pollution but Jacarepaguá (JC) and Camorim (CAM) are severely impacted. Mixing of sediments, seawater and continental freshwater make these ecosystem a dynamic habitat. Shifts in physical, chemical and microbiological properties of adjacent lakes and coastal marine environments occur over short periods of time, as a result of marine tides, raining water runoff and sewage flow. These alterations reflect on the local microbiome that responds to the most subtle changes.

For a more accurate picture of microbial diversity in environmental samples a polyphasic approach is recommended. Therefore, to investigate the lagoon system we performed a multi-

step analysis that included construction of 16S rRNA gene libraries, of both free-living (metagenomic) and cultured (enrichment culture) bacterial communities. Additionally, we isolated bacteria from the lagoon system through selective agar plating. These isolates were submitted to antibiotic susceptibility tests through disc diffusion method. This technique consists in placing discs embedded with antibiotics on pure bacterial cultures streaked on agar. Antibiotics diffuse through the culturing medium unevenly, i.e. closer to the discs antibiotic concentrations are higher. By measuring the halo of inhibition around the discs, bacteria are classified as resistant or susceptible.

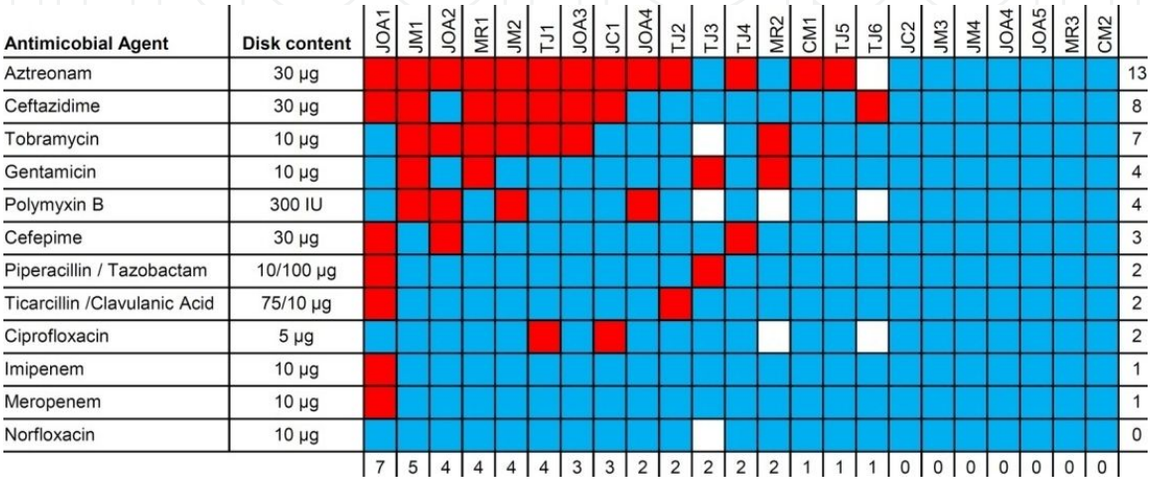

Figure 4. Antibiotic-susceptibility profiles of 23 isolates obtained from Jacarepaguá lagoon system determined by disc diffusion method. Red squares indicate resistance; blue squares indicate susceptibility and unavailable data is represented in white. Numbers in the right end of the table indicate how many isolates where resistant to the antibiotic of each line. Numbers in the bottom of the table indicate to how many antibiotics each isolate was considered resistant.

Through metagenomics, enrichment cultures and isolation, we evaluate three different portions of the bacterial diversity from the studied area, as evidenced by the very small number of operational taxonomic units found in common between the three datasets. *Vibrio cholerae* was detected in lakes by all methodological approaches. Phylogenetic analysis revealed great diversity of fecal bacteria and pathogens dwelling in the lagoons. ARB represented 50% of all isolates, which included species of known human pathogens such as *Pseudomonas aeruginosa* and *Vibrio cholerae*, as well as opportunistic pathogens like *Enterococcus gallinarum* and *Vibrio fluvialis*, all showing patterns of multi-resistance [55]. The resistance profile of 23 isolates obtained from the Jacarepaguá lagoon system is shown in Figure 4.

Our polyphasic experimental approach provided community-based indicators to assess health risks associated with aquatic environments in urban areas. Opportunistic pathogens are common in polluted lakes. Nevertheless, when these bacteria harbor resistance genes against conventional drugs, such environments pose a more serious risk to the well-being of local residents. Jacarepaguá lagoons are a source of food, water and leisure, despite the inadequacy of the lakes for human contact. In this study, we suggest complementary techniques for analyzing microbial diversity, aimed at evaluating water quality based on physical and chemical parameters; metagenomics and microbiological data.

5.3. Antibiotic resistance in *Pseudomonas aeruginosa* isolated from hospital wastewater treatment system

P. aeruginosa is a highly adaptable opportunistic pathogen. The capacity of this bacterium to survive antimicrobial therapy represents a major challenge regarding treatment of infectious diseases [56]. Resistance in *P. aeruginosa* is attributed to chromosomal mutations or acquisition of ARG by genetic exchange mediated by plasmids, transposons or bacteriophages [57]. An increase in the incidence of multi-drug resistant hospital infections caused by this bacterium has been described [58-60].

Most reports surveying antimicrobial resistance have focused on clinical isolates but few studies assessed the antimicrobial resistance profiles of bacteria from hospital wastewater treatment plants [61-63].Such plants have been recognized as a reservoir of resistant organisms and resistance genes [30,53].

We evaluated the antimicrobial resistance patterns of *P. aeruginosa* isolated from a hospital situated in Rio de Janeiro city. These isolates were retrieved either from clinical samples or from the treatment plant that processes the hospital's wastewater discharges. The wastewater treatment system performs an extended aeration-activated sludge process followed by post-treatment (i.e. disinfection of final effluent by chlorination). The whole process includes five stages: wastewater arrival; aeration tank; settling tank; chlorination tank and output of chlorinated sewage (Figure 5). These stages are designed so that the majority of bacteria are eliminated during treatment. The process is considered adequate, unlike that of some hospitals around the globe, which release their effluents without any treatment.

With the exception of Polymyxin B, resistance was detected for all antibiotics tested. Resistance rates among clinical isolates were higher than those found in samples of the treatment system. By contrast, aztreonam resistance rates were higher among isolates from the treatment plant. A total of 21 resistance profiles were identified among our isolates. Of all clinical isolates, 89% were resistant to at least three classes of antibiotics, thus defined as multi-resistant. Only 18.5% of isolates from the wastewater treatment plant were classified as multi-resistant. The most surprising result obtained was the high resistance rates to aztreonam, in both clinical (50%) and wastewater isolates (63%) [51].

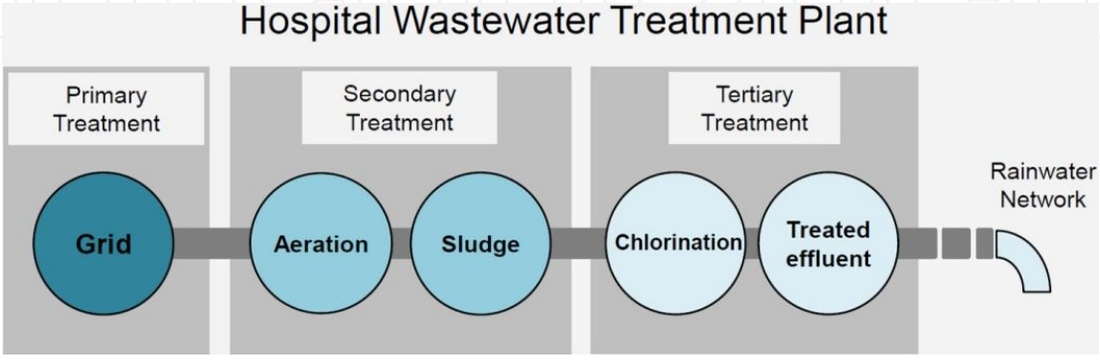

Figure 5. Schematic representation of the hospital's wastewater treatment plant.

Several studies indicate that hospital wastewater treatment systems could work as disseminators of antibiotic resistant bacteria into the environment [61,62,64-67] Resistance levels detected among clinical and treatment system isolates suggests that these organisms are a significant source of ARG. Bacteria resistant to aztreonam were proportionally very abundant. This antibiotic is commonly used for the treatment of *P. aeruginosa* infections.

Exposure to antibiotics is recognized as the major factor promoting acquisition of fluoroquinolone and beta-lactam resistance [68-69]. Also, the up-regulation of efflux pumps is known to convey fluoroquinolone and aztreonam resistance [70]. Triclosan and quaternary ammonium compounds are widely used in clinical practices. These substances are released in hospital effluents along with cells of *P. aeruginosa* that must make use of these efflux pumps to tolerate their presence [71,72]. Use of quaternary ammonium compounds exerts a selective pressure that promotes the dissemination of *qac* genes, responsible for conferring resistance to these disinfectants. *qac*E genes are usually located in class 1 integrons, which are involved in the HGT of antibiotic resistance genes. Thus, these disinfectants can induce AR by co-selection. [73-75].

Our data indicates that the dissemination of aztreonam resistant bacteria in aquatic ecosystems may be compromising the effectiveness of that drug. Also, these results are further evidence that precarious conditions of basic sanitation and low efficacy of hospital effluent treatment plants can contribute to the spread of multi-resistant bacteria into aquatic ecosystems [76-78].

6. Prospects for reversing resistance

One of the most important actions needed to combat propagation of resistance is more research. Even though the sources ARG and ARB have been characterized, our understanding of the step-by-step process by which bacteria acquire and spread resistance is limited. Moreover, very little is known about the emergence of new resistance mechanisms. Advances in these fields are necessary for the development of comprehensive strategies to interfere with the dissemination of resistance traits [30,79]. These strategies should focus on both environmental and clinical perspectives of the problem to be successful.

It is important to determine which environments are potential sources of resistance traits that pose a threat to human health [11,80]. It is also relevant to elucidate how pollutants can work to enhance these risks. Among free-living communities, antibiotics are considered signaling molecules, but the mechanisms by which small substances act upon microbial communication are still poorly characterized. Efforts in this research field will yield valuable knowledge about the functioning of these drugs. This information will contribute to the development of new drugs and to optimize the use of antibiotics [33].

Ecosystems may be contaminated by ABs through wastewater discharges. Even so, it is unlikely that these substances reach inhibitory concentrations in aquatic environments. Moreover, these drugs are subjected to various degradation mechanisms such as photolysis, thermolysis, and enzymatic inactivation. Therefore, it is improbable that the presence of

antibiotics in the environment causes bactericidal effects on microbial communities. Although it has been suggested otherwise, scientific evidence to support this statement is still missing. Nevertheless, sub-inhibitory concentrations of ABs can affect communities by modifying bacterial transcription patterns [81,82]. Resistant bacteria are able to multiply and spread resistance genes [42]. Therefore, the propagation of these organisms, instead of environmental contamination by antibiotics per se, is the most relevant factor contributing to the dissemination of resistance.

Providing adequate hygiene and sanitation conditions for human populations is essential, as untreated water can spread resistant pathogens [79]. Particular care should be taken regarding water treatment in hospital effluents, and any other places where antibiotics are widely consumed. Those are places where resistant strains are likely to be proportionally abundant. Therefore, effluents from those sites demand a more efficient method to eliminate bacteria prior to release [36].

Reduction of selective pressure by regulating the use of antibiotics is a key step to undermine the spread of resistance. This could be achieved by suspending the use of antibiotics when not strictly necessary (i.e., animal husbandry, agriculture and aquaculture), limiting the use of these drugs for medical and veterinary uses. Even for that, antibiotics should be controlled so that resistant strains are not favored [79]. In order to preserve the effectiveness of these molecules some countries have banned the use of antibiotics as growth promoters for animal raising. As previously stated, antibiotic resistance traits are often associated with lower relative fitness. Exploiting these fitness costs to favor susceptible strains is another possibility to reduce the number of bacteria capable of tolerating anti-microbials [83].

The enormous diversity within the resistome makes AR inevitable [12,25]. Maybe there are no effective methods to completely prevent the propagation of resistance. Therefore, the development of alternative treatments for bacterial infections may be required. Making use of new pharmacological compounds to which bacteria cannot resist or take longer to develop resistance could be the only alternative to antibiotic based treatments [79]. Also, phage therapy is a promising alternative to antibiotics.

7. Conclusions

Dissemination of antibiotic resistant bacteria has been investigated mainly due to its risks posed to public health. However, these investigations were focused on the medical perspectives of the problem and often ignored the role of ecosystems. It has been demonstrated that natural habitats are reservoirs of resistance traits, which drew attention to the importance of these sites for the evolution and ecology of antibiotic resistance [29,30]. Mankind has intensively impacted aquatic habitats, favoring the emergence of ARB [46-52].

The results from our analysis of aquatic environments from Rio de Janeiro are an example of how anthropogenic impacts can promote the spread of resistance in aquatic habitats. We could not detect resistant bacteria in pristine sites of Ilha Grande. Meanwhile, impacted environ-

ments of the city had high diversity of ARB. Many resistant pathogens were detected in 16S rRNA gene libraries and among isolated bacteria. The abundance of these organisms indicates an alarming phenomenon: pollution is promoting the spread of both pathogens and antibiotic resistance traits. In addition, even treated wastewater still harbors multi-resistant *P. aeruginosa*, suggesting that some of our efforts to mitigate the propagation of resistance are not completely efficient.

Unless preventive measures are taken to combat the spread of antibiotic resistance, by reducing pollution and making conscious use of these drugs, humanity may be faced with the end of the antibiotic era [5,15]. As previously mentioned, several approaches have been proposed to counteract the spread of AR, but very few of them have been implemented globally until 2012. Risks to human well-being brought by pollution of natural habitats are likely to be among the most convincing arguments for the preservation of Earth's ecosystems. Therefore it is important to raise awareness to those risks by disseminating this information among the members of our society. Also, antibiotic resistance should be taken into consideration in discussions of sustainable development like Rio+20, held in Rio de Janeiro city during 2012. These strategies can ensure the benefits brought by antibiotics, which significantly increased the welfare of humans and animals, certainly saving countless lives.

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