Chapter 12
Antibiotics and Resistance in the Environment

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12.1 Introduction

The discovery and use of antibiotics was one of the greatest public health achievements of the twentieth century. Antibiotics have saved millions of human and animal lives, reduced agricultural losses, and contributed to increased food production. These agents have extended the lives of people with genetic conditions and have become indispensible in modern medicine. The majority of antibiotics currently in use were originally produced by living microbes that were then modified by man. Antibiotics either inhibit growth of other microbes or kill them by interacting with specific microbial targets. Most of the targets are unique to microbes, which has led to the agents being safe enough to use with eukaryotic organisms.

In the mid-twentieth century, antibiotics became the foundation for treating bacterial infections in both humans and animals. Antibiotic-resistant bacteria [ARB] and antibiotic resistance genes [ARGs] were recognized within a year after penicillin was first used in humans, and soon after it was seen with agricultural use [1, 2]. ARB infections now contribute to thousands of deaths each year plus increased morbidity and medical cost. Currently, it is estimated that ~10 million deaths due to antibiotic-resistant infections occur each year; this number is expected to rise in coming years [3]. In essence, antibiotic resistance has changed treatable infections into untreatable diseases, thereby moving us closer to the "post-antibiotic era."

Multidrug-resistant pathogens were first identified in the 1950s [4]. ARB were initially limited to hospital settings and few outbreaks occurred; ARB were not seen as a major concern for general community medicine. Today it is known that antibiotic use in humans and agriculture results in increased antibiotic resistance in

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all types of bacteria, ranging from pathogenic to environmental species. A major paradigm shift occurred in the 1970s, with the identification of ampicillin-resistant *Haemophilus influenzae* and penicillin-resistant *Neisseria gonorrhoeae*, both community-acquired pathogens. Resistance to the preferred therapy led to changes in the recommended therapies for disease arising from these pathogens. The need for monitoring ARB and ARGs and periodic changes of first-line therapies has become an ongoing issue for many different pathogens. Resistance has also lead to a new industry of diagnostics in which new methods and techniques continue to be developed for rapid identification of resistance in clinically important bacteria.

In the past, surveillance of the environment locally, nationally, and internationally has not been a priority, but that has changed [5], as we are beginning to examine the issue and assess the impacts of ARB/ARGs on human and animal health, agricultural and food production, aquaculture, human and animal waste management, and the impact and contamination of the environment globally [6–11].

Antibiotic uses and abuses are directly responsible for the increases in the level of ARB and ARGs isolated in agricultural as well as aquacultural settings, the food chain, man, and built and natural environments [12–14]. Much has been said about the uses of antibiotics as growth promoters in Europe and the USA as being a major source of antibiotic resistance. In June 2015, the US Food and Drug Administration published a final rule known as the veterinary feed directive (https://www.gpo.gov/fdsys/pkg/FR-2015-06-03/pdf/2015-13393.pdf), which limits the use of antibiotics as feed additives for growth promotion. The rule became effective on October 1, 2015, and may have widespread impact on use and prescribing of medically important antibiotics in food animals, both in the years leading up to implementation and after implementation (Jan 2017).

In the early years of antibiotic usage, there were new antibiotics available to replace the older antibiotics as bacteria became resistant. Thus, when one antibiotic failed to work, another was available to take its place. Today there are very few new antibiotics in development to replace the less effective, older antibiotics [3]. The current lack of new and novel antibiotics coming into the market, along with the high cost of newer antibiotics, has led researchers to anticipate a time when there will be no useable antibiotic for many common bacterial diseases. Thus, animals, plants, and people will die of infections that were once easily treated with antibiotics but are now resistant to all available therapies [11]. The factors that contribute to emergence and dissemination of bacterial resistance are complex and require attention in both industrialized and developing countries [12, 13].

Concerns over the spread of antibiotic resistance have stimulated several groups to assess the impact of ARB/ARGs on human and animal health, agricultural and food production, and agricultural and human waste management [15, 16]. One of the primary outcomes of emerging reports is a call for increased surveillance of ARB and ARGs in agricultural and environmental settings, with a particular interest in identifying transmission routes of ARB and ARGs throughout the world [11, 15]. Keys to the success of current and future surveillance efforts are strategies to determine which types of resistant genes to monitor and how to support the surveillance effort, especially for environmental settings and in developing and resource-poor

countries. This is a major task given that in the USA there is no national surveillance program for the most common pathogens across most states. Instead, the Centers for Disease Control and Prevention (CDC) has used surveillance systems that focus on nine representative states [17]. The European Union does a more comprehensive job of covering their member states (http://ecdc.europa.eu/en/healthtopics/antimicrobial-resistance-and-consumption/antimicrobial_resistance/EARS-Net/Pages/EARS-Net.aspx); other parts of the world have varying success with human surveillance systems [17]. The problem is difficult, because ARGs are not randomly distributed among bacterial species. Data suggests that a clear link exists between bacterial taxonomy and specific types of ARG [18, 19]. This phenomenon has been particularly well documented with tetracycline resistance genes [20–22].

The environmental dissemination of ARGs and the development of ARGs are thought to be primarily due to horizontal gene transfer. The most common way bacteria exchange ARGs is by conjugation, which allows rapid transfer of ARGs between species and genera within and between ecosystems [21]. However, our knowledge is limited in regard to how the environment contributes to transmission between the environment, wildlife, domesticated animals, plants, and humans. It is critical when examining specific antibiotic resistance genes to know whether a given gene is normally associated with a mobile element and whether that element has a narrow or broad host range. Clearly a mobile element with a broad host range will allow for wider transmission across multiple genera than a narrow host range element [23, 24]. It is important to identify the specific ARGs associated with specific bacterial species and/or genera within the environment. Durso et al. [18] suggested that the same antibiotic resistance gene might have different risks for environmental transmission that depends on the specific bacterial taxa within which it is found. For example, if the bacteria are widely distributed among a variety of environments, the ARGs associated with them are more likely to spread widely. If, on the other hand, the bacteria have a limited environmental range, the ARGs will tend to remain associated with them specifically. If they have a limited host range, they may also not be widely distributed. It is equally important to know how ARGs and ARB are distributed among human and animal populations and how these ecosystems interact with various environments. Moreover, we need to know how microbial distribution differs by region, nation, and worldwide [25]. Other issues include the fact that most environmental studies look at a selected group of ARGs by qPCR, which determines the presence or absence of particular genes [26], or they use microbiome studies that usually do not look specifically for selected ARGs [25]. Thus, environmental studies should include bacterial culturing, in addition to molecular studies, to fully understand the distribution within the bacterial ecosystem of various environments. The more comprehensive analysis is especially important because many of the new ARGs are coming from the environment rather than from either human or animal sources, which makes it difficult to know the bacterial source of a given ARG (http://faculty.washington.edu/marilynr/).

Organized environmental surveillance of ARGs/ARB will hopefully allow identification of major gaps in our understanding of the forces that act on selection and transmission of bacterial resistance. This effort in turn may lead scientists in direc-

tions that could either slow or stop the march to a time when common infections and minor injuries kill, as they did prior to the introduction and widespread use of antibiotics (this phenomenon is well illustrated by the recent spread of NDM-1 β-lactamase carrying bacteria [27]). It is clear that a global "One Health" approach is needed in which animal and human usage and environmental contamination are considered together, along with an understanding of how ARGs and ARB move between the ecosystems.

12.2 Antibiotics Used for Conditions Due to Non-bacterial [Noninfectious] Conditions

Some antibiotics have non-bacterial effects on humans and animals and have been used to treat non-bacterial conditions, especially skin diseases. A review of the non-antibiotic properties of minocycline by Garrido-Mesa et al. [28] is a useful guide to other properties that this antibiotic has and the non-bacterial conditions for which minocycline is used as treatment. A 2013 paper [29] reported that minocycline improves symptoms of fragile X syndrome when given to children and adolescents. Another study explored the use of tetracyclines with cancer targets through a randomized phase II trial [30]. In a third case, the macrolide azithromycin stimulated immune and epithelial cell modulation of transcription factors AP-1 and NFκB with subsequent delayed inhibitory effects on cell function and may cause lysosomal accumulation of the macrolide with disruption of protein and lipid transport through the Golgi apparatus and effects the surface receptor expression, including macrophage phenotype changes and autophagy [31]. In addition, azithromycin inhibits quorum sensing and biofilm formation by *Pseudomonas aeruginosa*, even though the drug does not inhibit growth. Moreover, azithromycin, given prophylactically, can reduce the incidence of ventilation-associated pneumonia [31]. It is important to note that the use of antibiotics for non-bacterial conditions increases the exposure of individuals' microbiomes to the selective pressures underlying the emergence of bacterial resistance (M. Roberts unpublished results). It also increases the potential for environmental contamination by the antibiotic, its residues, and ultimately selection of ARGs and ARB resistant to these antibiotics.

12.3 The One Health Approach

The One Health approach contrasts with the traditional practice of human and animal medicine, which have been studied and practiced in isolation rather than as part of an ecosystem. The environmental contribution to global health has not been generally considered, or if studied, rarely, until recently in connection with the health of man and/or animals. The world microbial ecosystem includes the microbiomes associated with each domain of life and the direct and indirect mixing of

the different microbiomes that, in some cases, may lead to disease. The human-animal interface is ancient, but it has expanded with the development of farming animals and fish. It is a continuum of contacts and interactions that allow for barrier breaches of pathogens to occur and an increased driver of infections. This is illustrated by the estimation that ~75% of emerging infectious diseases in humans over the last 20 years have been zoonotic, i.e., the pathogen spread from animals or insects to people. In some cases, the pathogen becomes established and then spreads within human populations. However, more commonly, there are recurrent events of transmission from an animal/insect reservoir to humans, with limited human-to-human transmission. An example of this situation is observed with the Zika virus [32, 33]. Other examples include many foodborne bacterial infections, such as those caused by *E. coli* O157:H7 and enterotoxigenic *E. coli* O114:H4. *E. coli* O114:H4 caused a huge outbreak in 2011, which, besides causing death and infections, created tension among EU members involving boycotts of vegetables within the EU [34]. Dealing with emerging and reemerging infections that cross species barriers not only impacts humans but also impacts livestock, pets, wildlife, crops, and aquaculture. These pathogens can contaminate the environment, and in worse cases, they may impact food resources and food security. The importance of global ecological changes due to human impact on the environment and technological changes in society, along with important changes in how food is produced, processed, and transported, combines to increase the potential risk of disease transmission [32]. With environmental contamination as a major by-product of these endeavors, changing the downward spiral of increasing global contamination can only be addressed by improved communication, cooperation, and collaboration across disciplines and the realization that there are multiple ways contamination can enter the food chain.

How a particular antibiotic can influence where and how antibiotic resistance and ARB develop and spread from one domain to all three has been illustrated in the literature. One good example is the development of vancomycin-resistant *Enterococcus faecium* (VRE) in North America, the EU, and the rest of the world. In Europe and other parts of the world, a vancomycin-related drug avoparcin was used as a growth promoter in livestock. Over time, VRE developed in chickens and swine to where it could be readily detected in processed meat [35]. Transmission of VRE genes, or the intact bacterium, from animals to humans occurs in the EU setting. Once VRE was established in livestock populations, farmers and those slaughtering the VRE+ animals acquired VRE in their intestinal tracts. VRE ultimately was isolated from hospitalized persons [36]. In contrast, avoparcin was never used as a food additive in the USA. Early studies suggested that VRE was not found in chickens in the USA, and there was little evidence to suggest transmission of VRE in healthy adults prior to 2000 [35]. In contrast to the EU, which did not use vancomycin heavily in the hospital setting, vancomycin was used extensively in US hospital. The result was the emergence of VRE as a major nosocomial pathogen within US hospitals [38]. This was due, in part, to the persistence of viable VRE on contaminated surfaces within the hospital for weeks and even months. Rooms housing patients colonized or infected with VRE were difficult to clean.

Consequently, these rooms served as reservoirs for transmission of VRE to new patients [37]. More recently, VRE has been cultured from the general community environment in the USA, as illustrated by VRE recovered from wild crows and recreational beach sand and water in North America [39–41]. Currently, if a patient enters a hospital and exhibits a VRE infection within 48 h of entrance, the infection is considered community acquired rather than nosocomial. The occurrence of outpatient VRE depends on geographic location, occupation of the people, differences between urban or rural settings, and/or recent attendance at a medical/dental outpatient clinic or office. VRE in the USA spread from hospitalized humans to the community and environment, while in the EU and other parts of the world, VRE developed in livestock receiving avoparcin and then spread to the farm workers, local communities, and ultimately hospitalized patients.

12.4 The Environment

Most studies on ARGs, over the last 70 years, have focused on clinically important bacteria found in humans and animals. It is estimated that there are ~5×10^{30} bacteria on earth, with only a small subset adapted to live either in or on humans and animals. More striking is the estimate that <1% of the total number of bacteria in the world have been cultured [42]. The natural world is rich in chemicals made by living organisms and human activity – antibiotics are not the only compounds that have influenced the evolution of microbiomes [43].

As stated above, knowing which type of bacterium carries a particular ARG can be critical in designing studies of the environment. For example, many in the field use *E. coli* as a model system for ARG carriage. Yet many ARGs found in *E. coli* are unique to Gram-negative facultative aerobes and not found in anaerobes, other Gram-negative bacteria, or Gram-positive species [22]. Thus, when *E. coli* is the model, most acquired ARGs are associated with plasmids that independently replicate and tend to have a host range limited to Gram-negative bacteria. In contrast, many ARGs in Gram-positive and anaerobic species are on mobile elements that are normally found in the chromosome; thus, they have a different host range dynamics that can be much broader than classical Gram-negative plasmids. Therefore, by looking for both classical Gram-negative and Gram-positive ARGs, researchers can select ARGs that are likely to be most important for a particular ecosystem from a large set that confers resistance to the same class of antibiotic. This is especially important when molecular methods of detection are used, because only a limited number of genes can be assayed, and if the rare genes are chosen, it will bias the results leading to an unrealistic picture in that ecosystem.

Environmental studies are moving away from culturing bacteria, instead of determining which ARGs are present by using either PCR or qPCR. These molecular assays have now been used for direct detection of ARGs in food [44], animal feeding facilities, and agricultural soils amended with manure [45] and as indicators for water quality changes [46]. In these studies, known ARGs were used without

determining their likely distribution in the particular sample source, which can lead to biased results. For example, if only ARGs present in Gram-negative bacteria are used for screening, then no information will be obtained regarding the Gram-positive and anaerobic component of the sample source. Such studies have a very limited ability to identify novel resistance genes.

To overcome the shortcomings of nucleotide sequence-dependent methods, soil bacteria were screened for the ability to degrade or inactivate antibiotics. In one study, strains were randomly isolated from 11 diverse rural and urban soils, and they were then tested for the ability to utilize 18 different antibiotics as sole sources of carbon and nitrogen [47]. Many of the bacteria were *Burkholderia* spp. and *Pseudomonas* spp., which are naturally part of the soil microbiome and only rarely cause disease. These bacteria could grow on antibiotic-supplemented media and were resistant to multiple antibiotics at clinically relevant concentrations, suggesting the presence of an unappreciated reservoir of antibiotic resistance genes in these soils [47]. This work led to development of the functional metagenomic approach (described below) in which the antibiotic resistome of an environment is examined. This has led to identification of novel resistance genes in addition to know ARGs [22].

Functional genomic studies have also been used to study a variety of microbial environments [48]. This assay determines whether the cloned DNA can be expressed and confer resistance when transferred into a host *E. coli*. When the cloned DNA allows the host bacteria to grow in the presence of antibiotic-supplemented media, the resistance-conferring DNA fragments can be sequenced and compared to known ARGs. The Antibiotic Resistance Genes Database now lists ~20,000 potential resistance genes([49]; http://ardb.cbcb.umd.edu/), while the Comprehensive Antibiotic Resistance Database [CARD] also has a large number of resistance genes that can be used to screen sequences [50]. A variety of new potential ARGs have been identified using this method ([51]; http://faculty.washington.edu/marilynr/). One issue with these databases is they rely on GenBank information, which can be confusing and inaccurate because the system allows authors to name their own genes rather than going through a system. They also make it difficult to change names. Thus many of the ARGs in GenBank do not have the correct nomenclature for specific ARGs. Consulting other sources such as http://faculty.washington.edu/marilynr/ and http://www.lahey.org/studies/ should be used to get the correct names for tetracycline, for macrolide-lincosamide-streptogramin genes, and for β-lactamase genes, respectively. Recent reviews are also good sources for the current nomenclature [22].

The term antibiotic "resistome" is defined as the collection of all genes that can either directly or indirectly contribute antibiotic resistance to its bacterial host [52]. Research groups have been examining the microbial resistome of natural and clinical environments [51, 53–55]. Studies have looked for ARGs in samples linked to human activity, such as food production [56, 57], polluted waterways, and wastewater treatment plants [26]. Resistome studies suggest that environmental bacteria may be antibiotic resistant by virtue of both previously characterized, known genes and unknown resistance genes, mutations, and resistance genes on

mobile elements [55, 56]. A number of new ARGs have been identified from these studies; in most cases, the bacterial host is unknown (http://faculty.washington.edu/marilynr/). One example of a ARG identified in a molecular study is the *tet*(43), which encodes an efflux protein. It was isolated from metagenomic analysis of soil taken from an apple orchard that had been repeatedly treated with streptomycin [56]. It is unknown what type of bacterium actually carries *tet*(43), and little is known about the distribution of this gene. Similarly, the nine genes [*tet*(47)-*tet*(55)], identified more recently, code for enzymes that inactivate tetracycline. These were identified, cloned, and sequenced from soil samples where functional metagenomic analysis was done [58]. Again the bacterial sources of each of the genes are not known. This later work increased the number of characterized enzymes that inactivate tetracycline from 3 to 13 (http://faculty.washington.edu/marilynr/), clearly showing that a variety of new ARGS may be present in environments.

Many bacteria, including environmental bacteria, encode β-lactamases, which hydrolyze and inactivate β-lactam antibiotics (http://www.lahey.org/studies/). They are the most widely distributed of all ARGs [3]. One example is *ampC*, which was originally an inducible chromosomal cephalosporinase found in a variety of Enterobacteriaceae. This gene has been found in opportunistic pathogens belonging to normal intestinal floral of humans and animals, in bacterial species that normally live in either soil or water, and in both pathogenic and nonpathogenic bacteria [59, 60]. It has been proposed that *ampC* originated in environmental bacteria. The first AmpC-positive clinical strains were *E. coli* isolated in the 1940s, just as the first antibiotics were being developed and used. In a host background that has porin deficiencies, *ampC*, when expressed, confers carbapenem resistance due to increased production of the AmpC β-lactamase. This increased production of the AmpC β-lactamase is usually due to mutations that up-regulate expression of the enzyme. Today the chromosomal AmpC β-lactamases are associated with plasmids, which was first noticed in the 1980s. These mobile plasmids often tend to be large, and they carry multiple ARGs. Plasmid-mediated AmpC β-lactamases have greatly expanded the host range of this group of enzymes that are now found in epidemic human pathogens such as *E. coli* ST131. This *E. coli* strain has been isolated from fresh vegetables, food-producing animals, fish farms, pets, and water environments [61, 62].

Many ARGs are associated with soil antibiotic producers such as *Streptomyces*. Some of these natural ARGs have the same mode of action as those found in clinically resistant bacteria [3]. In the past, it was assumed that most environmental bacteria were poorly adapted for life in humans and animals. However this idea is changing, as progress in medical science allows severely immunocompromised patients to live in the community where they can be infected with environmental organisms. Other susceptible persons include those who have foreign objects permanently implanted in their bodies and persons with various types of occupational exposure [63]. Moreover, the distinction between environmental and non-environmental bacteria has become difficult, because the mixing of the two sources of bacteria has become increasingly common as human and agricultural contamination of the environment has become widespread. Indeed, very few

ecosystems around the world have not been touched by the activities of human civilization – whether it is in polar regions or the Amazon jungle [64, 65]. The continual mixing of environmental and non-environmental bacteria provides opportunities for horizontal genetic exchange of ARGs between man, animal, and environmental bacteria.

Antibiotics, antibiotic residues, ARBs, and ARGs move by water and wind [66], wastewater treatment discharges [26, 67], biosolids, and manure applications [68], isolated from recreational beaches [40]. They are also moved along with the transportation of goods and people around the world [69, 70]. One result of this movement has been the global spread of specific strains, such as *Clostridium difficile* NAP1/027/BI [71]. *C. difficile* spores are robust, and they can survive in hospital dust for extended time periods. *C. difficile* was originally classified as a nosocomial pathogen. Today *C. difficile* is known to be a foodborne and community pathogen. Similarly, 25 years ago, *Acinetobacter baumannii* was a rarely identified human pathogen. At that time, *Acinetobacter* spp. were primarily found in the environment. They are well adapted to grow at a wide range of temperature and pH values, can use a variety of carbon and energy sources, and persist in both moist and dry locations for extended time periods. Today multidrug-resistant *A. baumannii* is considered an opportunistic pathogen that has become a major concern for military trauma patients and causes infections that are very difficult to treat due to limited therapeutic options [72].

12.5 Agriculture

Antibiotics are used for both human and agricultural activities for prevention and treatment of infections. They are also used as food additives and growth promoters in food production in the USA. However this widespread use is changing. In June 2015, the US Food and Drug Administration published a final rule, known as the veterinary feed directive (VFD), that extends the use of veterinary feed directives to an increased number of medically important antimicrobials used in food animal production [73]. The rule became effective on October 1, 2015, and may have impacted use and prescribing of medically important antibiotics in food animals in years prior to implementation because evidence supporting this idea is derived from the experience of the EU. On July 1, 1989, an EU-wide ban on the use of four growth-promoting antibiotics, spiramycin, tylosin, bacitracin zinc, and virginiamycin, came into effect. The result of this ban was a dramatic drop in the sales of antimicrobial growth-promoting agents. In 2006, the remaining antibiotics used as growth promoters (monensin, avilamycin, salinomycin, and flavomycin) came under an EU-wide ban. It is projected that a further dramatic decrease in sales of growth promoters will occur [74]. Therefore, it is hoped that the US FDA ruling will reduce overall uses of antibiotics used annually in livestock raised in the USA.

Antibiotics can be found in domestic animal manure, which may be transferred when manure is applied to fields or stored in lagoons. In the USA, manure, regardless of whether the host animals are treated with antibiotics or not, is considered an organic product. Domestic animal manure can be placed on crops that will be grown organically. In addition, "organic farms" are usually on land that was originally farmed conventionally. Therefore, antibiotics, antibiotic residues, ARGs, and ARB are normally present in the farm environment. The ARB can colonize the "organic" livestock, while the ARGs may be incorporated into the livestock microbiome. As a result, both organic and conventionally grown meats may have ARB [75].

Antibiotics are sprayed onto crops which contaminates the surrounding soil, sediment, and groundwater. This practice exerts selective pressure on the associated microbiomes and increases the prevalence of resistance in bacterial pathogens of fruit orchards. Antibiotics may also be incorporated into food given to farm animals and fish, which will, in turn, contaminate the surrounding area and ultimately enter the water system.

Antibiotics from human therapeutic use, especially from hospital effluents, are a continual source of pollution and are considered part of the "emerging contaminants" in municipal waste (concentrations of tetracycline vary from ng/L to μg/L). At these levels, antibiotics may select for tetracycline-resistant environmental bacteria which, once present, may persist in the environment for long times. The environment may become a reservoir for tetracycline resistance genes and for other antibiotic resistance genes, since co-selection with other ARGs is common. Antibiotic-resistant bacteria and residues have been identified in tap water, urban water supplies, milk, meat, vegetables, and processed and unprocessed foods [76]. All of these sources contaminate both built and natural environments, either directly or indirectly, and provide selective pressure on the resident environmental bacteria to become antibiotic resistant [3]. In some cases, transfer of specific antibiotic resistance genes is increased with exposure to low levels of antibiotics [77].

12.6 Human Influences on Environments

Human activity may directly influence the development of ARBs in built-up environments. For example, several studies have recovered antibiotic-resistant *E. coli* and *S. aureus* from air in homes that are enriched relative to samples from outside of the home, even though the latter have higher bacterial levels. However, there was variability both in study design and results [66]. Potentially, ARB may contaminate the environment either directly, as occurs when manure is applied to enrich agricultural fields, or indirectly due to sewage contamination of receiving waters where the final effluent is deposited such as a river, lake, or ocean. The first description of the *tet*(M) gene in *Bacillus* spp. and of Tc^r*Bacillus cereus* strains carrying the *tet*(M) gene, on a Tn*916* mobile element, was found in animal manure and in fields where the manure was spread. These results suggested that the presence of *tet*(M)-carrying *B. cereus* in fields was a direct result of manure application to the soil.

Whether *tet*(M)-carrying *B. cereus* will act as a donor and transfer the *tet*(M) gene to either related *B. anthracis* or *B. thuringiensis* is unknown. However, some toxin-encoding plasmids are shared among these three species [68].

An example of human wastes increasing ARB was illustrated by a 1980's study that observed three groups of wild baboons in Kenya. Two of the groups lived in their natural habitat with either limited or no human contact; these groups had low levels of antibiotic-resistant Gram-negative enteric bacteria. The third group lived close to a tourist lodge that provided opportunities for daily contact with unprocessed human refuse. From these animals, high levels of antibiotic-resistant Gram-negative enteric bacteria were recovered with >90% tetracycline resistant [Tc^r]. These results suggested that contact with human refuse greatly increased the carriage of Tc^r bacteria in these wild primates [78]. Unfortunately, the surrounding environmental bacteria were not sampled. One could speculate that the level of environmental ARB was likely higher around the human refuse site than in areas where the two other baboon groups lived in a more natural setting. Other studies have recovered antibiotic-resistant *E. coli* from arctic and subarctic seals [79], wild boars [80], and wild rabbits [81]. More recently, bacteria carrying extended-spectrum beta-lactamases (ESBLs) have been isolated from water birds in remote locations [82]. Birds and wild animals can also be found feeding either in or around wastewater treatment ponds, waste landfill sites, and septic tank discharges. Birds have the potential for long-distance dissemination of ARB and ARGs to remote environments. Such transmission sources may explain why ARB and ARGs can be found in environments having little anthropogenic activity, such as the remote arctic [66].

In many studies it has been assumed that ARG flowed from humans and animals to the environment. But in other cases, the use of antibiotics for food production has created antibiotic-resistant bacteria in animals and farm environment that has spread to man. One classic example of animal-to-human spread is the use of avoparcin in farm animals in the EU [83]. Vancomycin-resistant enterococci [VRE] develop on these farms, contaminating the farm ecosystem, including animal, environmental, and human microbiomes. The VRE strains were passed to farm workers and families living on the farm. In other cases, the plasmids carrying the *vanA/vanB* genes were transmitted from animal to human enterococci [40]. In contrast, VRE development in hospital settings in North America has occurred because vancomycin was commonly used in hospitalized individuals but not in the general community population. More recently, VRE strains have spread to the environment in the USA where they are now isolated in a variety of settings, from recreational beaches to birds to farms [39, 40, 84].

12.7 Aquaculture

As the taste for seafood and shellfish increases, the use of aquaculture around the world, especially in Asia, has increased. Integrated aquaculture is a traditional practice used by small-scale farmers in Asia. The fish are raised in ponds along

with livestock. The livestock manure is used to feed the fish. This system allows for mixing of ARGs and ARB, as well as for creating recombinant influenza viruses [85]. Other parts of the world are less likely to practice integrated aquaculture. Varying sizes of fish farms, both of the fresh water and marine type, grow many types of fish for global export. Tilapia (*Oreochromis niloticus*) is among the most cultured and internationally traded food fish, with an estimated 1.45 million tons produced in China in 2013 [85].

ARGs are enriched in sediments below fish farms in Finland, even though selective pressure from antibiotics was low. A new study, which looked at 364 PCR primer sets for detecting ARGs, mobile genetic elements, and 16S rRNA genes, detected 28 genes in fish feces and fish farm sediments. The ARGs included aminoglycoside (*aadA1*, *aadA2*), chloramphenicol (*catA1*), macrolide (*mef*(A), *msr*(A)), sulfonamide (*sul1*), trimethoprim (*dfrA1)*, and tetracycline ribosomal protection genes [*tet*(32), *tet*(M), *tet*(O), *tet*(W)]. The same ARGs were found in fish feces, suggesting that fish contribute to the ARG enrichment of the farm sediments even though no antibiotic treatment of the fish in the farms was performed. Individual farms had their own unique resistome compositions [86]. The Baltic Sea has no tide, and water circulation is slow; thus, ARGs in the sediment underneath the fish pens and up to 200 m from the fish farms were expected to reflect activity in the farm. Muziasari et al. [86] concluded that their findings provide indirect evidence for the hypothesis that selected ARGs are introduced into the sediment underneath fish farms in the Northern Baltic Sea by farmed fish. The antibiotic concentrations in the sediments were ~1–100 ng/g of sediment.

Tetracyclines have been used extensively in aquaculture, and Tc^r bacteria have been characterized from numerous sources, including fish pathogens and environmental bacteria associated with finfish aquaculture from around the world [87–91]. Tc^r bacteria can be found in fish feed, in the sediment under the fish pens, as well as in the water entering and leaving fresh water ponds [92]. Some of the greatest diversity in Tc^r genes has been identified in the aquaculture environment. In one of our studies, ~40% of the Tc^r bacteria isolated from Chilean salmon fish farms carried previously unidentified Tc^r genes, suggesting that the diversity in the types of *tet* resistance genes is higher than routinely found in collections from either man or other food animals [57]. Some of these bacteria were later found to carry *tet*(39), while other genes are still unknown [93]. It is common to find previously characterized *tet* genes in new bacterial genera. Many of these *tet* genes were not readily transferred under laboratory conditions, thereby raising the question of how some of the genes are transferred to bacteria across the world and from very different environments [57]. The diversity of type and number of Tc^r bacteria found in the aquaculture setting suggests that this may be one environment where there is rapid evolution of Tc^r bacteria and a hotspot for ARG transmission.

12.8 Wastewater Treatment Plants (WWTP)

Municipal wastewater is a mixture of everything that is flushed down a toilet or washed down a drain. This can include commercial, industrial, hospital, and residential waste, in addition to stormwater. The latter is especially important when excessive rain leads to floods. Flooding is expected to become more common, as the climate continues to change. Contamination of the sewer system by stormwater may also occur when storm and sanitary sewers are combined. Previously, municipal wastewater and biosolids were considered waste products that required disposal. However, as drought conditions continue, there has been a paradigm shift. Municipalities are increasingly considering the final wastewater and biosludge as resources to be utilized, rather than as waste products to be disposed of [94, 95]. This change is occurring throughout the world, although it is not a new idea ([96]; https://woods.stanford.edu/news-events/event/wastewater-resource-focus-bay). WWTP do not specifically have a goal of reducing the level of ARGs and ARBs in their final waste products.

Relatively little is known about the risk to farmers, exposed community members, and WWTP workers to the pathogens, ARGs, and ARB present in WWTP products. In most cases, a link between the presence of WWTP products and human health has not been established. However, one study looking at the reuse of wastewater found higher levels of intestinal parasitic infections among Uganda farmers than in other persons [97]. Fenollare et al. [98] found that sewage workers were more likely to be colonized with *Tropheryma whipplei*, the causal agent of Whipple's disease, than nonexposed people. Few other studies have looked at occupational risk of WWTP products.

Human pathogens, including shiga toxin-producing *E. coli* and enteric viruses, typically die off within a 3-month period in WWTP products, while *Clostridium* spp. can persist for years as dormant endospores [99]. Spores include those from *C. perfringens* and *C. difficile*, with the majority of work focused on *C. perfringens* [100]. Several examples of the human opportunistic/pathogens associated with WWTP effluents and biosolids are discussed below.

Wastewater treatment plants and their by-products [biosludge and effluents] have been considered potential reservoirs, amplifiers, and transmitters of ARGs and ARB in a variety of settings [95, 101, 102]. This is of concern because biosludge is an important by-product of the WWTP process and is now considered an economically important resource. Biosludge has been used for a variety of agricultural purposes, including growing food for public consumption; effluent has been used to recharge aquifers, for water landscaping and agriculture, and as a contributor to drinking water [94]. These uses suggest that ARGs/ARB found in biosludge and effluent may be transferred to food products, including shellfish. They can also contaminate waterways, lakes, rivers, recreational waters, and oceans worldwide. Some studies have speculated that the wastewater treatment process may increase the proportion

of ARB in outlets [102]. Hotspots of ARGs and ARB may be at WWTP outflows where wastewater effluents are discharged into bodies of water. Thus, WWTP effluent may contribute to the dissemination of specific ARGs in the natural environment [102, 103]. Similarly, other studies have shown that use of reclaimed water is a reservoir for ARGs which increase in the soils after repeated irrigation with reclaimed water. This has potential implications for human health [104].

Residual ARB/ARGs in the final effluent are normally deposited into bodies of water where they can then be taken up by fresh water and marine wildlife and ultimately cycle back to humans, land animals, and/or marine life [105]. Preliminary data supports this hypothesis. High levels of ARGs were detected where WWTP and CSO outflows discharged into Puget Sound WA USA (Dr. L. Rhodes personnel communications). This release may be one reason why the southern resident killer whales carry Gram-negative and Gram-positive resistant and multiresistant bacteria in their respiratory tracts, as determined by cultures from exhaled breath samples [106]. Similarly, antibiotic-resistant enterococci have been isolated from feces of sea turtles, seabirds, and marine mammals from the southern coast of Brazil [105]. We conclude that the major waterways are sources and reservoirs of ARGs and ARB worldwide.

Conventional wastewater treatment does reduce the total number of fecal bacteria, but it does not necessarily reduce the fraction of ARGs/ARB present. Over 30 years ago, Walter and Vennes [107] showed that between 0.35% and 5% of the coliforms from a domestic sewage system were resistant to ≥1 antibiotics, with ~75% of the multiple resistant strains capable of resistance gene transfer. Other studies have isolated and characterized multidrug-resistant fecal coliforms and/or enterococci from municipal water from multiple geographical areas [108, 109]. To complicate the issue, wastewater effluent is now being used for urban landscaping and to replenish urban aquifers. Thus what is in the effluent can make its way into the drinking water ([110]; http://www.ocwd.com/what-we-do/water-reuse/).

The wastewater treatment process, besides increasing the abundance of ARGs and the diversity of ARBs, may also provide selective pressure to increase the diversity of antibiotic-resistant phenotypes and transmission of ARGs to new bacterial species. These final WWTP products can ultimately contaminate a variety of ecosystems, with particular impact on health through aquaculture, agriculture, the human workers in these industries, and persons who consume these products [104]. Occupational exposure risk to human and animal health is just now being recognized [110]. ARGs and ARB have been found throughout the wastewater treatment process, from raw influent, primary and secondary effluent, aeration tanks, activated sludge, and residual biosolids [111, 112]. The biosolids represent the majority of the biomass and thus the highest concentration of the ARGs and ARB from the treatment process. This material is now widely used to enrich both urban and agricultural environments. This can lead to environmental contamination of soil and water and, most importantly, the potential to contaminate food consumed by the general public [101]. This potential contamination needs to be considered when

trying to determine where the bacteria causing an outbreak were introduced into the food product of interest. Moreover, knowing which specific ARG(s) are found in which bacterial species and/or genera in WWTP products is critical when selecting specific ARGs for regional, national, and international surveillance studies. It is likely that there are common microbes in most WWTP systems (*E. coli* and enterococci), but they may differ in the carriage of ARGs. Thus, unlike isolating bacteria, which can also lead to biases, determining which ARGs are carried by specific bacteria is key to the success of future surveillance efforts using molecular methods. The use of whole genome sequencing of WWTP products with emphasis on a large number of different ARGs would be extremely useful in determining which suite of ARGs should be examined when screening various components of the WWTP. This needs to be done in different types of WWTP systems in both rural and urban setting and both economically advantaged and disadvantaged nations.

Few studies have been conducted concerning metagenome analysis of plasmids [113] or the microbiome of human sewage [114]. More research needs to be done to determine whether there are variations by geographical location, seasons, and other factors. Thus most studies in the literature that screen for specific ARGs and/or resistant plasmids are inherently biased, because of the very large number of different ARGs that are known. This bias should be taken into account when reviewing the literature, including studies cited below.

A variety of studies have looked for specific ARGs in influent wastewater, after primary settling, treated effluent, activated sludge, and treated biosolids. Most of these studies select a small subset of the known antibiotic resistance genes characterized by conferring resistance to a particular antibiotic class. For example, one study looked at 10 different *tet* genes out of 59 that are known ([22]; http://faculty.washington.edu/marilynr/). The genes included Gram-negative-specific efflux genes *tet*(A), *tet*(E), and *tet*(G) and ribosomal protection genes *tet*(M), *tet*(O), *tet*(Q), and *tet*(S) that are found in both Gram-negative and Gram-positive bacteria [115] from the 18 samples over a 12-month period. The Gram-negative efflux *tet*(A) and *tet*(C) genes were identified from all samples (n = 18). The other Gram-negative efflux genes were isolated from 9–16 of the samples. The least common Gram-negative efflux gene, *tet*(D), was identified in 9 of the 18 samples. The results are not surprising, given the distribution of the different *tet* genes (http://faculty.washington.edu/marilynr/). It is interesting that most common efflux gene, *tet*(L), which is isolated in similar numbers of Gram-negative ($n = 19$) and Gram-positive ($n = 22$) bacteria, was not examined ([22]; http://faculty.washington.edu/marilynr/). This is a common issue with many of the environmental sample studies published. The authors selected tetracycline resistance genes to survey based on what previous studies have used rather than base the work on abundance or on those most widely distributed ARGs among different genera in the system they are studying. This approach provides a significant bias to many of the environmental studies, including those on WWTP products [101, 116].

12.9 Selective Examples of ARGs Found in Environmental Bacteria

Bacteria carrying Tc^r are widely distributed throughout the world. They have been isolated from deep, subsurface trenches; in wastewater, surface water, and groundwater, sediments, and soils; and in pristine environments untouched by human civilization, such as penguins in Antarctica and seals in the Arctic [42, 56, 65, 79]. Seventeen (39%) of the 43 known *tet* genes including 12 (44%) of the efflux, 3 (25%) of the ribosomal protection, and 2 (66%) of the enzymatic *tet* genes are uniquely ascribed to environmental bacteria. Whether this is an accurate representation, with some *tet* genes being truly "unique" to environmental bacteria, or whether these genes have not been used in surveillance studies of either animal or human bacteria is unclear. As of 2017, there are 59 *tet* genes with many of the new genes not having been identified in specific bacteria (http://faculty.washington.edu/marilynr/).

Five different resistance genes from *Streptomyces*, designated *otr*(A), *otr*(B), *otr*(C), *tcr3*, and *tet*, have been identified in the chromosome of antibiotic-producing strains. Today the *otr*(A) and *otr*(B) are now found in classical *Bacillus* and *Mycobacterium* species that were primarily environmental bacteria but recently have caused animal and human disease. It is possible that over time other environmental "*tet* genes" will move into bacteria of clinical importance and become associated with animals and man. For example, *Clostridium* spp. are found in the environment, but they are also associated with the intestinal tract of humans and animals. The *tetA*(P) and *tetB*(P) genes appear to be unique to *Clostridium* spp. Other environmental genes included are the *tet*(V) gene that has been found in *Mycobacterium smegmatis*, which is thought to be an environmental bacterium; the *tet*(30) gene in *Agrobacterium*; the *tet*(33) that has been found in environmental *Arthrobacter* and *Corynebacterium* spp.; the *tet*(35) gene in *Vibrio* and *Stenotrophomonas* spp., which can cause human disease; and the *tet*(41) gene in *Serratia* spp. which rarely causes human disease. The *tet*(42) gene found in *Bacillus*, *Microbacterium*, *Micrococcus*, *Paenibacillus*, and *Pseudomonas* spp. was isolated from a deep-sea trench. The *tet*(34) gene was first described in *Vibrio* spp. and more recently identified in *Pseudomonas* spp. and *Serratia* spp.(http://faculty.washington.edu/marilynr/). To determine if these genes are truly environmental will require new surveillance studies in human and animal bacteria to determine if some of genes currently assigned as "uniquely environmental" are really only associated with bacteria isolated in the environment.

Among the 97 genes that confer resistance to one or more macrolide, lincosamide, and streptogramin (MLS) antibiotics, there are a number of resistance genes that are exclusively identified in the *Streptomyces* spp. including rRNA methylase genes [*erm*(H), *erm*(I), *erm*(N), *erm*(O), *erm*(S), *erm*(U), *erm*(Z), *erm*(30), *erm*(31), and *erm*(32)], ATP-binding transporters [*car*(A), *ole*(C), *srm*(B), *tlr*(C)], and a major facilitator [*lmr*(A)] gene. Other rRNA methylases are found innately in vari-

ous environmental *Mycobacterium* spp., [*erm*(37) to *erm*(41)], while environmental bacteria carry a variety of the known MLS resistance genes (http://faculty.washington.edu/marilynr/). Other than genes associated with *Streptomyces* spp. and *Mycobacterium* spp., there are relatively few genes exclusively associated with environmental bacteria. Why a difference occurs in the distribution between *tet* and MLS genes in environmental bacteria is not clear.

β-Lactamases are enzymes that provide resistance to β-lactam antibiotics such as penicillins, cephamycins, and carbapenems (ertapenem). These β-lactamase enzymes have random mutations that modify the spectrum of resistance to varying classes of this antibiotic group. There are hundreds of these modified β-lactamase genes. β-Lactamase genes are ancient and have been identified in remote and isolated environments, suggesting that β-lactamases occur in nature [66]. Another class of β-lactamases, the CTX-M genes, which hydrolyze expanded-spectrum cephalosporins, originated in environmental *Kluyvera* spp. Bacteria with CTX-M genes were first identified in 1989. Today these genes can be found across the world [3]. The *qnr* genes originated in waterborne *Aeromonas*, *Shewanella*, and *Vibrio* spp. [52]. Data from a 30,000-year-old permafrost sample showed that the sample carried genes conferring resistance to a variety of different classes of antibiotics [β-lactams, tetracycline, and glycopeptides]; thus, resistance existed in the environment before antibiotics were used by man.

12.10 Conclusions

The environmental microbiome, which is difficult to define, remains largely unexplored. However, a few studies suggest the wide distribution of ARB and ARGs in the environment. For example, antibiotic-resistant marine bacteria have been isolated 522 km offshore and at depths of 8200 m [117]. The degree of pollution in the environment correlates with the prevalence of resistance, suggesting that over time even the more "pristine" environments will become increasingly contaminated with ARGs and ARB. This phenomenon will ultimately increase resistance among opportunistic and pathogenic bacterial species having human and animal importance. Increased selection pressure for antibiotic resistance in environmental microorganisms is likely to continue, since human activities will likely continue to pollute the environment. Natural forces, such as wind and movement of water, will continue to contaminate areas of relatively uninhabited environments.

The One Health concept is a worldwide strategy for expanding interdisciplinary collaborations and communications in all aspects of health care for humans, animals, and the environment. The aim is for inclusive collaborations dedicated to improving the lives of all species through the integration of human medicine, veterinary medicine, and environmental science. This concept recognizes that using compartmentalized (silo) mentality to approach the three disciplines is not adequate, since the distinction of environment from non-environment, especially at the bacterial level, has become increasingly difficult. It is clear that the introduction of

a new ARG into a human, animal, agricultural, or environmental microbial ecosystem often leads to cross-transmission and dissemination of ARGs and ARB within and between the ecosystems [3].

The data summarized in this chapter indicate that the environment is an important reservoir for ARGs and ARB; it needs to be considered in future studies. There is a large diversity of resistance genes in the environment, and many of these genes have yet to be identified or characterized. Horizontal gene transfer within the microbial world knows few boundaries, and our ability to experimentally mimic what occurs in nature has significant limitations. Indeed, the role that the natural environment plays in the evolution, maintenance, and transmission of ARB and ARGs is just now being examined. However, it is generally agreed that human anthropogenic changes are impacting natural ecosystems that will ultimately impact human and animal health.

It is clear that ARB and ARGs are spread among animals, the environment, and humans and from one geographic location to another throughout the world. The environment is an important reservoir for these resistance genes, with WWTP products being an important component as reservoirs, potential amplifiers, and/or transmitters of ARB and ARGs in the environment. These contaminants not only degrade the local environment but ultimately influence the health of humans and animals associated with that environmental landscape. The environment has provided an increasing number of novel ARGs that have not been found in bacteria traditionally associated with animals or humans (http://faculty.washington.edu/marilynr/). It is unclear whether these "new genes" will impact the treatment of animal and human infections in the future, but NDM-1 and CTX-M genes have been associated with bacterial pathogens. Evidence also exists that WWTP plays a role in the evolution of multidrug-resistant opportunistic and pathogenic bacteria. WWTP is thought to be a hotspot for the contamination of environments including receiving waters of effluent and of soil and agricultural lands where biosolids are utilized. This is very important, as WWTP biosolids and final effluents are considered to be resources that should be used for agricultural purposes and, in some communities, as water resources. Thus it is plausible that there is a human health risk associated with WWTP products; however, data backing this hypothesis is currently very limited. Reducing the levels of ARGs/ARB in WWTP by-products before they are recycled is an important component in the multipronged approach to reduce the global spread and distribution of ARGs. Advanced wastewater treatments using ozone, UV, ultrafiltration, chlorination, dry-air beds, and membrane bioreactor processes are effective in reducing the number of bacteria. These processes may be useful in reducing the level of ARB/ARGs in effluents and biosolids before they are utilized by communities, thereby reducing the risk to humans (113). Unfortunately, recent studies report that UV/H_2O_2 disinfection processes do not eliminate the possible spread of antimicrobial resistance in the receiving environment [118]. Moreover, cost-effectiveness is an important consideration with advanced wastewater treatment options. To comprehensively assess AMR-related impacts on risks to human health, we need to gain a better understanding of the role that biosolids and effluents play as amplifiers, reservoirs, and transmitters of these bacteria and genes.

It is important that members of human communities understand that they contribute to the contamination of their environment – practices such as discarding food and food waste products inappropriately may have downstream consequences. Thus education of the general community, from young children through adults, is an important mission that many scientist in the field neglect – it is potentially the most cost-effective use of resources.

Major Points

Limited work on various environmental ecosystems limits our understanding the relationships between environmental bacteria and the stressors that lead to selections and retention of ARGS/ARB in only one system. Preliminary data indicates that certain places such as WWTP and the receiving waters of this material along with the biosolids produced in the WWTP are hotspots for the exchange of ARGs among the bacterial microbiome. How to deal with these products to reduce the number and diversity of ARGs and ARB is not clear. Using a One Health approach, it is clear that ARGs and ARB can flow from humans and/or animals into the environment and environmental bacteria, and genes can flow back into human and/or animal bacteria. Looking at the complete picture will provide better information for specific ARGs and ARB and with this knowledge perhaps ways of reducing overall transmission from one sector to the other. This requires resources and science at all levels to stabilize and hopefully reduce the human-generated impact on the environment including contamination as well as changes in climates which can disturb the natural web of life as well as increase food insecurity to millions.

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