Chapter 7
Spread of Antibiotic Resistance in the Environment: Impact on Human Health

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Abstract Antibiotic-resistant pathogenic bacteria pose a high threat to human health, but the environmental reservoirs of resistance genes are poorly understood. The origins of antibiotic resistance in the environment are relevant to human health because of the increasing importance of zoonotic diseases as well as the requirement for predicting emerging resistant pathogens. Only little is known about the antibiotic resistomes of the great majority of environmental bacteria, although there have been calls for a greater understanding of the environmental reservoirs of antibiotic resistance. The data on antibiotic resistance before the antibiotic era and in soil show how far away we are from a complete picture about the ecology of antibiotic resistance genes (ARGs). Most of the natural antibiotic producers reside in soil, but soil is a particularly challenging habitat due to its chemical and physical heterogeneity. The prevalence and diversity of ARGs in the environment led to hypotheses about the native roles of resistance genes in natural microbial communities.

This chapter gives an overview on the occurrence of antibiotic resistance determinants in different environments, discusses the environmental sources, the functions and roles of resistance determinants in microbial ecology, and the ways by which those genes may be disseminated in response to human antibiotic use. It also describes molecular methodologies used to study antibiotic resistance dissemination in the environment and attempts to assess the risks associated with resistance spread in the environment for human health.

Keywords Antibiotic resistance · Antibiotic use · Horizontal gene transfer · Resistance monitoring · Human health

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7.1 Introduction

Antibiotics are probably the most successful family of drugs so far developed for improving human health. Besides this fundamental application, antimicrobials have also been used for preventing and treating animals and plants infections, as well as for promoting growth in animal farming (Martinez 2009; Cabello 2006; Singer et al. 2003; McManus et al. 2002; Smith et al. 2002). All these applications caused the release of large amounts of antibiotics in natural ecosystems. However, little is known on the overall effects of antibiotics on the population dynamics of the microbiosphere (Martinez 2009; Sarmah et al. 2006). Large amounts of the antibiotics administered for therapeutic reasons are only partially metabolized. They are discharged along with the excreta from humans and animals to sewage treatment plants and those used in animal husbandry are directly released without any treatment into the environment, particularly to waters or soils.

It is well accepted that antibiotics at therapeutic concentrations select for resistant microbes; however, there is only scarce information and in some cases, contradictory data are available on the effect of antibiotics at subtherapeutic concentrations or concentrations below the minimal inhibitory concentrations (MICs; Rodríguez-Rojas et al. 2013; Andersson and Hughes 2012; Hughes and Andersson 2012; Gullberg et al. 2011; Liu et al. 2011).

The debate on what was originally the major role of antibiotics in the environment is even more controversial: One well-accepted argument is that their role in nature is to inhibit microbial competitors. An alternative hypothesis states that antibiotics could be primarily signal molecules that shape the structure of microbial communities (Martinez 2009; Fajardo and Martinez 2008; Yim et al. 2007; Linares et al. 2006). Under this view, antimicrobials will have a hermetic effect, beneficial at low concentrations that are likely found in most natural ecosystems, and harmful at the high concentrations used for therapeutic reasons (Martinez 2009; Davies et al. 2006; Calabrese 2005).

For decades, the general opinion of medical doctors, clinicians, and scientists was that antibiotic resistance and the occurrence of the associated genetic determinants are a problem restricted to hospitals and health-care centers. Only recently it has been recognized that antibiotic-resistant microorganisms and the associated resistance determinants are ubiquitous and are also present in pristine environments which have never been in contact with antimicrobials (Allen et al. 2010), as evidenced clearly by the detection of antibiotic resistance determinants in soils conserved in a frozen state from the pre-antibiotic era (Knapp et al. 2011; Knapp et al. 2010).

Additionally, it has been stated that some genetic elements that serve to resist high concentrations of antimicrobials have distinct functional roles (e.g., cell homeostasis, signal trafficking, metabolic enzymes, etc.) in their original hosts (Martinez et al. 2009; Martinez 2009; Martinez et al. 2007). The strong increase of antimicrobial concentrations in natural ecosystems, as a consequence of human activities (human antibiotic therapy, farming), might have shifted the original functions of antimicrobials and resistance determinants to the threatening role they nowadays play in hospitals or farms (Martinez 2009, 2008). These changes might influence not just the

selection of antibiotic-resistant bacteria, but also the structure of the natural bacterial populations and may as well change the physiology of bacteria (Martinez 2009).

The chapter will focus on the antibiotic resistance problem in the environment and the major sources of pollution by antibiotic resistance determinants and suggest ways to relieve the problem. Furthermore, we will give an overview on the major ways of antibiotic resistance spread in the environment and try to assess the risks associated with the occurrence and spread of resistance determinants for human health.

7.2 The Antibiotic Resistance Problem

7.2.1 State of the Art of the Problem

It is now well accepted that antibiotic resistance genes (ARGs) are found everywhere, in clinical settings, tertiary care centers, pets, wildlife, surface waters, and soils, basically in all locations which have been or are in contact with microbes. The major mechanisms conferring resistance to antibiotics are also known (Fig. 7.1). Concentrations of ARGs and the classes of antibiotics to which they confer resistance differ between sites. One thumb rule which holds true for most locations is: The closer the environment is to anthropogenic influence, the higher the incidence is of antibiotic-resistant bacteria and the prevalence of the respective ARGs. The major ways of antibiotic resistance spread in the environment are also known. However, their contribution in different habitats and between different microbes varies considerably and is still a cause for debates in the scientific community.

Ways to slow down the development of antibiotic resistance include: (i) prudent use of antibiotics in therapy (human and animals); (ii) worldwide ban of all antimicrobials which are generally used in human therapy from growth promotion in animal husbandry; (iii) strong worldwide reduction of the use of antibiotics in aquaculture and mariculture; (iv) separation and separated treatment of hospital waste and wastewater (ww) from sewage; (v) application of treated or at least partially treated ww for crop irrigation (never without any treatment); and (vi) application of advanced technologies for water purification for drinking water purposes.

The World Health Organization (WHO) and many national health authorities are now aware of the problem of the occurrence as well as of the dissemination of antibiotic resistance in the environment. However, to efficiently tackle the problem and to install countermeasures, systematic studies are required worldwide to assess the impact of ARGs in the environment on human health.

7.2.2 Relationship to Antibiotic Usage

Antibiotic utilization for clinical or farming purposes selects for resistant microorganisms (Martinez 2009; Livermore 2005; Teuber 2001). Thus, it can be predicted that residues from hospitals or farms contain both types of pollutants: antibiotics

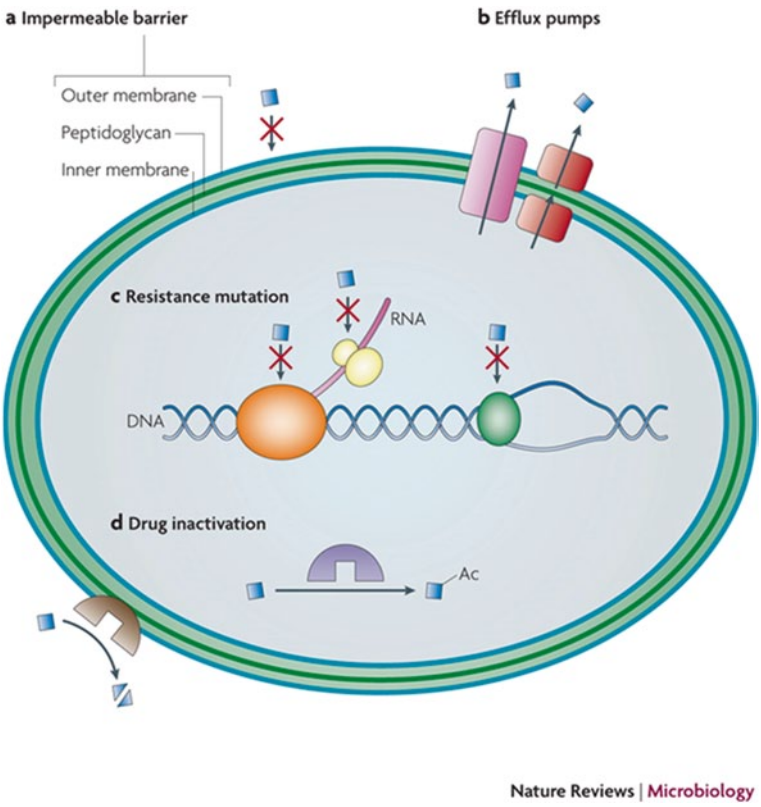

Fig. 7.1 Mechanisms of antibiotic resistance in a Gram-negative bacterium (adapted from Allen et al. 2010). **a** Impermeable barriers. Some bacteria are intrinsically resistant to certain antibiotics (*blue squares*) because they have an impermeable membrane or lack the target of the antibiotic. **b** Multidrug resistance efflux pumps. These pumps secrete antibiotics from the cell. Some transporters, such as those of the resistance–nodulation–cell division family (*pink*), can pump antibiotics directly outside the cell, whereas others, such as those of the major facilitator superfamily (*red*), secrete them into the periplasm. **c** Resistance mutations. These mutations modify the target protein, for example, by disabling the antibiotic-binding site but leaving the cellular functionality of the protein intact. Specific examples include mutations in the gyrase (*green*), which cause resistance to fluoroquinolones, in RNA polymerase subunit B (*orange*), which cause resistance to rifampicin, and in the 30S ribosomal subunit protein S12 (encoded by *rpsL*; *yellow*), which cause resistance to streptomycin. **d** Inactivation of the antibiotic. Inactivation can occur by covalent modification of the antibiotic, such as that catalyzed by acetyltransferases (*purple*) acting on aminoglycoside antibiotics, or by degradation of the antibiotic, such as that catalyzed by β-lactamases (*brown*) acting on β-lactam antibiotics. Ac, acetyl group

and ARGs. Nevertheless, the fate of both types of pollutants is most likely different. Several antibiotics are natural compounds that have been in contact with environmental bacteria for millions of years and are thus biodegradable; some can even serve as food resource for several microorganisms (Martinez 2009; Dantas et al. 2008). Synthetic antibiotics such as quinolones can be more refractory to biodegradation.

Recent work has shown that the binding of quinolones to soil and sediments delays their biodegradation (Martinez 2009). Nevertheless, ww treatment of quinolone-polluted waters efficiently removes these antibiotics through biodegradation and photodegradation (Sukul and Spiteller 2007). Consistent with these data, it has been demonstrated that most antibiotics are usually below detection limits in ground water samples, although they are more stable upon adsorption to sediments (Hirsch et al. 1999; Halling-Sorensen et al. 1998). Due to this fact, sediment samples from antibiotic-polluted environments contain higher antibiotic concentrations than water samples from the same site (Martinez 2009; Kim and Carlson 2007). The fact that antibiotics are degraded in natural ecosystems does not mean that they are not relevant pollutants, as the degradation process is slow at low temperatures in winter (Martinez 2009; Dolliver and Gupta 2008). Furthermore, some environments suffer a constant release of antibiotics (e.g., hospital effluents and farm residues); they are constantly polluted irrespective of antibiotic degradation. The consequence is that the organisms are continuously exposed to antibiotics at subtherapeutic concentrations (Martinez 2009; Lindberg et al. 2007). Since sub-inhibitory concentrations of antibiotics can trigger specific transcriptional responses in bacteria (summarized in Martinez 2009), the presence of antibiotics will necessarily modify the metabolic activity of the microorganisms present in these polluted environments. However, in any case, the fate of antibiotics in natural ecosystems is their degradation (Pei et al. 2006) in such a way that if the utilization of a given antibiotic is banned, it will sooner or later disappear as a pollutant from natural ecosystems.

In contrast, antibiotic resistance determinants present in gene transfer units on mobile genetic elements such as plasmids or integrative conjugative elements (ICEs) are auto-replicative elements that can be maintained in microbial populations unless they confer a fitness cost to the recipient bacteria (Martinez 2009). Some studies have clearly shown that reducing the antibiotic load in natural environments may reduce the amount of pollutant ARGs, e.g., it has been shown that sewage dilution in river waters reduced the number of plasmid-encoded ARGs in *Escherichia coli* (Martinez 2009; Gonzalo et al. 1989). In another well-known example, the ban of the utilization of some antibiotics in farming has significantly reduced antibiotic resistance in animals and its transfer to humans (Martinez 2009; Aarestrup et al. 2001). However, unfortunately the situation is not that simple. It has been observed that even though the incidence of antibiotic resistance declines, the decline is slow and part of the resistant population remains (Andersson 2003), a situation which is consistent with predictions based on mathematical models (Levin 2002). Furthermore, the presence of the same ARGs currently present in human pathogens has been reported in ecosystems without a history of antibiotic contamination (Pallecchi et al. 2008). These ecosystems include remote human and animal populations without known antibiotic exposure which can present a high prevalence of resistance despite not receiving any antibiotic (Bartoloni et al. 2009; Martinez 2009; Grenet et al. 2004; Gilliver et al. 1999). This indicates that ARGs can be resilient to elimination even in the absence of antibiotic selective pressure (Salyers and Amabile-Cuevas 1997). Several efficient mechanisms exist that allow the maintenance and the spread of ARGs in the environment. Thus, as opposed to antibiotic contaminations, pollution by antibiotic

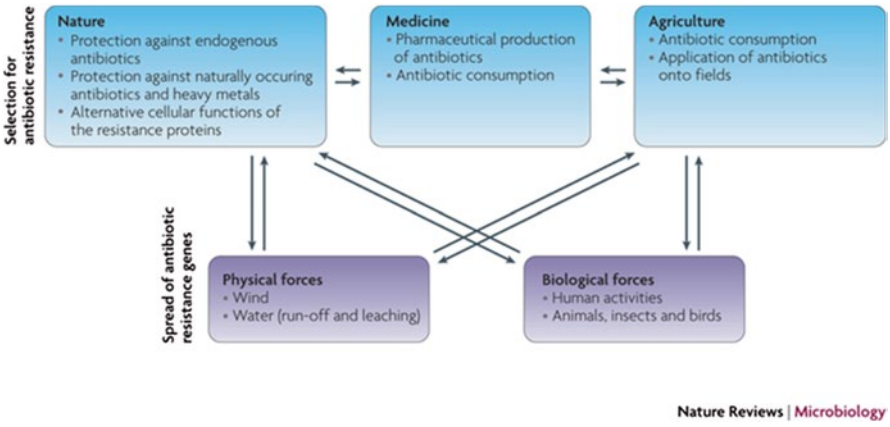

Fig. 7.2 Sources and movement of ARGs in the environment (adapted from Allen et al. 2010). ARGs exist naturally in the environment owing to a range of selective pressures in nature. Humans have applied additional selective pressure for ARGs because of the large quantities of antibiotics that we produce, consume, and apply in medicine and agriculture. Physical and biological forces also cause widespread dissemination of ARGs throughout many environments

resistance determinants will not necessarily disappear even if the release of ARGs in the environment is stopped (Martinez 2009). Sources and movement of ARGs in the environment are summarized in Fig. 7.2.

7.3 Environments of Particular Concern: Major Sources of Antibiotic Resistance Genes

We will focus here, by choice, on natural environments under anthropogenic influence and on anthropogenic environments excluding hospitals and health-care centers as a plethora of excellent articles are available on antibiotic resistances in hospitals and on their impact on human health (Arias and Murray 2012; Hollenbeck and Rice 2012; Yezli and Li 2012; Gould 2008; Witte et al. 2008; Koch et al. 2004; Klare et al. 2003). Additionally, we will consider the influence of the increased mobility of the human population on the spread of infectious diseases and resistant microbes. Wilson published an excellent review article on the traveler and emerging infections (Wilson 2003). The movement of populations shapes the patterns and distribution of infectious diseases globally. The consequences of travel are seen in the traveler and in places and populations visited and may persist long after travel. The traveler can be seen as an interactive biological unit who picks up processes, and carries and drops off microbial genetic material (Wilson 2003). Travelers can also be seen as couriers who inadvertently transfer pathogens and microbial genetic material to regions where researchers can perform detailed analyses that can help to map the location and movement of strains, genotypes, and resistance patterns. The

connectedness and mobility in today's world facilitate the emergence of infectious diseases in humans and also in animals and plants. Population size and density favor spread of many infections. The rapid generation time of microbes and their adaptability to changes in the physico-chemical and immunological environment will pose continuing challenges to mankind (Wilson 2003).

Travelers regularly and effectively move antibiotic-resistant bacteria across borders (Wilson 2003; Okeke and Edelman 2001; Harnett et al. 1998; Slavin et al. 1996; Brown and Linham 1988). In 1987, Murray and co-workers examined fecal specimens from persons before, during, and after traveling to Mexico (Murray et al. 1990). They observed that resistance in *E. coli* increased to multiple antibiotics, including ampicillin, trimethoprim-sulfamethoxazole, sulfonamides, and chloramphenicol, in association with travel. This occurred even in persons who had taken no antibiotics. A multidrug-resistant methicillin-resistant *Staphylococcus aureus* (*S. aureus*; MRSA) clone is thought to have spread from Brazil to Portugal, presumably carried by one or more persons who were colonized or infected (Wilson 2003; Aires de Sousa et al. 1998). An ARG may emerge once on a single plasmid and subsequently be carried to multiple locations, where it may continue to spread, e.g., a gentamicin-resistance gene appears to have been spread on a conjugative plasmid (O′Brien et al. 1985). Highly resistant bacteria carried by travelers can also spread after the travelers had returned home, particularly in a clinical setting (Wilson 2003; M′Zali et al. 1997).

The industrialization of food animal production, specifically the widespread use of antimicrobials, not only increased pressure on microbial populations, but also changed the ecosystems in which antimicrobials and bacteria interact. Davis and colleagues defined industrial food animal production (IFAP) as an anthropogenic ecosystem (Davis et al. 2011).

7.3.1 Farms: Spread of Antibiotic Resistance Genes in the Food Chain

Today, the magnitude of human impacts on natural systems makes consideration of anthropogenic changes to ecosystems important. Agriculture is one such activity, because it inherently creates anthropogenic ecosystems (Jackson and Piper 1989), which are defined as collections of organisms and physical structures under human control and manipulation (Davis et al. 2011). The adoption of an industrialized model in modern food animal production (Martinez 2002) has been successful in increasing global food production, but it also has intensified its impact through the expansion of anthropogenic ecosystems (Tilman et al. 2002; Jackson and Piper 1989). Davis and coworkers argued that IFAP creates anthropogenic ecosystems wherein the use of antibiotics inevitably selects for antibiotic resistance in bacterial populations within animal hosts and the environment. Consequently, this alters microbial communities (microbiomes) and the collection of available mobile resistance determinants (resistome) dispersed into the surrounding ecosystems

(Davis et al. 2011; Wright 2007, 2010; Martinez 2009). Davis and colleagues have studied the role of anthropogenic ecosystems on the emergence of drug-resistant bacteria from agricultural environments on the example of US industrial poultry production. The anthropogenic ecosystems generated by IFAP practices have extensive direct impacts on the microbial ecology of poultry hosts and the environment, and probably have indirect impacts on consumers through poultry products (Davis et al. 2011). In nature, microorganisms are known to both produce and develop resistance to antimicrobials, resulting in a set of complex interactions now thought to contribute to the signaling and regulation in natural microbial ecosystems (Davis et al. 2011; Aminov 2009). However, the extent and magnitude of antimicrobial use in IFAP far exceed, in volume and impact, those of naturally occurring antimicrobials (Davis et al. 2011; Martinez 2009; Kumar 2005). The US **Food and Drug Administration** (FDA) reported that 13 million kg of antimicrobials were sold or distributed for use in food-producing animals during 2009 (FDA report 2010). Particularly, the practice of using nontherapeutic concentrations of broad-spectrum antimicrobials to feed (Baurhoo et al. 2009) creates an ideal environment for selecting individual bacterial cells or populations that have acquired resistance through mutations or horizontal gene transfer (HGT) (Love et al. 2011; Lees et al. 2006).

The process of natural selection by antimicrobial use in IFAP is reflected in observations of antimicrobial-resistant isolates from livestock, including poultry, shortly after the introduction of routine use of antimicrobials as feed additives in the 1950s and 1960s (Davis et al. 2011; De Soet 1974; Smith 1970; Starr and Reynolds 1951). As resistant populations replace susceptible populations at the community level, ARGs in one population/species are available to other populations/species through HGT. Consequently, the development of novel multidrug-resistant bacteria and/or multidrug resistance conferring Mobile Genetic Elements (MGEs) is enabled (Davis et al. 2011; Davies and Davies 2010; Wright 2007). M′ikanatha and colleagues typed *Salmonella* cultured from retail chicken purchased in Pennsylvania and compared the chicken isolates with human isolates. Applying molecular methods, an identical isolate was found in a retail chicken and in a patient (M′ikanatha et al. 2010).

Davis and colleagues focused their review on research along the pathways that connect the commercial poultry intestinal microbiome with microbiomes in surrounding environments. The impact of natural selection exerted by antimicrobial use within the intestine of individual poultry hosts can be further scaled up to the inter-microbiome and inter-ecosystem level (Fig. 7.3). Agricultural ecosystems interact with other ecosystems directly at both local and regional levels, and more broadly through global movement of dusts and water (Peterson et al. 2010), as well as economic trade in feeds, animals, and animal waste (Davis et al. 2011; Sapkota et al. 2007). Although the industrial poultry house often is assumed to be biocontained and biosecure, multiple pathways connect it with surrounding ecosystems (Silbergeld et al. 2008). These are ventilation systems required to keep crowded animals alive; movement of rodents (Henzler and Opitz 1992), wild birds (Leibler et al. 2009), and insects (Graham et al. 2009) in and out of confinement facilities; and transfer of wastes (Davis et al. 2011; Graham and Nachman 2010). These

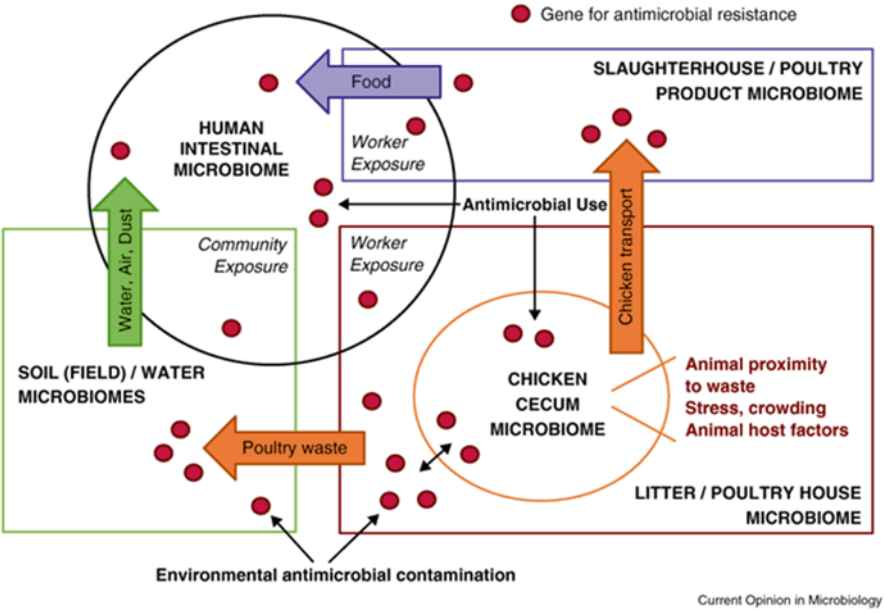

Fig. 7.3 Potential role of antimicrobial selective pressure in the environment (from Davis et al. 2011). Conceptual, potential role of selective pressure of antimicrobial use and other anthropogenic ecosystem alterations that impact microbiomes in the chicken cecum, poultry house environment, local soil and water environments, processing plant environment, and human intestine

conditions release viable bacteria and ARGs into surrounding environments, water systems, and wild animal reservoirs (Davis et al. 2011; Chee-Sanford et al. 2009; Baquero et al. 2008; Silbergeld et al. 2008).

Genetic analysis of the US commercial broiler cecum microbiome has shown that it contained a wide array of ARGs and genes enabling HGT (Davis et al. 2011; Qu et al. 2008). Recent Canadian studies also have found widespread prevalence of virulence and resistance genes from *Enterococcus* spp., *E. coli*, and *Clostridium perfringens* isolated from enteric samples from conventional broilers that were fed antimicrobials (Davis et al. 2011; Diarra et al. 2007, 2010; Bonnet et al. 2009). Furthermore, antimicrobial-treated broilers, compared to those not fed antimicrobials, were significantly associated with increases in the presence of ARGs and class 1 integron genes in cecal and environmental *E. coli* isolates (Davis et al. 2011; Diarra et al. 2007). Especially class 1 integrons are known to shuffle ARGs and are known to be able to promote transfer of ARGs among bacteria (Davies and Davies 2010; Diarra et al. 2007).

Much like the chicken cecum, poultry waste contains a significant number of resistance integrons, particularly within gram-positive bacteria (Diarra et al. 2007; Nandi et al. 2004). Some resistance patterns appear to persist in bacteria even after cessation of antimicrobial use, for example, fluoroquinolone resistance in *Campylobacter* (Price et al. 2007) and sulfonamide resistance in *E. coli* (Davis et al. 2011; Furtula et al. 2010).

Much of the impact of antimicrobial use on the environmental microbiome is exerted through poultry waste disposal. Application of litter onto open fields can impact the soil microbiome locally to regionally through run-off and air-borne drift. The USA has no regulatory requirements for treating animal wastes, leading to uncontrolled waste storage before land disposal (Davis et al. 2011; Graham and Nachman 2010). Simple storage methods do not affect prevalence of pathogens nor drug-resistant pathogens (Graham et al. 2009). Most of the antimicrobials in feeds pass largely unchanged through the broiler gut into the excreta (Kumar et al. 2005). Some antimicrobials, such as oxytetracycline and fluoroquinolone analogs, can persist in the soil environment with half-lives as long as 150–250 days with undiminished potency (Davis et al. 2011; Chee-Sanford et al. 2009; Kumar et al. 2005).

Spread of antimicrobial-resistant bacteria and resistance determinants represents the inter-ecosystem effects of antimicrobial usage in industrialized food animal production. Human links include vehicles, animal transport, and networks of social and commercial contact (Davis et al. 2011; Leibler et al. 2010; Rule et al. 2008). Cross-contamination of poultry during transport and at slaughter contributes to greater microbial diversity in retail chicken than in live birds (Hastings et al. 2011; Colles et al. 2010). Contamination during the harvest process can impact poultry house (Price et al. 2007) and slaughter workers (Mulders et al. 2010), as well as retail chicken consumers in the global market (Davis et al. 2011). Compelling evidence for the impact of antimicrobial use in industrialized food animal production comes from molecular analyses of bacteria in live poultry and/or on poultry products in conjunction with analysis of human isolates (Davis et al. 2011; McEwen et al. 2010; Denis et al. 2009; Gupta et al. 2004). Numerous studies demonstrated the presence of very similar or identical ARGs (Diarra et al. 2010; M′ikanatha et al. 2010; Simjee et al. 2007), identical strains of antimicrobial-resistant bacteria, such as MRSA (Smith and Pearson 2011; Bystroń et al. 2010), and related or identical resistance plasmids (McEwen et al. 2010) in humans and poultry (Davis et al. 2011).

Witte and coworkers performed an experiment with the antibiotic nurseothricin which is not used in humans; strains resistant to it were recovered from both animals and farm workers (Acar and Moulin 2006; Witte et al. 1984). More recent studies dealing with enterococci and Enterobacteriaceae confirmed transfer of resistant bacteria from animals to humans (Acar and Moulin 2006; Hershberger et al. 2005; Aarestrup and McNicholas 2002; Frey et al. 2000; Van den Bogaard et al. 1997).

7.3.2 Aquatic Environments

Basically, all aquatic environments can be considerably affected by pollution through antimicrobials, antimicrobial degradation products, by antimicrobial-resistant microbes and the genes conferring antimicrobial resistance. In the following section, aquatic environments especially affected by the occurrence of ARGs are discussed.

7.3.2.1 Aquaculture

Any agricultural or aquacultural farming operation that relies on the routine and regular use of antimicrobials to control losses is, on the long run, unsustainable. The continued usage of antimicrobials will lead to the emergence of resistance in the target bacteria. Thus, such a dependence on antimicrobials not only represents an unacceptable and imprudent use of these valuable agents, but it will almost certainly prove to be self-defeating (Smith 2008). In any population of farmed animals, maintaining appropriate living conditions, employing appropriate husbandry practices, and using vaccines, whenever available, against enzootic or frequently encountered infections are the primary and most effective methods by which losses due to infectious diseases can be limited (Smith 2008). However, the aim of all these prophylactic procedures is to limit the occurrence of infectious disease and it is unrealistic to expect them to entirely prevent any occurrence of these diseases (Smith 2008). Thus, the inevitability that disease emergencies will occur requires that we learn how to use antimicrobials in such a way so as to maximize their efficacy while minimizing the pressure for increased frequencies of resistant strains (Smith 2008).

Smith presented estimates of antimicrobial use in the aquaculture industries of different countries. The estimated antimicrobial use (g/t production) differs tremendously between the listed countries. Norway and Sweden apply 1 and 2 g/t production, whereas Greece and Canada apply 100 and 156 g/t production, respectively. At the end of the list are two countries applying enormous quantities, namely Chile (200 g) and Vietnam (700 g) per ton production (Smith 2008). There are three methods, medicated feed, bath, and injection, by which antimicrobials are routinely administered to aquatic animals. For the majority of farmed species, administration occurs via medicated feed.

In the following section, we will focus on the negative consequences of antimicrobial use in aquaculture as experienced in human and public health contexts. The most significant public health risks associated with increased frequencies of resistance due to the use of antimicrobial agents in aquaculture can be summarized by two major issues: (i) concerns associated with the selection of resistant variants of bacteria capable of inducing infections in humans that would require antimicrobial therapy and (ii) concerns associated with the movement of ARGs from bacteria in the aquatic environment to those in the terrestrial environment that are capable of infecting humans or other land-based animals (Smith 2008).

Selection for Resistance in Bacteria Associated with Human Disease

It has been assumed that the major risks associated with the use of antimicrobials in land-based agriculture are those leading to selective enrichment of resistant strains of zoonotic bacteria (Smith 2008; Helmuth and Hensel 2004). There is an ongoing debate on the size of this risk, with some arguing that it is relatively small (Wassenaar 2005; Bywater 2004) and others that it might be significant (Angulo et al. 2004). Bacteria capable of infecting humans are found much less frequently in aquaculture than in agriculture. Thus, the risks associated to the selection of

resistant zoonotic bacteria by the use of antimicrobial agents will be significantly smaller in aquaculture than in agriculture (Smith 2008; Smith 2001).

The WHO/Food and Agriculture Organization (FAO)/World Organisation for Animal Health (OIE) expert working group (WHO 2013) identified two groups of bacteria that might be encountered in aquaculture and might also be capable of infecting humans, enteric pathogens such as *Salmonella*—due to contamination of aquaculture by human or animal wastes—and aquatic bacteria such as *Vibrio parahaemolyticus* and *V. cholerae* (Smith 2008).

Selection for Transmissible Resistance

The WHO/FAO/OIE expert group reached the following conclusion: "The greatest potential risk to public health associated with antimicrobial use in aquaculture is the development of a reservoir of transferable resistance genes in bacteria in aquatic environments from which such genes can be disseminated by HGT to other bacteria and ultimately reach human pathogens." There is a plethora of data available (recently reviewed by Sørum (2006)) demonstrating that ARGs capable of being transferred to terrestrial bacteria have been regularly detected in bacteria associated with disease of aquatic animals (Smith 2008). There are also ample data demonstrating that transmissible ARGs are present in the bacteria found in the vicinity of aquaculture operations (Smith 2008; Miranda et al. 2003; Schmidt et al. 2001; Rhodes et al. 2000). Surprisingly, there are only few papers that have convincingly linked the use of antimicrobials in aquaculture with an increase in the frequency of these transmissible genes. The available data support the hypothesis that a reservoir of transmissible ARGs will develop as a consequence of the use of antimicrobials in aquaculture. What is less certain is the size of this reservoir and its public health significance (Smith 2001, 2008).

Movement of Transmissible Resistances Between Terrestrial and Aquatic Microorganisms

Molecular studies have shown that the resistance genes in bacteria associated with aquaculture are significantly similar to those that have been found in terrestrial bacteria causing human and land-based animal disease (Smith 2008; Sørum 2006; Kim et al. 2004; Bolton et al. 1999). Confirmation that these genes can move between bacteria in these two environments has been provided through laboratory studies by Kruse and Sørum (1994) and Sandaa and Enger (1994), which have demonstrated that these genes can be transferred from aquatic to terrestrial bacteria with relatively high frequencies (Smith 2008).

The current monitoring and surveillance programs of the use of antimicrobials in aquaculture have to be considerably improved to be able to assess the impact of antimicrobial resistance as a consequence of antimicrobial use in aquaculture on human health. In addition, laboratory methods used to identify resistance and to quantify the frequencies of resistance that result from antimicrobial use in aquaculture have to be harmonized to enable comparison of results from different laboratories (Smith 2008).

7.3.2.2 Wastewater and Wastewater Treatment Systems

Urban wastewater treatment plants (UWTPs) are among the main sources for the release of antibiotics into the environment. The occurrence of antibiotics may promote the selection of ARGs and antibiotic-resistant bacteria, which shade health risks to humans and animals (Rizzo et al. 2013). Rizzo and colleagues reviewed the fate of antibiotic-resistant bacteria and ARGs in UWTPs, focusing on the different processes typically included in UWTPs, e.g., mechanical, biological, physical, chemical, and physical–chemical processes, which may affect the fate of antibiotics, antibiotic resistant bacteria, and ARGs in different ways and consequently the development and spread of resistance into the environment (Rizzo et al. 2013).

Over the past years, a renewed interest on the antibiotic resistance phenotypes in UWTPs was obvious in the scientific literature (Rizzo et al. 2013; Manaia et al. 2012; Kümmerer 2009; Baquero et al. 2008). Human and animal commensal bacteria and other of environmental origin have been the major focus of the studies on antibiotic resistance in ww. Due to their close contact with humans and the easiness to isolate and identify, the fecal indicators, coliforms and enterococci, have been the most studied groups (Rizzo et al. 2013; Araùjo et al. 2010; Sabate et al. 2008; Boczek et al. 2007; Ferreira da Silva et al. 2007; Martins da Costa et al. 2006; Reinthaler et al. 2003). To establish a relationship between the most severe cases reported in clinical settings and environment, a search for the last-generation antibiotic resistance determinants has also been reported in UWTP studies (Rizzo et al. 2013; Czekalski et al. 2012; Figueira et al. 2011a, b; Araùjo et al. 2010; Parsley et al. 2010; Szczepanowski et al. 2009; Gajan et al. 2008). In particular, the presence of MRSA, vancomycin resistant *Enterococcus* spp. (VRE), and gram-negative bacteria producing extended spectrum beta-lactamases (ESBL) has been studied.

Although the occurrence of antibiotic-resistant superbugs in the effluents may be an issue of particular concern, the numbers of common bacteria harboring ARGs that are continuously discharged in receiving waters are impressive (Galvin et al. 2010; Łuczkiewicz et al. 2010; Ferreira da Silva et al. 2007; Martins da Costa et al. 2006). The final effluent of UWTPs can discharge approximately 10^9–10^{12} colony forming units (CFU) per day, per inhabitant equivalent; among these, at least 10^7–10^{10} could have any kind of acquired antibiotic resistance (Rizzo et al. 2013; Novo and Manaia 2010). Moreover, these estimates only include the culturable fraction of the bacterial population, and might only represent 1 % of the total. Indeed, the numerous unculturable bacteria dwelling in ww and related systems (sludge, biofilms) can host an immense number of ARGs (Rizzo et al. 2013; Szczepanowski et al. 2009). Szczepanowski and coworkers found, in a study performed with ww samples in Germany, 140 different clinically relevant ARGs, encoding resistance to the different classes of antibiotics (aminoglycosides, β-lactams, chloramphenicol, fluoroquinolones, macrolides, rifampicin, tetracycline, trimethoprim, and sulfonamides, as well as efflux pumps) (Rizzo et al. 2013; Szczepanowski et al. 2009). The majority of the studies have focused on the selection and relative prevalence of antibiotic resistant bacteria and ARG transfer in UWTPs irrespective of the biological process, technology, and operating conditions. Only a few studies investigated the effects of the

operating parameters (Kim et al. 2007a, b, c) and different ww treatment technologies (summarized in Rizzo et al. 2013; Munir et al. 2011; Mezrioui and Baleux 1994) on the occurrence and release of ARGs and antibiotic-resistant bacteria.

The *E. coli* strains isolated from the effluent of an aerobic lagoon showed higher antibiotic resistance (35 %) than those isolated from domestic sewage (23 %). In the activated sludge, the percentage of antibiotic resistant strains (resistance to at least one antibiotic) showed seasonal changes in the inflow and outflow ww samples. The increase of the percentage of antibiotic-resistant strains of *E. coli* in the effluent of the aerobic lagoon was probably related to the selection of antibiotic-resistant strains by this treatment (Rizzo et al. 2013). Furthermore, survival experiments comparing *E. coli* strains resistant to seven antibiotics and *E. coli* strains susceptible to 15 tested antibiotics demonstrated that resistant bacteria had higher survival rates than susceptible ones in ww treated in lagoons (Rizzo et al. 2013).

Advanced treatments aim at improving the quality of the secondary effluent of ww treatment plants before disposal or reuse. Sand filtration, adsorption membranes, and advanced oxidation processes are among the most applied and studied advanced treatment technologies. In contrast to a myriad of studies available on the effect of advanced processes on bacteria inactivation, only very few studies exist regarding the effect on antibiotic resistance (summarized in Rizzo et al. 2013). Öncü and colleagues compared ozonation and TiO_2 heterogeneous photocatalysis with conventional chlorination in terms of effects on DNA structure and integrity (Öncü et al. 2011). In contrast to chlorine, which did not affect plasmid DNA structure at the studied doses, ozone and photocatalytic treatment resulted in conformational changes and the damage increased with increasing oxidant doses (Rizzo et al. 2013; Öncü et al. 2011). This finding is of particular interest taking into consideration that most of the ARGs are encoded on plasmids and the most applied disinfection process in ww treatment is chlorination, but ultraviolet (UV) radiation also finds extended applications.

In a recent study, the inactivation of tetracycline-resistant *E. coli* and antibiotic-sensitive *E. coli* by UV irradiation was investigated to assess their tolerance to UV light (Huang et al. 2013). The authors did not find any difference in the inactivation of tetracycline-resistant and antibiotic-sensitive *E. coli* after disinfection treatment. The general lack of data concerning the effect of UV-dependent DNA damage on antibiotic resistance makes this topic worthy of investigation (Rizzo et al. 2013). Iwane and colleagues found out that chlorination treatment did not significantly affect the percentage of resistance in *E. coli*, randomly isolated from ww samples, to one or more antibiotics (from 14.7 to 14.0 %) or specifically to ampicillin (constant at 7.3 %) and tetracycline (from 8.0 to 6.7 %) (Rizzo et al. 2013; Iwane et al. 2001). Munir and coworkers investigated the effect of five different UWTPs located in Michigan, USA on the occurrence and release of ARGs and antibiotic-resistant bacteria into the environment. They observed that disinfection by chlorination and UV radiation processes did not significantly reduce ARGs and antibiotic-resistant bacteria (Rizzo et al. 2013; Munir et al. 2011). In summary, in light of the available data, the effect of chlorine on bacterial DNA may be achieved only for high disinfectant dose compared to those typically used in ww disinfection (Rizzo et al. 2013; Dodd 2012).

7.3.2.3 Other Water Environments

Zhang and coworkers recently published an excellent review on antibiotic resistance in water environments (Zhang et al. 2009). As a result of extensive use of human and veterinary antibiotics, hospital ww and livestock manure are considered as the major sources of environmental ARGs. ARGs can enter into aquatic environments by the direct discharging of untreated ww or into sewage treatment plants through ww collection systems and subsequently into the environments with effluents and discharged sludge (Zhang et al. 2009; Auerbach et al. 2007). ARGs are transferred into soils by amending farm land with animal manure and processed biosludge from sewage treatment plants and subsequently can leach to groundwater or be carried by runoff and erosion to surface waters (Yang and Carlson 2003). Surface water and shallow groundwater are commonly used as sources of drinking water; thus, ARGs can go through drinking water treatment facilities and enter into the water distribution system (Schwarz et al. 2003).

Untreated Sewage

During the past years, various bacterial species isolated from untreated sewage were found to contain a variety of ARGs encoding resistance to aminoglycosides, β-lactam antibiotics, trimethoprim, tetracyclines, and vancomycin (reviewed in Zhang et al. 2009). Sewage receives the bacteria previously exposed to antibiotics from private households and hospitals and is considered as a hotspot for ARGs. ARGs enter sewage treatment plants with sewage water, and most of them cannot be effectively removed with traditional treatment processes before being released into the environment (Zhang et al. 2009; Auerbach et al. 2007; Volkmann et al. 2004). In addition, environmental conditions of activated sludge or biofilms facilitate horizontal transfer of the ARGs from one host to another because of the nutritional richness and high bacterial density and diversity (Zhang et al. 2009; Schlueter et al. 2007; Tennstedt et al. 2003).

Sewage Treatment Plant Activated Sludge and Biofilms

Several previous studies have shown that sewage treatment plants serve as important reservoirs for various ARGs (Zhang et al. 2009; Schlueter et al. 2007; Tennstedt et al. 2003; Smalla and Sobecky 2002). Sewage treatment plants receive the antibiotic-resistant bacteria with the inflow sewage water originating from hospitals, private households, industry, and agriculture. So, they play important roles in recombination, exchange, and spread of environmental ARGs (Zhang et al. 2009; Szczepanowski et al. 2004). Sewage treatment plants are known as important interfaces between different water bodies, such as hospital ww, domestic ww, surface water, and groundwater; therefore, they may facilitate gene exchange and spread between these environments (Zhang et al. 2009; Schlueter et al. 2007). It is also well known that the presence of antibiotics in sewage selects for the maintenance of ARGs conferring resistance in activated sludge (Kümmerer 2003). Many ARGs, such as *vanA*

and *vanB*, are not effectively removed by activated sludge process commonly used in sewage treatment plants, as the genes are being found in both influent and effluent water (Zhang et al. 2009; Caplin et al. 2008; Iversen et al. 2002). ARGs enter into other water bodies with effluent water and can be transferred horizontally to the indigenous bacteria in these water environments (Schwartz et al. 2003).

Natural Water

Different ARGs have been found in bacterial isolates or microbial communities in natural waters which were not or only slightly polluted (Zhang et al. 2009; Mohapatra et al. 2008; Rahman et al. 2008; Jacobs and Chenia 2007). ARGs in surface water and soils can leach to groundwater close to agriculture areas of animal production or aquaculture. Tetracycline resistance genes encoding both ribosomal protection proteins and efflux pumps have been detected in the groundwater as far as 250 m downstream from waste lagoons of swine farms (summarized in Zhang et al. 2009). Besides, in fresh waters, some ARGs conferring resistance to aminoglycosides (Heuer et al. 2002) and chloramphenicol (Dang et al. 2008) have also been detected in marine waters with no evidence for pollution (Zhang et al. 2009).

Sediments

It is evident that ARGs in sediments are acquired from water environments or generated and/or spread due to selection by the antibiotics present in the sediments. Sediments of aquaculture farms are important antibiotic resistance reservoirs where various antimicrobials and ARGs are concentrated (Zhang et al. 2009; Agersø and Petersen 2007; Dalsgaard et al. 2000). Marine sediments were shown to contain many different tetracycline resistance genes (Rahman et al. 2008). Nonaka and colleagues found that the numbers of oxytetracycline-resistant bacteria increased in sediments around a marine aquaculture site after oxytetracycline therapy, the *tet*M resistance gene was detected in different genera of gram-positive and gram-negative bacteria in the sediments of this marine environment (Zhang et al. 2009; Nonaka et al. 2007).

In rivers running through pristine, urban, and agriculturally impacted areas, ARG detection frequency correlated with the degree of pollution by antibiotic compounds (Zhang et al. 2009; Pei et al. 2006; Yang and Carlson 2003).

Drinking Water

Prevalence and resistance patterns of various microbial genera from drinking water distribution systems have been recently reported (Zhang et al. 2009; Ram et al. 2008; Koksal et al. 2007). Multiple antibiotic-resistant *E. coli* strains isolated from drinking water were found to carry ARGs conferring resistance to aminoglycosides, β-lactams, tetracyclines, and trimethoprim-sulfamethoxazole (Alpay-Karaoglu et al. 2007; Cernat et al. 2007), as well as class 1 integrons which are known as ARG shuffling units (summarized in Zhang et al. 2009; Ozgumus et al. 2007).

To investigate possible ARG transfer from ww and surface water to the drinking water distribution network, Schwartz and colleagues and Obst and colleagues analyzed biofilms in hospital and municipal ww, as well as drinking water from river bank filtrate. They found *vanA* and *ampC* conferring resistance to vancomycin and ampicillin resistance, respectively, both in ww and drinking water biofilms (Zhang et al. 2009; Obst et al. 2006; Schwartz et al. 2003).

7.3.3 Soils Impacted by Wastewater Irrigation

Sewage treatment plant effluent and sludge application to agricultural fields are recognized as important sources of ARGs to surface waters and soils and subsequently into groundwater (Rizzo et al. 2013; Yang and Carlson 2003).

Dalkmann and coworkers investigated the effect of ww irrigation on the occurrence of antibiotics or their degradation products as well as on the prevalence of the corresponding ARGs in soils from the Mezquital Valley in Mexico, which have been irrigated with untreated ww for distinct periods of time (Dalkmann et al. 2012). Long-term irrigation of soils with untreated ww led to an accumulation of antibiotics (e.g., sulfamethoxazole) and the regular input of ww increased the concentrations of *sul1* and *sul2* resistance genes in irrigated soils relative to soils under rain-fed agriculture.

7.4 Mechanisms of Spread and Maintenance of ARGs

There exist three major mechanisms of HGT within and among bacterial populations; all three of them contribute significantly to the horizontal dissemination and persistence of ARGs in the environment.

7.4.1 Conjugative Transfer

The conjugative plasmid systems are the largest and most widely distributed subfamily of type IV secretion systems, with systems described for most species of the *Bacteria* and some members of the *Archaea* (Alvarez-Martinez and Christie 2009). The overall process of conjugative DNA transfer can be dissected into three biochemical reactions: DNA substrate processing, substrate recruitment, and translocation (Alvarez-Martinez and Christie 2009; Christie et al. 2005; Schröder and Lanka 2005; Ding et al. 2003; Pansegrau and Lanka 1996). In the DNA processing reaction, DNA transfer and replication (Dtr) proteins initiate processing by binding a cognate origin of transfer (*oriT*) sequence on the conjugative element. The Dtr proteins include a relaxase and accessory factors (for some plasmid systems, such as the broad-host-range plasmid pIP501, no accessory factors have been found

so far (Kurenbach et al. 2006; Kopec et al. 2005)) and when bound to *oriT*, the resulting DNA–protein complex is termed the relaxosome (Alvarez-Martinez and Christie 2009). Accompanying the nicking reaction, the relaxase remains bound to the 5'-end of the transferred plasmid strand (T strand). The bound relaxase, probably together with other relaxosome components, mediates recognition of the DNA substrate by a cognate T4SS. The relaxase guides the T strand through the translocation channel. In the recipient cell, the relaxase catalyzes the re-circularization of the T strand and may also be involved in second strand synthesis or recombination into the chromosome (Alvarez-Martinez and Christie 2009; César et al. 2006; Draper et al. 2005). The self-transmissible plasmids are only one of the two major subgroups of conjugative elements. The second group of conjugative elements, originally denominated "conjugative transposons" and more recently termed Integrating Conjugative Elements (ICEs), is also present in many bacterial and archaeal species (Alvarez-Martinez and Christie 2009; Juhas et al. 2008, 2007; Burrus and Waldor 2004; Burrus et al. 2002). These elements are excised from the chromosome through the action of a recombinase/excisionase complex and followed by the formation of a circular intermediate. Then, the circularized intermediate is processed at *oriT* in the same way as described for conjugative plasmids. In the recipient cell, ICEs reintegrate into the chromosome by homologous recombination or through the action of an integrase encoded by the ICE itself (Alvarez-Martinez and Christie 2009). Conjugative plasmids and ICEs are recruited to the transfer machinery through interactions between the relaxosome and a highly conserved adenosine triphosphatase (ATPase) termed the type IV coupling protein. This protein interacts with the translocation channel, which consists of the mating pair formation proteins (Alvarez-Martinez and Christie 2009; Schröder and Lanka 2005; Christie 2004). In gram-negative bacteria, the mating pair formation proteins build the secretion channel as well as a pilus or other surface filaments to achieve attachment to target cells (Alvarez-Martinez and Christie 2009; Christie and Cascales 2005; Lawley et al. 2003). In gram-positive bacteria, surface adhesins rather than conjugative pili mediate attachment (Alvarez-Martinez and Christie 2009; Grohmann et al. 2003); for the majority of gram-positive bacteria, the origin and nature of the surface adhesins or other surface located factors involved in attachment and/or recognition of the recipient cell have not been elucidated so far.

7.4.2 Transformation

DNA transformation is based on the uptake of free DNA from the environment and, therefore, does not rely on MGEs; it is only encoded by the acceptor bacterium. Natural competence is the developmental state of the bacterium in which it is capable of taking up external DNA and of recombining this DNA into the chromosome, thereby undergoing natural transformation (Seitz and Blokesch 2013). A wide variety of bacterial species can develop natural competence and consequently take up external DNA (for recent reviews, see Chen and Dubnau 2004; Lorenz and Wackernagel 1994). The principal steps to take up the external DNA include:

(i) binding of double stranded (ds) DNA outside the cell to a (pseudo-) pilus structure elaborated by the acceptor cell; (ii) extension and retraction of the pilus, driven by ATP-dependent motor proteins, that mediate the uptake of the ds DNA through the secretin pore spanning the outer membrane of the acceptor cell; (iii) binding of the ds DNA by the DNA-binding protein ComEA, which takes place in the periplasmic space; (iv) transport across the inner membrane by ComEC concomitantly with the degradation of one DNA strand by a so far unidentified nuclease; (v) single stranded (ss) DNA reaches the cytoplasm and is immediately protected against degradation by DNA processing protein A (DprA) and a single strand binding protein; and (vi) DprA recruits RecA, which catalyzes homologous recombination within the genomic DNA of the acceptor cell (Seitz and Blokesch 2013).

7.4.3 Transduction

Transduction is the process in which bacterial DNA gets erroneously packaged into the heads of bacteriophages. When the phage infects another bacterial cell, the packaged DNA is incorporated into the new host's genome (Roberts and Mullany 2010).

Bacteriophages are highly specific to their bacterial hosts, able to infect even after significant periods of hiatus, and reproduce rapidly when their ecosystem allows to. The viral genome is stored encapsulated in the protein "head" until the virion attaches itself to a bacterial host cell for genome insertion (Brabban et al. 2005). This attachment process is highly specific involving the precise recognition of cell surface components, such as proteins and lipopolysaccharide elements, by specialized bacteriophage recognition structures. When the viral genome has been introduced into the host, the lifecycles of the lytic and temperate bacteriophages diverge determined by both the bacteriophage's biology and the cellular environment. Lytic bacteriophages only reproduce via a lytic lifecycle, whereas temperate bacteriophages can either reproduce lytically or enter lysogeny. Therefore, bacteriophages are historically classified based on their lifecycle (lytic vs. temperate), although finer subdivisions are based on their morphological characteristics (tailless vs. tailed), nature of the genome (e.g., DNA vs. RNA, single-stranded vs. double-stranded), and other factors (Brabban et al. 2005). Nowadays, it has become more common to classify bacteriophages at a molecular level through the comparison of specific genes with the well-characterized T-4-like bacteriophages (Tétart et al. 2001).

7.5 Monitoring of Occurrence of Antimicrobial Resistance and Spread

Based upon the knowledge that ARGs are widespread in aquatic and terrestrial environments, there is a need for the development and application of molecular methods to investigate the occurrence, spread, and fate of ARGs in the environment.

So far, the methods used for detection, typing, and characterization of ARGs have covered, but have not been limited to specific and multiplex polymerase chain reaction (PCR), real-time PCR, DNA sequencing, and hybridization-based techniques, including microarray (Zhang et al. 2009).

7.5.1 DNA Hybridization

Molecular hybridization has been used to detect the presence/absence of specific ARGs for more than 30 years (Zhang et al. 2009; Mendez et al. 1980). Many improvements have been made on molecular hybridization, in particular in probe design and synthesis, so that the technique, especially Southern blot, is still often applied to distinguish different ARGs of one group (e.g., *tet* genes) from each other (Levy et al. 1999; Robert and Kenny 1986) or to prove the presence of specific ARGs in certain environments (Zhang et al. 2009; Malik et al. 2008; Agerso and Petersen 2007).

With a number of non-radiolabeled systems commercially available, radioactive labeling of probes is no longer a reasonable option. As an important non-radiolabeled method, fluorescence in situ hybridization (FISH) has been successfully established and implemented for clinical detection of antimicrobial resistance. The application of the FISH technique has been described for the rapid identification of macrolide resistances due to ribosomal mutations (Rüssmann et al. 2001). Werner and coworkers have performed a study to assess the reliability of FISH for clinical detection of linezolid-resistant enterococci. They report that FISH, along with DNA probes containing locked nucleic acids with point mutation, demonstrated 100 % sensitivity for the detection of phenotypic linezolid resistance and even allowed detection of a single mutated 23S rRNA gene allele in phenotypically linezolid-susceptible enterococci (Werner et al. 2007). Although FISH has been often applied for clinical detection of antibiotic resistance, only few reports so far exist about its use in the identification of bacteria harboring ARGs in environmental samples (Zhang et al. 2009).

7.5.2 PCR (Simple and Multiplex PCR)

PCR assays have been widely applied in both pure cultures and environmental samples for the detection of ARGs encoding resistances to aminoglycosides (Mohapatra et al. 2008; Taviani et al. 2008), chloramphenicol (Dang et al. 2008), β-lactams (Taviani et al. 2008), macrolides (Chen et al. 2007; Patterson et al. 2007), sulfonamides (Agerso and Petersen 2007), tetracycline (Jacobs and Chenia 2007), vancomycin (Caplin et al. 2008), and other antibiotics as summarized in Zhang et al. (2009). Environmental target DNA or RNA at low concentrations can be amplified and detected by PCR. However, false-positive results sometimes occur in the PCR assays. These false-positive results can be avoided by application of a second method, namely Southern hybridization of PCR products labeled and used as DNA probes on plasmid or genomic DNA samples from strains putatively harboring antibiotic resistance

target genes (Zhang et al. 2009; Akinbowale et al. 2007; Ahmed et al. 2006). In addition, DNA sequencing is another common method to verify the PCR products of different ARGs (Thompson et al. 2007). To save time and effort, multiplex PCR methods have been developed and often used for simultaneous detection of various environmental ARGs (summarized in Zhang et al. 2009). With various primer pairs in the same PCR system, multiplex PCR can amplify the DNA fragments of several ARGs at the same time (Gilbride et al. 2006). However, the method also has its drawbacks due to compromise conditions applied to simultaneously amplify different ARGs. This can include inhibition of the amplification of some genes and/or generation of false-positive results. Therefore, the cycling and reaction conditions of multiplex PCRs have to be carefully adjusted prior to the application on complex environmental samples. Despite these drawbacks, multiplex PCR is still considered a rapid and convenient method for the detection of multiple ARGs in isolated bacteria or environmental DNA (Zhang et al. 2009; Agersø et al. 2007; Gilbride et al. 2006).

7.5.3 Quantitative PCR

The quantitative real-time PCR (qPCR) is usually used to quantify target DNA on basis of the principle that the initial target gene concentration can be estimated by determining the number of amplification cycles to obtain a PCR product concentration above a certain defined threshold. Among the fluorescent reagents developed for qPCR, SYBR Green is the most common method used for the amplification of ARGs (summarized in Zhang et al. 2009). Recently, the technique has been frequently used to quantify ARGs in environmental samples, including *tet* genes in beef cattle farms (Yu et al. 2005), groundwater (Mackie et al. 2006), river sediments (Pei et al. 2006), sewage treatment plants (Auerbach et al. 2007), *sul* genes in river sediments (Pei et al. 2006), *npt* genes in river water (Zhu 2007) and *qnr* genes in water and soil samples (Dalkmann et al. 2012; Siebe et al., unpublished data).

TaqMan probe has also been applied to quantify *tetO*, *tetW*, and *tetQ* (Smith et al. 2004), *vanA*, *mecA* and *ampC* genes (Volkmann et al. 2004) in ww and *sul* genes in ww-irrigated soils and water samples (Siebe et al., unpublished data; Dalkmann et al. 2012).

qPCR is not only used for the quantitative analysis of the distribution of ARGs in the environment, but also often applied to study the effects of environmental factors or treatment processes on removal of ARGs (Zhang et al. 2009), such as *tet* genes (Auerbach et al. 2007; Mackie et al. 2006), *sul* genes (Pei et al. 2006), and *erm* genes (Chen et al. 2007). Through qPCR, Mackie and coworkers found that the detection frequency of *tetM*, *O*, *Q*, and *W* genes was much higher in wells located closer to and down gradient from swine lagoons than in wells more distant from the lagoons (Mackie et al. 2006). Also by qPCR, Chen and colleagues observed that the abundance of *erm* genes in composted swine manure samples was significantly lower than those in swine manure, indicating that manure storage probably decreases the persistence of environmental ARGs (Zhang et al. 2009; Chen et al. 2007).

7.5.4 DNA Microarray

The DNA microarray technique is a genomic analysis technique with high throughput, high speed, and high dedicacy. For detection of antibiotic resistances, DNA microarrays can provide detailed, clinically relevant information on the isolates by detecting the presence or absence of a large number of ARGs simultaneously in a single assay (Zhang et al. 2009; Gilbride et al. 2006). Microarrays allow detection of antibiotic resistance determinants within several hours and can be used as a time-saving, convenient method supporting conventional resistance detection assays (Antwerpen et al. 2007). Although microarrays have been successfully applied to assess the antibiotic resistances of clinical samples, only few reports exist applying this technique to detect ARGs in environmental samples (Zhang et al. 2009). The first factor hampering its application in environmental samples is the low detection limit of the method, but microarray coupled with PCR can enhance the detection limit for environmental ARGs (Gilbride et al. 2006). Patterson and coworkers designed a microarray system based on PCR amplification of 23 different *tet* genes and ten different *erm* genes to screen environmental samples for the presence of these ARGs (Patterson et al. 2007) and found that *tetW*, *O*, and *Q* were the most abundant ARGs found in swine fecal samples, and *ermV* and *ermE* were the most frequent ones detected in farm and garden soil samples (Zhang et al. 2009; Patterson et al. 2007). Another reason for the poor application of microarray in most environmental samples is the complexity of the samples and the required pretreatment. The presence of contaminants, such as humic substances and humic acids in environmental samples, inhibits DNA extraction and/or target gene amplification, therefore, a complicated pretreatment of environmental samples is necessary and crucial to get satisfactory detection results (Zhang et al. 2009; Call 2005). However, the microarray technique can provide a detailed description of bacterial antibiotic resistance and can reveal global changes in the expression of ARGs in response to environmental changes (Gilbride et al. 2006; Call et al. 2003). The information on gene expression levels can provide insights into the mechanisms of antibiotic resistance and into general responses of ARGs to environmental changes (Zhang et al. 2009).

7.5.5 Biosensors

The development of biosensors and their application for the detection of antimicrobials in environmental samples have made fundamental progress in the past years. Reder-Christ and Bendas recently summarized the applications of biosensors in the field of antibiotic research in an interesting review (Reder-Christ and Bendas 2011). In general, there are two main principles for the recognition of antimicrobials by biosensor systems. The first one comprises the widespread use of immobilized RNA or DNA aptamers as recognition elements (so-called aptasensors) (Rowe et al. 2010; Zhang et al. 2010; de-los-Santos-Alvarez et al. 2009; Kim et al. 2009). Their

sensitivity is comparable to that of antibodies. The second principle of antibacterial recognition for bio-sensing is given by antibody-mediated binding processes. Those immunosensors have been widely used for antibacterial detection (summarized in Reder-Christ and Bendas 2011; Cha et al. 2011; Dong et al. 2009; Giroud et al. 2009; Rebe Raz et al. 2008; Ionescu et al. 2007; Ferguson et al. 2002). It is possible either to immobilize antimicrobial-specific antibodies at the sensor surface to directly detect the binding of the antimicrobial or to invert the assay and detect the binding of antibody-spiked samples onto immobilized antimicrobials in terms of a competitive assay (Reder-Christ and Bendas 2011). In summary, biosensors are comparable to conventional methods with respect to sensitivity and specificity of antimicrobial detection and thus fulfill international regulatory requirements. As biosensors represent fast, simple, and cost-efficient methods that can be used without additional sample preparation, they offer large advantages compared to conventional analytical techniques and will, therefore, hold great promises for a wide application in the near future (Reder-Christ and Bendas 2011).

7.6 Risk Assessment of Antibiotic Resistance Spread

Several reviews with the intention to assess the impact of the occurrence and spread of clinically relevant bacteria and/or ARGs in the environment on human health have been published recently. Most of them deal with ww habitats (Varela and Manaia 2013), ww treatment plants (Rizzo et al. 2013), and aquaculture (Smith 2008). Bacteria in ww habitats play a plethora of different roles; the beneficial ones include their participation in the waste degradation processes (those will not be reviewed here) and the harmful ones with potential impact on human health include the carriage and potential spread of virulence genes and ARGs.

Several chemical contaminants present in the ww (heavy metals, disinfectants and antibiotics) may select for these bacteria and/or their genes (Varela and Manaia 2013). Worldwide studies showed that treated ww can contain antibiotic-resistant bacteria or genes encoding virulence or antimicrobial resistance, demonstrating that treatment processes may fail to eliminate efficiently these bio-pollutants. The contamination of the surrounding environment, such as rivers and lakes receiving ww treatment plant effluents, has also been documented in several studies (summarized in Varela and Manaia 2013). The current state of the art suggests that still only part of the antibiotic resistance and virulence potential in ww is known, as well as only some of the factors that trigger their maintenance and spread in the environment (Varela and Manaia 2013). Although there is much uncertainty concerning the transmission of ARGs or virulence genes from ww bacteria to human commensal and pathogenic bacteria, the current knowledge recommends the application of the precautionary principle regarding the discharge and particularly the reuse of ww. Varela and Manaia recommended going one step further in relation to the current recommendations (APHA 1995; Council Directive 91/271/EEC 1991). They urgently recommended the regular detection and quantification of ARGs or virulence genes, as well as the presence of heavy metals or antimicrobial residues in ww-

impacted areas. Furthermore, the assessment of negative impacts due to long-term exposures to the discharge of treated ww should be a priority (Varela and Manaia 2013). The accumulation of apparently very small concentrations of harmful bacteria, genetic determinants encoding for ARGs or virulence genes or micropollutants may generate measurable and relevant effects after some years as demonstrated by Dalkmann et al. (2012) and Aleem et al. (2003). It is also important to consider that risk assessments carried out in one world region cannot be simply used or transposed to regions with distinct geological and climate conditions, since it cannot be taken for granted that conditions such as temperature, precipitation, insolation, or properties of the soil will not interfere with the accumulation of potential hazardous pollutants discharged by ww treatment plants (Varela and Manaia 2013).

Recently Rizzo and colleagues published a comprehensive review on UWTPs as hotspots for antibiotic-resistant bacteria and ARG spread into the environment (Rizzo et al. 2013). They concluded that in spite of intense efforts made over the past years to find solutions to control antibiotic resistance spread in the environment, there are still important gaps to fill in. In particular, it is important to: (i) improve risk assessment studies to allow accurate estimates about the maximum abundance of antibiotic resistant bacteria in UWTP effluents that would not pose risks for human and environmental health and (ii) elucidate the factors and mechanisms that drive maintenance and selection of antibiotic resistance in ww habitats (Rizzo et al. 2013). The final objective should be to implement ww treatment technologies that are able to assure the production of UWTP effluents with an acceptable level of antibiotic resistant bacteria (Rizzo et al. 2013). In the opinion of Rizzo and colleagues, one of the most important questions to address to advance towards ww treatment plants generating effluents with an acceptable level of bio-pollutants would be the setup of a public database with information on ww habitats such as: (i) antibiotic resistant bacteria and their phylogenetic lineages; (ii) ARG and respective nucleotide sequences and genetic environment as well as (iii) sampled sites and their major characteristics. Such a public database would represent a valuable tool to a better understanding of antibiotic resistance ecology and control measures (Rizzo et al. 2013).

Recently, Smith published an interesting review on antimicrobial resistance in aquaculture. Appropriate antimicrobial therapy represents one of the most effective management responses to emergencies associated with infectious disease epizootics. The use of these agents, however, has the potential to increase the frequencies of bacterial resistance and this would result in a negative impact on the subsequent use of these antimicrobials to control infectious disease in aquaculture. It is also possible that the enrichment of resistant bacteria or ARGs could negatively influence the use of antimicrobials to control diseases in humans and other land-based animals (Smith 2008). Attempts to apply formal risk analysis to this problem have failed due to the extreme diversity of aquaculture and the general shortage of relevant data. Smith argued that not only do we lack relevant data to perform this exercise but we also lack validated methods to collect those data in the first place (Smith 2008). Due to the lack of any significant risk assessment, current attempts at risk management are focused on the development of lists of critically important antimicrobials for the

various users of these agents. Smith argued that studies of gene ecology and models of gene flow in the environment are urgently needed if we should be able to evaluate this risk management approach, to predict its consequences or to generate more appropriate strategies (Smith 2008).

The two most valuable outcomes that can be expected from any risk assessment are the definition of rational, evidence-based risk mitigation strategies and the identification of the future requirements for additional research (Smith 2008). A risk assessment should enable the identification of key areas where intervention could minimize the risk. The identification of these key areas would consequently allow the development of effective risk mitigation strategies. To the extent that risk analysis can provide some estimate on the size or significance of a risk, it will also provide us the basis for a cost–benefit analysis of any intervention (Smith 2008). Smith concluded that we urgently need to develop evidence-based management strategies that will enable us to minimize the impact of bacterial resistance, selected by the aquacultural use of antimicrobials, both on the control of diseases encountered in aquaculture itself and in those encountered in humans and land-based agriculture (Smith 2008).

7.7 Conclusions and Perspectives

Bacteria resistant to antimicrobials are widespread. Humans, animals, and environmental habitats are all reservoirs where bacterial communities live that contain bacteria that are susceptible to antimicrobials and others that are resistant (Acar and Moulin 2006). Farm ecosystems offer a particular environment in which resistant bacteria and ARGs can emerge, amplify, and spread. Dissemination can occur via the food chain and via several other pathways, such as sewage and manuring of agricultural fields. Ecological, epidemiological, molecular, and mathematical approaches are currently used to study the origin and expansion of the antimicrobial resistance problem and its relationship to antibiotic usage (Acar and Moulin 2006). Prudent and responsible use of antibiotics is an essential part of an ethical approach to improving animal health, food safety, and consequently human health (Acar and Moulin 2006). The responsible use of antibiotics during research is vital, but to fully contribute to the containment of antimicrobial resistance, prudent and responsible use must also be part of good management practices at all levels of farm life (land-based and aquaculture) and human antibiotic therapy.

ARGs can flow among different biological units of different hierarchical levels, such as integrons, transposons, plasmids, clones, species, or genetic exchange communities (Baquero 2012). Baquero argued that metagenomics would be the best-suited tool to explore the presence of ARGs in all these biological and evolutionary units, and to identify possible “high risk associations.” He is in favor of a multilayered metagenomic epidemiology approach which can help to understand and eventually predict and apply intervention strategies aiming to limit antibiotic resistance (Baquero 2012).

Another valuable approach would be the more frequent application of biosensors particularly destined to detect and quantify antibiotics and their degradation products in environmental samples (summarized in Reder-Christ and Bendas 2011).

The combination of both, sensitive and quantitative detection of antibiotic resistance determinants as well as of the corresponding antibiotics, would present a valuable innovative approach whose data could feed the modeling approaches that are urgently required to predict the spread of ARGs in certain habitats sufficiently well in advance to act and implement countermeasures.

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