Sanitation, Water Quality and Antibiotic Resistance Dissemination

M. Quintela-Baluja[1], **C.W. Chan**[2], **M. Ezzat Alnakip**[3], **M. Abouelnaga**[4] and **David W. Graham**[1]

[1] School of Civil Engineering & Geosciences. Newcastle University, Newcastle upon Tyne, UK
[2] Sir Joseph Swan Centre for Energy Research, Newcastle University, Newcastle upon Tyne, UK
[3] Department of Food Control, Faculty of Veterinary Medicine, Zagazig University, Egypt
[4] Department of Analytical Chemistry, Nutrition and Bromatology. University of Santiago de Compostela, Spain

Acquired antibiotic resistance (AR) is an evolutionary response in bacteria due to increased use of antibiotics in medicine and agriculture, which has become a serious worldwide public health concern with AR genes (ARGs) now viewed by some as emerging pollutants (Pruden et al.,2006). Consequently, the World Health Organization (WHO) has appealed for urgent concerted actions by governments, health professionals, industry, and civil society to (i) slow the spread of drug resistance; (ii) mitigate the potential fallout from AR outbreaks, and; (iii) preserve medical advances for future generations (WHO, 2014). The magnitude of the problem, which is estimated to cost at least 50,000 lives per year in Europe and US alone, has led to the implementation of antimicrobial resistance (AMR) surveillance systems in many countries at regional, national and supranational levels (ECDC/EMEA, 2009). However, monitoring AR bacteria (ARB) in health care systems does not wholly explain the spread of AR on global scales because they often do consider other sources, such as wider routes of spread, relationships among pathogenic and commensal microorganisms, and how organisms evolve under different environmental conditions. Specifically, researchers have primarily focused on identifying and understanding important AR mechanisms that directly affect clinical settings, which are functionally among the main location of critical AR detection. However, some recently researchers broadened their focus to include the wider environment as a source of ARGs and also as a site of AR evolution[4]. Within this context, although research is increasing, the role of municipal wastewater has received relatively limited attention as a reservoir of AR, even though several studies have shown that wastewater and animal waste can contain huge numbers of ARBs and ARGs, which can pass through wastewater treatment plants and reach the receiving water bodies. In reality, wastewater releases are almost certainly a major link between human gut and environmental strains, influencing the distribution and abundance of ARGs across aquatic compartments and promoting horizontal gene transfer (HGT) among exposed microbial strains. Nowadays, ARB can be found in almost any environment, leading to an increase in the natural resistance background levels and thus enhance the likelihood of ARGs being transferred back to human and animal commensals or even pathogens through water and food. HGT does not "respect" phylogenetic, geographical or ecological borders, therefore the use of antibiotics in one area, such as households can impact the broader resistome, such as human and veterinary medicine or the environment. Inadequate water sanitation allows the dissemination of bacterial pathogens, both resistant and non-resistant into the environment, where they may interact with indigenous microbial communities changing their species and genetic composition. While in developed countries, effective wastewater and water treatment infrastructures have produced a false sense of security among decision-makers with respect to waterborne pathogen dissemination; water quality has in contrast, declined in most emerging countries where increasing personal wealth permits greater purchase and use of antibiotics in health care and other sectors. Furthermore, waste management in emerging countries has not kept up with economic growth; i.e., contaminated water in such locations may dominate AR dissemination, contributing to and expanding AR mechanisms and pathways and making local AR into a global problem.

Keywords: Antibiotic resistance; Horizontal Gene Transfer; Water Sanitation

1. Introduction

Water (H_2O) is vital for all known life forms and the efficient function of ecosystems, communities and economies. This chemical compound was essential to agricultural development and the transformation of humans from a nomadic to a more stationary lifestyle, which resulted in the flourishing of large metropolises along the main rivers of the world. For example, the first civilization to appear in recorded history started on a river valley, Mesopotamia - the so-called cradle of civilization, situated between the major rivers (Tigris and Euphrates), and the ancient society of the Egyptians depended entirely upon the Nile. Over time, ancient civilizations learned to move water into their metropolises and the Romans constructed public water supply systems with aqueducts and pipes that supplied homes, public wells and fountains for public use: achievements not remotely matched until improved water engineering and sanitation in late 19th century Europe and North America.

The ability to move water on a mass scale allowed cities to grow to unimaginable size, but the earliest sewers of these cities carried street runoff away to inhabited areas and into surface waterways without treatment, leading to terrible over-polluted streets, which acted as a constant source of outbreaks of diseases. Higher population densities required progressively more complex sewer collection and conveyance systems to maintain sanitary conditions in crowded cities. Therefore, in the late 19th and early 20th centuries, many cities constructed underground sewer systems to help contain the wastewater and potential outbreaks of disease, such as typhoid and cholera. Initially, these systems discharged wastewater directly to surface waters without treatment, but they become a water pollution concern suspected to be linked to disease. However, as evidence linking wastes and health strengthened, water treatment and

especially wastewater treatment became essential because of the massive amount of wastewater being produced in growing cities. Thus, a new process to treat wastewater using air and biological floc composed of bacteria and protozoa was noted in 1912 in Manchester (United Kingdom), becoming a well-known biological wastewater treatment process (coined as activated sludge) and can be considered the most significant improvement in public health and the environment during the course of the century. Initially, activated sludge treatment was thought to be a chemical process, but as microbial detection methods improved, it became apparent that microorganisms were key to treatment rather chemical reactions. As such, biological waste treatment evolved, principally by harnessing metabolic activities of bacteria and protozoa to remove organic pollutants and pathogens, and together with improved drinking water treatment, has saved countless lives. In fact, it has been estimated that by breaking the cycle of waterborne diseases, more lives have been saved than by all medical interventions put together.

The discovery of the penicillin in 1928 by Alexander Fleming started the pharmaceutical golden era where the mass production and distribution in 1945 revolutionized the treatment of infectious bacterial diseases, curing illnesses such as tuberculosis, diphtheria and pneumonia for the first time in the human history. Although antibiotics have played a critical role in protecting the public health and are responsible for saving millions of human lives; anthropogenic overuse and misuse of antibiotics for medical treatment, veterinary practices, agriculture and aquaculture in the last six decades has produced the continuous release of antibiotics and antibiotic resistance bacteria (ARB) into the environment. Subsequently, the selection, the increase, and the spread of antimicrobial resistance (AMR), are compromising the effectiveness of antimicrobial therapy. The magnitude of the problem claims at least 50,000 lives each year across Europe and US alone, with many hundreds of thousands more dying in other areas of the world. However, the continued world rise in resistance by 2050 could lead to 10 million people dying every year and thus the reduction of 2% to 3.5% in Gross Domestic Product (GDP)[5]. Consequently, AMR surveillance systems are being implemented in many countries at regional, national and supranational levels[6]. The first warning came from World Health Organization (WHO) report "Antimicrobial resistance: global report on surveillance 2014" (WHO, 2014) that stated the world may be entering a "post-antibiotic" era in which even the most powerful antibiotics are becoming ineffective against infections that could have been treated easily in the past.

In the last 20 years, researchers have been focused on identifying and understanding the most important antibiotic resistance (AR) mechanisms affecting clinical settings, believed to be the main sites responsible for the spread of AMR, but studies have failed to wholly explain the development and the spread of AR on global scales. Such studies rarely included other sources, such as (i) wider environmental routes for spread (e.g., water), (ii) relationships amongst microorganisms, and (iii) organisms responses to different environmental conditions. Recently, researchers have broadened their focus to include the environmental microbiota as a source of resistance genes (ARGs) and as the site of antibiotic-resistance evolution[4]. Yet, inadequate local sanitation and poor water quality on global scales has still received limited attention as a major source of AMR, even though biological wastewater treatment processes have been identified as possible hotspots for ARB and ARGs[7]. They allow resistant organisms and genes to stray from humans to the environment and back, thus contributing to AMR persistence and spread.

2. Antibiotic Resistance

Antibiotics represent one of the most effective therapeutic categories used in the treatment of infectious diseases caused by bacteria, but successful treatment can be compromised by the development of tolerance or resistance in bacterial targets. Antibiotics and/or genes that confer resistance to bacteria is an outcome of Darwinian selection in the microbial world; the acquired ability of microbes to resist the effect of drugs is a natural phenomenon. However, accelerated resistance evolution has occurred in recent years by increased selective pressure exerted by the widespread use of antibacterial drugs in medicine, agriculture and aquaculture. When a drug is used on bacteria communities, non-resistant strains are impaired or die and do not produce offspring, whereas those that survive can pass on resistance traits to their next generation. Although bacteria can be naturally resistant to some types of antibiotics, they also can become newly resistant in two ways (Figure 1):

a) Chromosomal mutation

Genetic mutations are spontaneous changes in the genetic material of the bacteria, which yield different genotypes of resistance. Some mutations enable the bacteria to produce potent chemicals (e.g., enzymes) that inactivate antibiotics, whereas other mutations later the intracellular target on which the antibiotic attacks. Still other mutations can lead to blockage of entry ports that allow antibiotics into the cell, and others manufacture pumping mechanisms that export antibiotics outside the cell so it never reaches its target.

b) Acquiring resistance by mobile genetic elements (MGE)

Mobile genetic elements (MGEs) are segments of DNA that more readily move around genomes, traditionally being classified as (i) bacteriophages, which are viruses that infect and replicate within bacteria; (ii) plasmids, which are small, circular DNA molecules within a cell physically separated from chromosomal DNA and replicate independently, or (iii) transposons, which are DNA sequences that can change their position within the genome. Therefore, bacteria can directly and radpily acquire ARGs from other bacteria mediated by MGEs uncoupled from traditional replication, including: (i) simple mating called "conjugation", where bacteria can transfer genetic material including genes encoding

resistance to antibiotics by mobile elements (plasmids and transposons) during cell to cell contact; (ii) "transformation" by naked DNA, in the case of naturally competent state of some bacteria; and (iii) "transduction" by bacteriophages, where the resistance traits from one bacterium are packaged in the head portion of an infecting virus. The virus then injects the resistance traits in any new bacteria it attacks.

The most common method of antibiotic resistance transfer is believed to be plasmid conjugation[8]. However, it is possible to differentiate between narrow-host-range plasmids (NHR; i.e., "specialists") that cannot move to and replicate in all strains; and broad-host-range (BHR; "generalists" that can transfer and replicate in many different and distantly related bacterial species. Dissemination of antibiotic resistance plasmids in different environments depends on the survival of these plasmids in the environments they are released into, that is, plasmids have to remain functional in order to be transferred into recipient bacteria. Furthermore, antibiotic resistance plasmids can be carrying other accessory genes such as heavy metal resistance genes [9,10]. These accessory genes can fix the plasmids with non-antibiotic resistance selection [10–12].

Genetically, AR spreads through bacteria populations both "vertically," when new generations inherit ARGs, and "horizontally," when bacteria share or exchange sections of genetic material with other bacteria without an absolute requirement for cell division. The extent of such "horizontal gene transfer" (HGT) is substantial and continuous, where up 25% of some bacterial genomes are believed to be derived from HGT over evolutionary periods of time [8].

Nowadays, ARB can be found in almost any environment and HGT mechanisms does not respect phylogenetic, geographical or ecological borders; i.e., the use of antibiotics on one setting, such as a household, can impact the prevalence of ARGs and the resistome in other areas, such as across human medicine or the environment. Obviously, AR traits also can be lost by HGT (plasmid curing), but evidence suggests the reverse process occurs more slowly and requires the removal of the selective pressure for an extended time to prevail. The Antibiotic Resistance Database (ARDB, //ardb.cbcb.umd.edu/) indicated that 13,000 ARGs have been identified in more than 600 genomes of ARB[13]. However, antibiotic resistance determinants found in potential pathogens comprised only a small portion of the total ARGs surveyed[14], which implies that the major reservoir for ARGs is in non-pathogenic environmental bacteria. The main problem is that clinical researchers have only been interested in characterizing the function of individual antibiotic resistance mechanism responsible for nosocomial infections, leaving aside the primary MGEs responsible for facilitating ARGs transfer between different bacteria. The latter would be able to better explain the AR dynamics in the environment and how host range MGEs containing ARGs are disseminated from animal husbandry, aquaculture, and WWTPs via the water cycle and food chain into clinically relevant bacteria[14] (Figure 2). There is some evidence of the transfer of resistance elements to known human commensal bacteria and pathogens[15,16], and gene transfer in the human intestinal microbiome is extensive [16,17]. However, unlike clinic data, there are almost no epidemiological data of AR in the environment, especially geographically-based, making it hard to predict the spread and emergence of new ARs from environmental sources.

3. Identification of ARB and ARGs in Water Sanitation Systems

Identification of ARBs or ARGs in water sanitation systems is vital, specially to understand: (i) how AR arises, (ii) how resistant strains and resistant genes spread in nature, and (iii) the relative significance of sanitation and water quality for human and environmental health. The majority of studies are focused in evaluating the presence of specific ARGs and clinically relevant pathogenic and commensal strains can be found in WWTPs. Although such information is conditionally useful, the real problem is the presence and exchange of MGEs among environmental bacteria and pathogens, which is what we detect and define as the rapid dissemination of AR[18]; i.e., increasing genetic vectors for ARGs suggests increased HGT to and from the environment.

Detection of AR elements in WWTPs can be addressed by culture-dependent or culture-independent (molecular based) methods[19], each of them exhibiting specific advantages and drawbacks. While molecular-based methods are highly efficient for ARG detection, such methods do not replace phenotypic analysis of AR isolates because the presence of an ARG does not mean it is being expressed. Thus, a combination of culture-dependent and culture-independent methods should be applied for comprehensive evaluation of AR in WWTPs and other environments. Culture-dependent methods are currently used in clinical laboratories to determine resistance profiles as well as other physiological, genetic and biochemical characteristics to assist clinicians in a bacterial infection therapy. The use of selective growth media that enrich for particular groups of bacteria allows the isolation of specific resistant bacterial taxa. After purification, isolates are subjected to a wide range array of biochemical assays to characterize specific resistance phenotypes, allowing the calculation of resistance rates or the definition of resistance profiles. AR testing is frequently based on the disc diffusion or micro-dilution methods, which, according to standardized values, allows the distinction between resistant and susceptible organism (e.g CLSI, 2013; EUCAST, 2013; EUCAST, 2012). However, such methods for resistance determination in environmental samples are not standardized, i.e. the data on AR studies have used a range of media types, antibiotic concentrations, and incubation periods making it difficult to compare results. Furthermore, the limitations of isolation-based methods to detect uncultivable bacteria have been circumvented by a myriad of culture-independent methodologies that target nucleic acids extracted directly from natural environments. Such culture-independent approaches provide information on ARG prevalence, but often are completely

unrelated to clinical isolates of health and environmental importance. Common molecular methods for detection, typing, and characterization of ARGs are: (i) polymerase chain reaction (PCR), (ii) quantitative PCR (qPCR), (iii) DNA sequencing, and (iv) hybridization-based techniques, including microarray. Recently, new molecular approaches are used: (a) reverse transcription PCR coupled to transcriptomic analysis which enables to study which genes are expressed across the entire community; and (b) high throughput DNA sequencing technologies which enable deciphering the resistome in environmental compartments. However, all of the methods have limitations and the only way of truly "diagnosing" resistance, and resistance transmission and dissemination is by combining methods with an understanding of the value of each.

4. Effect of Water Sanitation and Antibiotic Trade in the Increase of Antibiotic Resistance

Safe drinking water, sanitation and good hygiene are fundamental to health, survival, growth and human development. Therefore, safe drinking water and basic sanitation is one of the eight Millennium Development Goals (MDGs) that were selected to the "International Decade for Action, Water for Life, 2005-2015" by the United Nations to challenge the global community to reduce by half the proportion of the population without access to both (WHO, 2005). In fact, the WHO/UNICEF recently published the Joint Monitoring Program (JPI) report on water supply and sanitation (WHO & UNICEF, 2014, 2015) and stated safe drinking water targets were generally met by 2010; i.e., 90% of the world's population has access to improved sources of drinking water. However, they indicated access to basic sanitation facilities was severely lagging behind with about 36% of the world's population not having access to improved sanitation facilities*. For example, in 2015, 610 million people still rely on open defecation in Southern Asia (decreased from 771 million in 1990), and in sub-Saharan Africa, 229 million people have no sanitation options (increased from 181 million in 1990). Unfortunately, regional overpopulation, increased urbanisation and uncontrolled pollution in these locations is causing a broad decline in water quality, which is most profound in countries with emerging economies. In these cases, waste management is not keeping up with economic growth with improved sanitation systems continuously lagging behind due to chronic under-investment.

The growing demand for water sanitation in developing countries has not yet become part of the priorities of the national governments, as evident by the municipal wastewater dataset (http://www.fao.org/nr/water/aquastat/main/index.stm) from the Food and Agriculture Organization of the United Nations (FAO). For example, only 50 countries have data on their municipal wastewater treatment in the last 10 years, of which only 24 countries treat > 60% of their municipal wastewater (Table 1). This is especially alarming relative to AMR because, although improved sanitation does not specifically remove AR strains and ARGs, having such some intervention can reduce the spread of both sensitive and resistant strains[25]. Therefore, the improvement in management of faecal waste (decrease open defecation, use of central sewers, increase the amount of treated wastewater, etc.) will prevent the transmission of bacteria, especially faecal, by the faecal-oral route, thus preventing their contamination into the food chain and the water cycle (Figure 2).

Furthermore, many pharmaceutical industry companies have relocated to emerging countries from western countries to ensure competitive economic advantage. For example, the Indian pharmaceutical industry supplies 20% of the total finished generics to the world and China exports > 60% of the total active pharmaceutical ingredients for the global pharmaceutical industry [26]. Recent investigations have revealed that certain Indian and Chinese production units are contributing to environmental pollution levels far above those previously reported [27]. Further, poorly controlled antibiotic consumption has being increasing in many emerging countries; e.g., increased use in China was between 1.5-2.5% and in India was between 2.5-6% per year between 2000-2010 [28]. This is especially worrying because, at consumption rates, greater amounts of antibiotics are released in faeces into municipal wastewater due to reduced absorption [29–34] and incomplete metabolism (e.g., fluoroquinolones) by humans and animals [35–37]. Additionally, arbitrary disposal of unused antibiotics, which occurs when they are overly available, can impacts exposed environmental microbial communities and aquatic ecosystem function, potentially serving as a new selective driver of AR evolution[14]. Simultaneously, (i) wastewater contains AR with other pollutants and, together, exert an additional selective pressure, facilitating the survival of ARB and-or acquisition of ARGs by HGT [38–40]; (ii) wastewater bio-products such as waste sludge are used to irrigate and fertilize crops [41,42]; (iii) wastewater may also be used to water plants in parks[43]; and (iv) pre-treated wastewater is used in fed aquaculture systems for wastewater treatment, reuse and resource recovery [44,45]. These factors together lead to almost unlimited spread of AR, where some studies suggested that the spread of ARB in natural fresh water systems can reach drinking water supplies and thus enter to human food chain[46].

Untreated wastewater produces the discharge of ARGs into the natural environment where they can persist even if the antibiotic selecting pressure disappeared [47,48]. Additionally, the presence of extraneous antibiotics can potentially affect in situ AR evolution within environmental microbial communities[49]. Although a few studies have documented positive relationships between antibiotic and the presence/persistence of AR in the environment [50,51], it is unclear whether antibiotic concentration at ppb (parts per billion) levels can reach effective threshold concentration towards proliferating AR in the environment [52,53,54,55]. In addition, the traditional selective pressure theory about antibiotics for

the proliferation of AR in the microbial community might not be appropriate in environmental conditions since most of the ppb level of antibiotics cannot efficiently inhibit the antibiotic sensitive microorganisms. It is therefore possible that there is an alternative mechanism such as HGT for the dissemination of AR traits in environments with such low levels of antimicrobial compounds[52].

5. Wastewater Treatment Plant as Redistributor of Antibiotic Resistance

The principal objective of wastewater treatment is to allow the save disposal of human and industrial wastes without danger to human health or causing unacceptable damage to the natural environment. This objective is achieved by reducing conventional pollutants, including suspended solids, nutrients (nitrogen and sometimes phosphorous), organic matter, and to some extent, also pathogens. Conventional wastewater treatment consists of preliminary, primary, secondary and sometimes tertiary stages, with different biological and physiological processes available for each stage of treatment. Preliminary treatment removes coarse solids and other damaging large materials to enhance the operation and reduce the maintenance needed for subsequent treatment units. Subsequently, primary treatment removes settleable organic and inorganic solids by sedimentation, and the removal of materials that float (scum) by skimming. This step is performed entirely mechanically by means of filtration and sedimentation and is common to almost all the WWTPs. Thereafter, the secondary treatment usually relies on a biological process to remove biodegradable dissolved and colloidal organic matter and/or nutrients with aerobic and-or anaerobic systems. Activated sludge is the most common method for secondary treatment, but membrane bioreactor (MBR), moving bed biofilm reactor (MBBR), or fixed-bed bioreactors (FBR) are also used. Finally, tertiary treatment can be employed beyond the secondary when specific wastewater constituents cannot be removed (e.g. phosphorous, residual nitrogen, additional suspended solids, heavy metals, dissolved solids and refractory organics), but such technologies can be very expensive, requiring higher level technologies, well-trained operators, ready and steady energy supplies, additional chemicals, and very specific equipment. Disinfection, such as chlorination or ultraviolet radiation, can be the final step before the discharge of the effluent, although this is employed in only some parts of world.

The diversity of microbial communities within WWTPs can be large depending on the bio-chemical ingredients received by the WWTPs [56,57], being represented gram positive and negative bacteria, including some environmental opportunistic and clinically significant bacterial pathogens. Moreover, the microbial community distribution differs among different wastewater treatments [56], and generally speaking, the majority of bacteria leaving the WWTP are associated with the biosolids that are not part of the liquid effluent stream [58–60]. However, there is considerable debate about influence of treatment on ARB or ARG concentrations and release to the environment; the total bacteria (including ARB) are almost always reduced, but some studies have shown treatment can increase levels of specific ARB/ARG in effluents [61–63]. Whatever the case, the amount of ARB/ARGs discharged in the final effluent would still appear to be too large, given that only the culturable fraction of the bacterial community is being considered (i.e., it only represents ~1% of the total population).

Among options, AS and biofilm reactors in WWTPs are believed to be most important as hotspots for ARG transmission because they combine two key factors: (i) recurrent exposure of contaminants in the wastewater that create stress and potentially select for MGEs, including conjugative transposable elements (transposons and insertion sequences) and integrative conjugative elements or integrons [12,64–66]; and (ii) very high microbial densities and diversity that promote cell-to-cell contact and HGT (Dionisio et al., 2002; Sorensen et al., 2005; Haines et al., 2007; Zhang et al., 2009). Therefore, the placement of AS sludges onto agricultural soils or using pretreated or raw wastewater to feed aquaculture systems almost certainly both ARGs and ARBs to such environments, and can have detrimental consequences if they are exposed to and mate with pathogens.

Recent studies have focused on WWTPs as an interface between human activities and environmental systems, where WWTPs facilitates gene spread to different water bodies. However, the fact that enteric pathogenic bacteria cannot survive for prolonged periods in the environment has led researchers to be more concerned on how WWTPs provide an opportunity for MGEs to mix among environmental, human, and/or animal related bacteria [67]. This mixing can accelerate gene recombination and transfer of prospective AMR mechanisms and pathways[10]. In fact, this new scenario may be behind the rapid spread of many types of AR, where clinical pathogens contact clinical opportunistic pathogens, such as *Pseudomonas aeruginosa, Acinetobacter baumannii, Stenotrophomonas maltophilia* and environmental microbes [39,67], including emergent multiple-resistance pathogens[39,67,68]. Broad-host range (BHR) plasmid-encoded ARGs or other accessories genes with clinical significance have been detected in the final effluent from WWTPs around the world [10,68–72], pointing to the ineffectiveness of the wastewater treatment to remove plasmids, whereby implying that there is possible dissemination of BHR plasmid-encoding ARG into the environment. Dissemination of BHR plasmids results in their presence in various solids and aquatic environments [68,73,74], which account for the dispersion of ARs among different environment that eventually re-contaminate humans and animals via drinking water or food. Therefore, human actions are accidentally behind MGEs in feedback loops, which broadly increase the risk of bad clinical outcomes and increases in opportunistic infections.

6. Concluding Remarks

Large-scale chemical production that commenced in the early Industrial Revolution has played a key role in increasing the world's population and our collective quality of life. However, this boom also has produced greater wastes, including toxic wastes, heavy metals, disinfectants, biocides, pharmaceutical compounds and residues of manufacturing processes. Only relatively recently have researchers begun to understand the true effect of such increased consumption, including antibiotics and antimicrobials, and how inadequate waste treatment worldwide can and has produced substantive environmental biochemical pollution. With the importance of antibiotics to treating human and animal infectious diseases, and stagnation in new antibiotic development, society has underestimated until recently how growing population and mass manufacturing have accelerated evolution as a microbial adaptation. As a consequence, AMR is now causing an enormous clinical and financial burden on health systems, while the world is heading towards a post-antibiotic era.

In recent years, AMR surveillance has been implemented in healthcare systems and policies to regulate, restrict and/or prohibit the use of antibiotics are improving, but mostly in developed countries. Unfortunately, such monitoring in healthcare systems does not wholly explain the spread of AR on global scales because other sources or pathways have not been considered, such relationships among environmental and clinical microorganisms, and microbial responses to changing environmental conditions. The spread of ARB and its contamination of the environment, mainly via because inadequate sanitation and locally poor water quality, is a serious public health problem since selection of ARB (following exposure to antibiotic residues) and HGT of ARGs from ARB (from human's gastrointestinal tract), represent routes by which AR can be transmitted. Consequently, our fight against AMR requires a comprehensive, sustained, and coordinated response at the national, sub-regional and international levels, but without forgetting that the lack to antibiotics access still kills more people than ARB. Therefore, humanity's reliance on antibiotics, although decreasing, will remain high in developed countries, but in developing and emerging countries will increase. Therefore, a more comprehensive understanding of the molecular, evolutionary and ecological mechanism associated with the acquisition and spread of AR is urgently required to develop Hazards Analysis and Critical Control Points (HACCP) policies. This will allow for the implementation of effective management strategies, mainly seeking to impose barriers against the dissemination of resistance across well-established resistance reservoirs. Unfortunately, the control of the emergence and dissemination of ARB and ARGs cannot replace the need to seek new therapeutic drugs, but it the rate of microbial evolution can be slowed, it will buy time and incentivise the discovery of new antibiotics that are needed for the future.

Table 1 Data of treated municipal wastewater in different countries (http://www.fao.org/nr/water/aquastat/main/index.stm).

Country	Year	Produced municipal wastewater	Treated municipal wastewater	% Treated municipal wastewater
Singapore	2013	0.5	0.511	100
France	2008	3.8	3.770	99
Italy	2007	3.9	3.902	99
Spain	2004	3.2	3.160	99
United Kingdom	2011	4.1	4.048	99
Germany	2007	5.3	5.183	98
Netherlands	2010	1.9	1.875	97
Ireland	2010	0.8	0.723	92
Canada	2009	6.6	5.621	85
Republic of Korea	2011	7.8	6.583	84
Estonia	2009	0.4	0.307	80
Switzerland	2011	1.4	1.084	77
Turkey	2010	3.6	2.719	76
Monaco	2009	0.0	0.006	75
Swaziland	2002	0.0	0.009	75
Slovenia	2010	0.2	0.126	73
Chile	2011	1.1	0.768	69
Saudi Arabia	2010	1.5	1.063	69
Belarus	2010	1.0	0.676	68
Japan	2011	16.9	11.560	68
United States of America	2008	60.4	40.890	68
Sweden	2010	0.7	0.436	65
Malaysia	2009	4.2	2.603	62
Poland	2011	2.3	1.356	60
Egypt	2012	7.1	4.013	57
South Africa	2009	3.5	1.919	54
Zimbabwe	2012	0.2	0.095	49
Lithuania	2009	0.3	0.128	49
Portugal	2009	0.6	0.270	47
Latvia	2009	0.3	0.128	45
Mexico	2011	7.5	3.081	41
Syrian Arab Republic	2012	1.4	0.550	40
Montenegro	2008	0.0	0.014	40
Algeria	2012	0.8	0.324	40
Nicaragua	2008	0.3	0.111	37
Brazil	2008	10.3	3.100	30
India	2011	15.5	4.416	29
Romania	2011	1.3	0.373	28
Turkmenistan	2010	1.3	0.336	26
Azerbaijan	2005	0.7	0.161	24
Thailand	2012	5.1	1.168	23
Iraq	2012	0.6	0.098	17
Armenia	2011	0.8	0.115	15
Argentina	2010	2.5	0.290	12
Viet Nam	2012	2.0	0.197	10
Ghana	2006	0.3	0.022	8
Bosnia and Herzegovina	2011	0.1	0.003	4
Tajikistan	2004	4.7	0.152	3
Dominican Republic	2011	0.4	0.008	2
El Salvador	2010	0.1	0.001	1

Figures

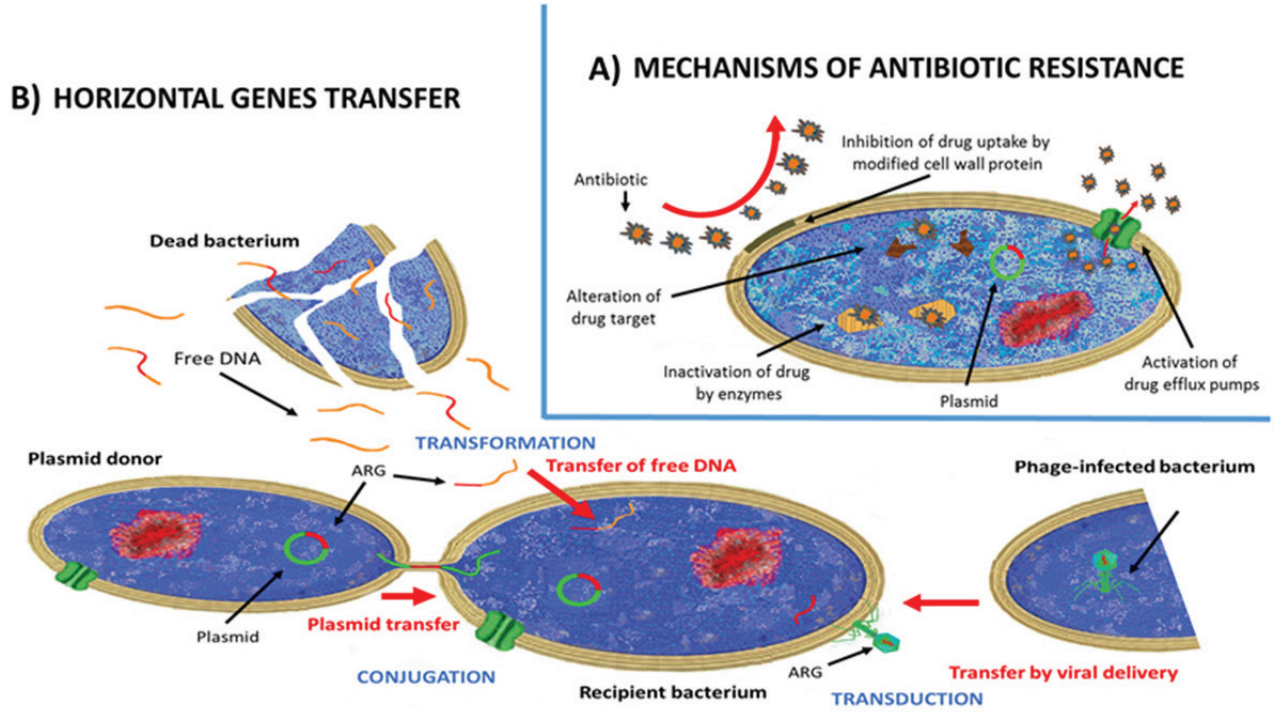

Fig. 1 Mechanisms that can give rise to antibiotic resistance: A) atributed to chromosomal mutation and B) acquiring resistance by horizontal gene transfer.

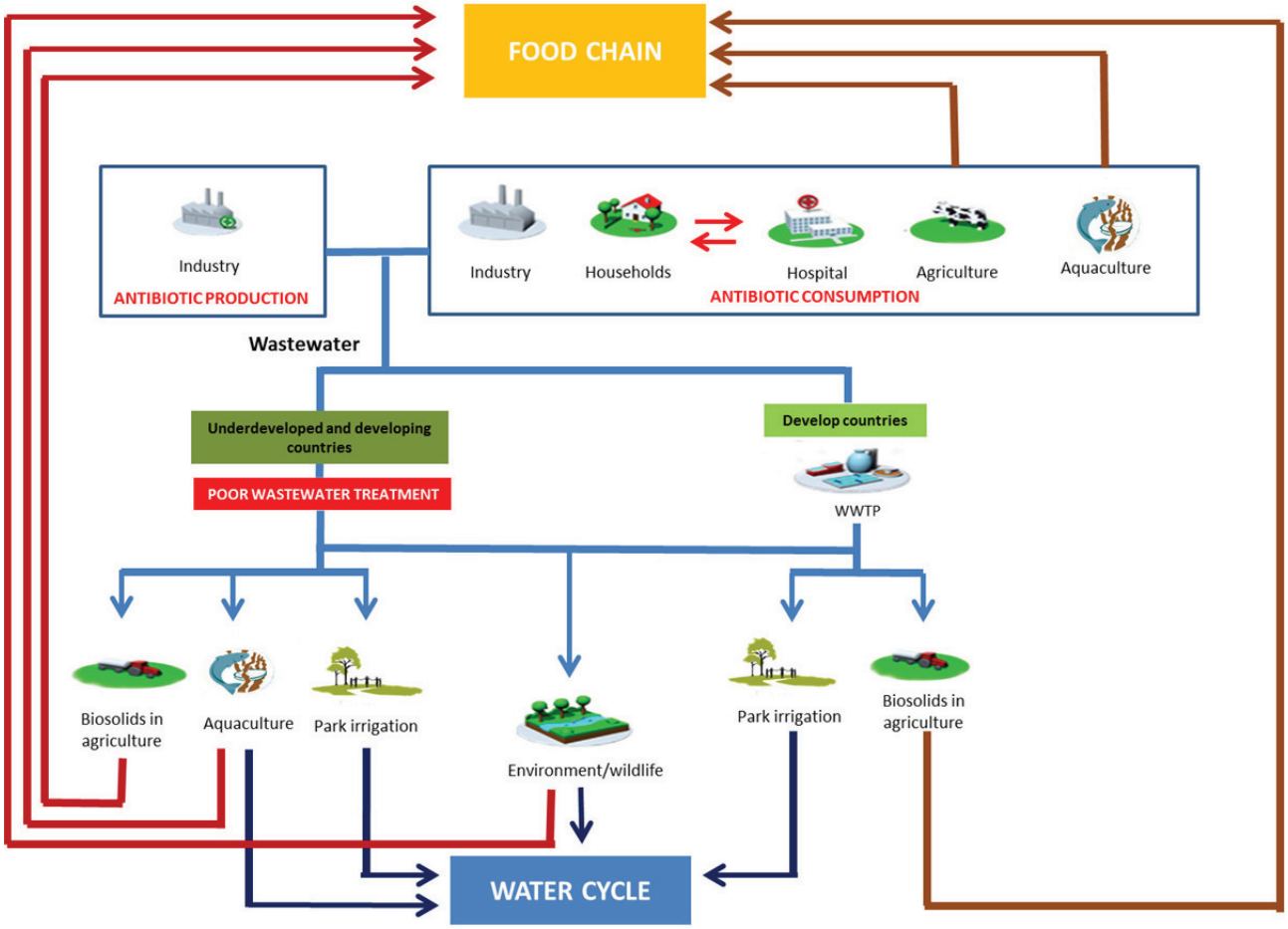

Fig. 2 Water sanitation and antibiotic resistance disemination through the water cycle and food chain.

Acknowledgements The work of Marcos Quintela Baluja is supported by the Mermaid, Marie Curie ITN. Grant number: 60749.

References

[1] Pruden, A., Pei, R., Storteboom, H. & Carlson, K. H. Antibiotic resistance genes as emerging contaminants: studies in northern Colorado. *Environ. Sci. Technol.* 40, 7445–7450 (2006).
[2] WHO | Antimicrobial resistance: global report on surveillance 2014. at <http://www.who.int/drugresistance/documents/surveillancereport/en/>
[3] ECDC/EMEA Joint group. *The bacterial challenge : time to react*. (2009).
[4] Cantas, L. *et al.* A brief multi-disciplinary review on antimicrobial resistance in medicine and its linkage to the global environmental microbiota. *Front. Microbiol.* 4, 1–14 (2013).
[5] O'Neill, J. Antimicrobial Resistance : Tackling a crisis for the health and wealth of nations. *Rev. Antimicrob. Resist.* (2014).
[6] Resistance, A. & Committee, S. National surveillance and reporting of antimicrobial r esistance and antibiotic usage for human health in Australia. (2013).
[7] Rizzo, L. *et al.* Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: a review. *Sci. Total Environ.* 447, 345–60 (2013).
[8] Ochman, H., Lawrence, J. G. & Groisman, E. a. Lateral gene transfer and the nature of bacterial innovation. *Nature* 405, 299–304 (2000).
[9] Szczepanowski, R. *et al.* The 120 592 bp IncF plasmid pRSB107 isolated from a sewage-treatment plant encodes nine different antiobiotic-resistance determinants, two iron-acquisition systems and other putative virulence-associated functions. *Microbiology* 151, 1095–1111 (2005).
[10] Schlüter, A., Szczepanowski, R., Pühler, A. & Top, E. M. Genomics of IncP-1 antibiotic resistance plasmids isolated from wastewater treatment plants provides evidence for a widely accessible drug resistance gene pool. *FEMS Microbiol. Rev.* 31, 449–477 (2007).
[11] Sørensen, S. J., Bailey, M., Hansen, L. H., Kroer, N. & Wuertz, S. Studying plasmid horizontal transfer in situ: a critical review. *Nat. Rev. Microbiol.* 3, 700–10 (2005).
[12] Allen, H. K. *et al.* Call of the wild: antibiotic resistance genes in natural environments. *Nat. Rev. Microbiol.* 8, 251–9 (2010).
[13] Liu, B. & Pop, M. ARDB - Antibiotic resistance genes database. *Nucleic Acids Res.* 37, 443–447 (2009).
[14] Davies, J. & Davies, D. Origins and evolution of antibiotic resistance. *Microbiol. Mol. Biol. Rev.* 74, 417–33 (2010).
[15] Marshall, B. M., Ochieng, D. J. & Levy, S. B. Commensals: Underappreciated Reservoir of Antibiotic Resistance. *Microbe* 4, 231–238 (2009).
[16] Salyers, A. a., Gupta, A. & Wang, Y. Human intestinal bacteria as reservoirs for antibiotic resistance genes. *Trends Microbiol.* 12, 412–416 (2004).
[17] Schjørring, S. & Krogfelt, K. a. Assessment of bacterial antibiotic resistance transfer in the gut. *Int. J. Microbiol.* 2011, (2011).
[18] Courvalin, P. Predictable and unpredictable evolution of antibiotic resistance. *J. Intern. Med.* 264, 4–16 (2008).
[19] Bush, K. *et al.* Tackling antibiotic resistance. *Nat. Rev. Microbiol.* 9, 894–6 (2011).
[20] Skov, R. & Skov, G. EUCAST guidelines for detection of resistance mechanisms and specific resistances of clinical and / or epidemiological importance. 1–47 (2012).
[21] Clsi. *Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Second Informational Supplement*. *Clin. Lab. Stand. Inst.* 32, (2013).
[22] Leclercq, R. *et al.* EUCAST expert rules in antimicrobial susceptibility testing. *Clin. Microbiol. Infect.* 19, 141–160 (2013).
[23] Guide, A. A. Celebrating Water for Life 2005-2015 THE INTERNATIONAL DECADE. *Sustain. Dev.* (2005).
[24] Who & Unicef. Progress on sanitation and drinking-water - 2014 update. ... *Monit. Program. water supply Sanit. ...* 1–78 (2014). doi:978 92 4 150724 0
[25] Christgen, B. *et al.* Metagenomics Shows That Low-Energy Anaerobic−Aerobic Treatment Reactors Reduce Antibiotic Resistance Gene Levels from Domestic Wastewater. *Environ. Sci. Technol.* 150202093155005 (2015). doi:10.1021/es505521w
[26] Rehman, M. S. U. *et al.* Global risk of pharmaceutical contamination from highly populated developing countries. *Chemosphere* (2013). doi:10.1016/j.chemosphere.2013.02.036
[27] Fick, J. *et al.* Contamination of Surface, Ground, and Drinking Water From Pharmaceutical Production. *Environ. Toxicol. Chem.* 28, 2522–2527 (2009).
[28] Van Boeckel, T. P. *et al.* Global antibiotic consumption 2000 to 2010: An analysis of national pharmaceutical sales data. *Lancet Infect. Dis.* 14, 742–750 (2014).
[29] Halling-Sorensen, B. *et al.* Occurence, fate and effects of pharmaceuticals substance in the environment - A review. *Chemosphere* 36, 357–393 (1998).
[30] Alcock, R. E., Sweetman, a. & Jones, K. C. Assessment of organic contanhnant fate in waste water treatment plants I: Selected compounds and physicochemical properties. *Chemosphere* 38, 2247–2262 (1999).
[31] Winckler, C. & Grafe, A. Use of veterinary drugs in intensive animal production: Evidence for persistence of tetracycline in pig slurry. *J. Soils Sediments* 1, 66–70 (2001).
[32] Heberer, T. & Heberer, T. Occurrence, fate, and removal of pharmaceutical residues in the aquatic environment: a review of recent research data. *Toxicol. Lett.* 131, 5–17 (2002).
[33] Bound, J. P. & Voulvoulis, N. Pharmaceuticals in the aquatic environment - A comparison of risk assessment strategies. *Chemosphere* 56, 1143–1155 (2004).
[34] Phillips, I. *et al.* Does the use of antibiotics in food animals pose a risk to human health? A critical review of published data. *J. Antimicrob. Chemother.* 53, 28–52 (2004).
[35] Nagulapally, S. R. *et al.* Occurrence of ciprofloxacin-, trimethoprim-sulfamethoxazole-, and vancomycin-resistant bacteria in a municipal wastewater treatment plant. *Water Environ. Res.* 81, 82–90 (2009).

[36] Karthikeyan, K. G. & Meyer, M. T. Occurrence of antibiotics in wastewater treatment facilities in Wisconsin, USA. *Sci. Total Environ.* 361, 196–207 (2006).
[37] Larsson, D. G. J., de Pedro, C. & Paxeus, N. Effluent from drug manufactures contains extremely high levels of pharmaceuticals. *J. Hazard. Mater.* 148, 751–755 (2007).
[38] Gaze, W. H. *et al.* Impacts of anthropogenic activity on the ecology of class 1 integrons and integron-associated genes in the environment. *ISME J.* 5, 1253–1261 (2011).
[39] Martinez, J. L. The role of natural environments in the evolution of resistance traits in pathogenic bacteria. *Proc. Biol. Sci.* 276, 2521–2530 (2009).
[40] Wellington, E. M. H. *et al.* The role of the natural environment in the emergence of antibiotic resistance in Gram-negative bacteria. *Lancet Infect. Dis.* 13, 155–165 (2013).
[41] Rahube, T. O. *et al.* Impact of Fertilizing with Raw or Anaerobically Digested Sewage Sludge on the Abundance of Antibiotic-Resistant Coliforms, Antibiotic Resistance Genes, and Pathogenic Bacteria in Soil and on Vegetables at Harvest. *Appl. Environ. Microbiol.* 80, 6898–6907 (2014).
[42] Negreanu, Y., Pasternak, Z., Jurkevitch, E. & Cytryn, E. Impact of treated wastewater irrigation on antibiotic resistance in agricultural soils. *Environ. Sci. Technol.* 46, 4800–8 (2012).
[43] Wang, F. *et al.* High Throughput Profiling of Antibiotic Resistance Genes in Urban Park Soils with Reclaimed Water Irrigation. *Environ. Sci. Technol.* (2014).
[44] Kumar, D., Hiremath, A. M. & Asolekar, S. R. Integrated Management of Wastewater through Sewage Fed Aquaculture for Resource Recovery and Reuse of Treated Effluent: A Case Study. *APCBEE Procedia* 10, 74–78 (2014).
[45] Sanyal, S. & Banerjee, S. Transferable drug-resistant coliforms in fish exposed to sewage. 29–39 (2013). doi:10.2478/aopf-2013-0004
[46] Walsh, T. R., Weeks, J., Livermore, D. M. & Toleman, M. A. Dissemination of NDM-1 positive bacteria in the New Delhi environment and its implications for human health: an environmental point prevalence study. *Lancet. Infect. Dis.* 11, 355–62 (2011).
[47] Mao, D. *et al.* Persistence of extracellular DNA in river sediment facilitates antibiotic resistance gene propagation. *Environ. Sci. Technol.* 48, 71–78 (2014).
[48] Barkovskii, A. L. & Bridges, C. Persistence and profiles of tetracycline resistance genes in swine farms and impact of operational practices on their occurrence in farms' vicinities. *Water. Air. Soil Pollut.* 223, 49–62 (2012).
[49] Thiele-Bruhn, S. & Beck, I. C. Effects of sulfonamide and tetracycline antibiotics on soil microbial activity and microbial biomass. *Chemosphere* 59, 457–465 (2005).
[50] Merlin, C., Bonot, S., Courtois, S. & Block, J. C. Persistence and dissemination of the multiple-antibiotic-resistance plasmid pB10 in the microbial communities of wastewater sludge microcosms. *Water Res.* 45, 2897–2905 (2011).
[51] Shakibaie, M. R., Jalilzadeh, K. a. & Yamakanamardi, S. M. Horizontal transfer of antibiotic resistance genes among gram negative bacteria in sewage and lake water and influence of some physico-chemical parameters of water on conjugation process. *J. Environ. Biol.* 30, 45–49 (2009).
[52] Knapp, C. W. *et al.* Indirect evidence of transposon-mediated selection of antibiotic resistance genes in aquatic systems at low level oxytetracycline exposures. *Environ. Sci. Technol* 42, 5348–5353 (2008).
[53] Anderson, J. C. *et al.* Performance of a constructed wetland in Grand Marais, Manitoba, Canada: Removal of nutrients, pharmaceuticals, and antibiotic resistance genes from municipal wastewater. *Chem. Cent. J.* 7, 54–69 (2013).
[54] Ohlsen, K. *et al.* Impact of antibiotics on conjugational resistance gene transfer in Staphylococcus aureus in sewage. *Environ. Microbiol.* 5, 711–716 (2003).
[55] Barr, V., Barr, K., Millar, M. R. & Lacey, R. W. / J-Lactam antibiotics increase the frequency of plasmid transfer in Staphylococcus aureus In common with other pathogenic bacteria , antibiotic resistance in Staphylococcus aureus is often encoded by plasmid genes . Cultures of this organism resistant to. 409–413 (1986).
[56] Wagner, M. *et al.* Microbial community composition and function in wastewater treatment plants. *Antonie Van Leeuwenhoek* 81, 665–680 (2002).
[57] Zhao, Y. & Truhlar, D. G. The M06 suite of density functionals for main group thermochemistry, thermochemical kinetics, noncovalent interactions, excited states, and transition elements: Two new functionals and systematic testing of four M06-class functionals and 12 other function. *Theor. Chem. Acc.* 120, 215–241 (2008).
[58] Guardabassi, L., Lo Fo Wong, D. M. a & Dalsgaard, A. The effects of tertiary wastewater treatment on the prevalence of antimicrobial resistant bacteria. *Water Res.* 36, 1955–1964 (2002).
[59] Huang, X. Z. *et al.* Characteristics of plasmids in multi-drug-resistant enterobacteriaceae isolated during prospective surveillance of a newly opened hospital in Iraq. *PLoS One* 7, (2012).
[60] Munir, M., Wong, K. & Xagoraraki, I. Release of antibiotic resistant bacteria and genes in the effluent and biosolids of five wastewater utilities in Michigan. *Water Res.* 45, 681–93 (2011).
[61] Reinthaler, F. F. *et al.* Antibiotic resistance of E. coli in sewage and sludge. *Water Res.* 37, 1685–1690 (2003).
[62] Da Silva, M. F. *et al.* Antibiotic resistance of enterococci and related bacteria in an urban wastewater treatment plant. *FEMS Microbiol. Ecol.* 55, 322–329 (2006).
[63] Zhang, Y., Marrs, C. F., Simon, C. & Xi, C. Wastewater treatment contributes to selective increase of antibiotic resistance among Acinetobacter spp. *Sci. Total Environ.* 407, 3702–3706 (2009).
[64] Bennett, P. M. Plasmid encoded antibiotic resistance: acquisition and transfer of antibiotic resistance genes in bacteria. *Br. J. Pharmacol.* 153 Suppl 1, S347–S357 (2008).
[65] Slater, F. R., Bailey, M. J., Tett, A. J. & Turner, S. L. Progress towards understanding the fate of plasmids in bacterial communities. *FEMS Microbiol. Ecol.* 66, 3–13 (2008).
[66] Gillings, M. R. Integrons: past, present, and future. *Microbiol. Mol. Biol. Rev.* 78, 257–77 (2014).
[67] Baquero, F., Martínez, J. L. & Cantón, R. Antibiotics and antibiotic resistance in water environments. *Curr. Opin. Biotechnol.* 19, 260–265 (2008).

[68] Bahl, M. I., Oregaard, G., Sørensen, S. J. & Hansen, L. H. Horizontal Gene Transfer. 532, 257–268 (2009).
[69] Heuer, H. *et al.* The complete sequences of plasmids pB2 and pB3 provide evidence for a recent ancestor of the IncP-1β group without any accessory genes. *Microbiology* 150, 3591–3599 (2004).
[70] Bönemann, G., Stiens, M., Pühler, A. & Schlüter, A. Mobilizable IncQ-related plasmid carrying a new quinolone resistance gene, qnrS2, isolated from the bacterial community of a wastewater treatment plant. *Antimicrob. Agents Chemother.* 50, 3075–3080 (2006).
[71] Tennstedt, T., Szczepanowski, R., Krahn, I., Pühler, A. & Schlüter, A. Sequence of the 68,869 bp IncP-1α plasmid pTB11 from a waste-water treatment plant reveals a highly conserved backbone, a Tn402-like integron and other transposable elements. *Plasmid* 53, 218–238 (2005).
[72] Moura, H. *et al.* MALDI-TOF mass spectrometry as a tool for differentiation of invasive and noninvasive Streptococcus pyogenes isolates. *FEMS Immunol. Med. Microbiol.* 53, 333–42 (2008).
[73] Ansari, M. I., Grohmann, E. & Malik, a. Conjugative plasmids in multi-resistant bacterial isolates from Indian soil. *J. Appl. Microbiol.* 104, 1774–1781 (2008).
[74] Malik, A. *et al.* Detection of conjugative plasmids and antibiotic resistance genes in anthropogenic soils from Germany and India. *FEMS Microbiol. Lett.* 279, 207–216 (2008).