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Bacterial perspectives on the dissemination of antibiotic resistance genes in domestic wastewater bio-treatment systems: Beneficiary to victim.

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19 **Abstract**

20 Domestic wastes, ranging from sewage and sludge to municipal solid waste, are
21 usually treated in bioprocessing systems. These systems are regarded as main conduits
22 for the elevated levels of antibiotic resistance genes (ARGs) observed in the
23 environment. This paper mainly reviews recent studies on the occurrence and
24 dynamics of ARGs in wastewater bio-treatment systems and discusses the ins and outs
25 of ARG dissemination from the perspective of the microbial community. Our analysis
26 shows that concentration of antibiotics through adsorption to microbial aggregates
27 triggers the bacteria to acquire ARGs, which can be facilitated by the presence of
28 mobile genetic elements. Notably, the acquisition and flow of ARGs during the rapid
29 dissemination process is directed towards and for the best interests of the microbial
30 community as a whole, and is influenced by surrounding nutrient levels, toxicant
31 types and sensitivities of the species in the prevailing antibiotic-stressed conditions.
32 Furthermore, our review argues that predation of ARG-carrying bacteria by
33 bacteriophages does periodically enhance the accessibility of ARGs to bacteria, which
34 indirectly facilitates the recruitment of ARGs into environmental microbial
35 communities.

36 **Keywords** Domestic wastes • Antibiotic resistance genes • Waste
37 bio-treatment • Antibiotic resistance dissemination

38

39 **Introduction**

40 The classic story how Alexander Fleming discovered antibiotic-producing
41 microorganisms tells us that nature itself harbors reservoirs of antibiotics. Previous
42 studies have also shown that antibiotic resistance genes (ARGs) are ancient DNA
43 fragments (D'Costa et al. 2011), predating the use of antibiotics by *Homo sapiens* by
44 tens of thousands of years (Hardy et al. 2012). But notably, by analyzing soil samples
45 spanning the last 70 years, environmental scientists have shown that the basal levels
46 of ARGs are increasing in parallel with the modern massive production of antibiotics
47 (Graham et al. 2016; Knapp et al. 2010). From the local to the transcontinental scale,
48 the disposal of anthropogenic wastes is considered as a major factor fueling ARG
49 dissemination (Pehrsson et al. 2016; Zhu et al. 2017).

50 The rapid anthropogenic dissemination of antimicrobial resistant bacteria (ARB) and
51 ARGs across the world challenges the concept that antimicrobial resistance (AMR) is
52 just a “natural feature of diverse microbial ecosystems” (Crofts et al. 2017;
53 Wellington et al. 2013). This review aims to integrate studies addressing chemical
54 (antibiotics/metals) selection pressures versus the cost-effect balance of acquiring
55 ARGs (being resistant) from the perspective of bacteria and bacterial community. Our
56 conclusions are expected to serve as starting points for the development of ecological
57 approaches to reduce the spread of AMR issues in the environment.

58 **Dissemination of ARGs is not random**

59 **General benefits and costs of being antibiotic resistant**

60 In natural ecosystems, antibiotics produced by intrinsically resistant bacteria (in
61 pristine environments) are playing various functional roles, like signaling (mutual
62 beneficial signals), aiding dispersal and acting as toxicants (Ratcliff and Denison

2011), which give antibiotics-producing bacteria advantages over their competitors in the microbial community. Current studies generally presume that the genes for antibiotic resistance initially emerged in the wastes and body (e.g. intestinal microenvironments) under selective pressures of antibiotics-feeding animals or humans, or stem from intrinsic resistant bacteria (Pehrsson et al. 2016), whereas the subsequent dissemination of ARGs via horizontal gene transfer (HGT) is characterized with more randomness (Czekalski et al. 2014; Gaze et al. 2011). It seems plausible that ARGs or their fragments preserved in environments could be incidentally acquired by bacteria via HGT (Guo et al. 2017; Mao et al. 2014), and thereby facilitate the propagation of AMR. Previous studies have consistently pointed out that mobile genetic elements (MGEs) including integrons, insertion sequences, transposons and plasmids are responsible for the “promiscuity” of ARGs (Yu et al. 2016; Zhu et al. 2017). According to the evolution theory of Darwin, the overall increasing levels of ARGs in anthropogenic wastes and their impacted environments, whether in farming lands (Su et al. 2015), wastewater treatment plants (Yang et al. 2014), municipal landfills (Sun et al. 2016), or contaminated river (Jiang et al. 2013; Stepanauskas et al. 2006), should also confer a benefit to the bacteria that have acquired ARGs.

It is recognized that HGT allows distantly related bacteria (different species) to transfer genes (Koonin et al. 2001). This evolutionary phenomenon usually takes place during a period of adaptation to new environmental conditions, where HGT requires extra energy to capture extracellular DNA fragments, replicate and maintain these genetic materials (Baltrus 2013). In a long term, compared to conventional bacterial reproduction (**Fig. 1**), microorganisms will experience additional detrimental side effects known as “metabolic burdens” that are induced by increases in gene expressions of the HGT related DNA regions (Park and Zhang 2012). Therefore, if newly acquired ARGs are not fitted to an antimicrobial role in none antibiotic-stressed

conditions (**Fig. 1**), the HGT-related costs will be net energy losses. In this case, bacteria that host ARGs will have comparatively less energy in reproduction. This could inherently decrease the proportion of ARGs in the gene pools of any type of environment (Bjorkman and Andersson 2000). It is common to observe the loss and decreasing abundance of HGT related genes in environmental samples (Mao et al. 2014; Rysz et al. 2013). Thus, to unravel the puzzle why ARGs are massively sprawling from human to natural environments, we need to better understand how bacteria benefit from being antibiotic resistant.

Impacts of discharge and bio-adsorption of antibiotics

The total amount of antibiotics consumed by humans increased by 46% between 2000 to 2010 (Van Boeckel et al. 2014), reaching an annual consumption of 15g per capita (Zhang et al. 2008). Kümmerer et al. (2000) estimated that domestic sewer networks and sewage treatment plants receive 20–40% of all outdated medicaments. Although pharmaceuticals, classified as hazardous wastes (Environment Agency 2013), are not permitted to be disposed in sewer now, a wide range of pharmaceuticals is still found in anthropogenic wastes and waste treatment systems (Bound and Voulvoulis 2005). Studies show that 75% of antibiotics fed to animals (200,000 tons/year are used in livestock farming systems) and up to 50% of total amount prescribed to humans are discharged (e.g. urinary excretion) in an active form (Kemper 2008; Van Boeckel et al. 2014).

In general, the levels of antibiotics detected in domestic wastewater and solid wastes are at the nanogram per liter or per kilogram level (Marx et al. 2015; Miao et al. 2004; Sun et al. 2016; Wu et al. 2015; Zhou et al. 2013). This is one order of magnitude lower than the minimum selective concentration (MSC), which can cause inhibition (AMR mutations) on drug-hypersensitive strains (Andersson and Hughes, 2014). This

seems to suggest that the acquisition of ARGs would not confer an advantage, being resistant to antibiotics, to the recipient bacteria (**Fig. 1**). However, conventional biotreatment facilities utilizing densely agglomerated bacteria (active sludge flocs or biofilms) were not originally designed for the reduction of antibiotics (Marx et al. 2015). The observed reduction of most antibiotics in wastewater treatment effluent is more indicative of their “disappearance” via bio-adsorption than of them being biodegraded (Kümmerer 2008). This is because bacteria compete to rapidly adsorb organic matter from wastewater and then conduct a slower metabolic assimilation (Chua and Hua 1996). Consequently, a distinct gradient of organic matters (e.g. antibiotics) is formed around the radius of the sludge flocs (**Fig. 2**). Guellil et al. (2001).’s research shows that sludge’s biosorption rates of organic matters from range from 5-15 mg_{COD}TSS⁻¹min⁻¹.

In terms of antibiotics, compounds that are featured with high octanolewater partition coefficient ($K_{ow} > 2.5$), such as tetracyclines, macrolides (MLs) and fluoroquinolones (FQs), usually show high sorption potentials onto solids or sludge (Michael et al. 2013). From a wider perspective, a mass balance analysis conducted by Zhou et al. (2013) shows that on average 70% of the tetracycline entering domestic wastewater treatment plant was redistributed to the biosolids (sludge) phase. Lindberg et al. (2006) pointed out that, for FQs (norfloxacin and ciprofloxacin), this value can increase to 80%. A higher adsorption rate in wastewater treatment systems can be promoted by increasing the sludge concentration, which is achieved through increasing the sludge retention time (SRT) in practice (Abegglen et al. 2009). Similarly, extending SRT from 3 to 40 days resulted in a 50% higher transfer of MLs and FQs to sludge (Li et al. 2013).

According to Mao et al. (2014), the addition of plasmids doubled the ARG-uptake rate from the extracellular matrix under an antibiotics-stressed condition (kanamycin;

20mg/L), relative to the unstressed condition; however, under the same antibiotic-stressed condition, no ARGs transfer was observed when plasmids were not added. These observations suggest that a rapid ARG propagation through natural transformation from extracellular DNA to indigenous bacteria needs the i) sufficient amount of MGEs ; ii) presence of antibiotics that confer selective pressures. As shown in **Fig. 2**, the prevailing bioadsorption of antibiotics by sludge flocs provides these two prerequisites. This may imply that the widely disseminated ARGs, instead of being incidentally involved in HGT, do function in antibiotic resistance in wastewater bio-treatment systems. Luczkiewicz et al. (2010) reported that the abundances of tetracycline resistant *E. faecalis* and penicillin resistant *E. coliforms* increased by 10% and 15%, respectively, in activate sludge. Similarly, Szczepanowski et al. (2009) showed that bacteria in sludge from a domestic wastewater treatment plant exhibited a reduced susceptibility to antibiotics and meanwhile 140 clinically important ARGs, situated on MGEs, were enriched in the system.

Mediation of microbial community conditions

Bacterial communities in either natural environments or biotreatment systems have the tendency to act as a unit, where each species contributes to the function of the whole consortium. From this perspective, the dissemination of ARGs needs to fit the best interests of individual bacteria as well as the whole microbial consortium. For example, a metagenomic analysis shows that ARGs are rarely associated with MGEs (Chen et al. 2016) in pristine Tibetan soil samples, which are not contaminated by antibiotics. Also, environmental resistome is observed to structure with composition of microbial community, which is commonly defined by surrounding carbon or secondary nutrients contents (Forsberg et al. 2014). These findings suggest that the dissemination of ARGs in those environments is not facilitated by random or unregulated associations with MGEs (**Fig. 1**).

167 **Serving best interests**

168 The currently identified mechanisms of antibiotic resistance are comprised of efflux
169 pumps, target modification (resistance mutations that modify the target protein),
170 inactivation of the antibiotic, and target bypass (e.g. impermeable cell membrane) or
171 lack of target (Allen et al. 2010). Compared to other bacteria, the ARGs-carried ones
172 appear to have a wider choice of substrates if the integrated ARGs allow them to
173 consume antibiotics via their inactivation functions (Dantas et al. 2008). Nevertheless,
174 it is important to note that antibiotics are not a good source of carbon and energy. For
175 example, the presence of acetate can reduce the biodegradation rate of antibiotics
176 (Drillia et al. 2005).

177 In the case of domestic wastes and treatment systems, the easily degradable organics
178 are rarely depleted and therefore the microorganisms in these systems are reluctant to
179 switch to antibiotics as substrate. Metagenomics analysis also shows that the most
180 prevalent mechanism of antibiotic resistance is efflux pumps (Christgen et al. 2015;
181 Yang et al. 2014), which keeps antibiotics out of the cell, instead of decomposing
182 them. But notably, the dissemination of ARGs is coordinated by a whole microbial
183 community. Cordero et al. (2012) and Nguyen et al. (2011) state that bacterial
184 populations are not driven by gene-centric and “selfish” dynamics but represent
185 socially “cohesive units” regarding antimicrobial resistance in common microhabitats.
186 For example, a previous study has shown that, in an antibiotics stressed domestic
187 waste treatment system, denitrification by denitrifying bacteria proceeded smoothly
188 thanks to the presence and activity of ARGs in the non-denitrifying party of the
189 population (Wu et al. 2017). This means that not all bacteria affected by antibiotics
190 need to and indeed will acquire ARGs, and become resistant in the environment. In
191 mixed-species biofilms, Lee et al. (2014) has shown that the increased antibiotic
192 resistance/tolerance was conferred via cross-protection (**Fig. 3**), which was offered by

one resistant species (*Pseudomonas protegens* Pf-5) to all members in the microbial assembly; this study also found that the role of protector (resistant strains) can be switched to other species, under different cultivation (antibiotic types and nutrition levels) conditions, for the highest growth rate of biofilms. All these observations suggest that the aggregated bacteria are prone to serve the best interests of microbial communities, where the dissemination direction of ARGs is systematically oriented by antibiotic types, bacterial sensitivity and the most needed functions for microbial community to expand (**Fig. 3**).

The importance of metals

Apart from antibiotics, (heavy) metals are considered to impose relatively strong selection pressure on surrounding bacteria (Hu et al. 2017). Metals have physico-chemical characteristics that promote the build-up of resistance-selective concentrations, e.g. bio-adsorption, complexation, physical adsorption in the environment (Seiler and Berendonk 2012), more so than the other AMR selective agents (Czekalski et al. 2014). The links between metals and increasing tolerance to antibiotics have been studied extensively. Baker-Austin et al. (2006) systematically grouped the resistance mechanisms induced by metals into three classes. Briefly, they are (i) cross-selection resistance, where one gene encodes resistance to both antibiotics and metals, (ii) co-selection resistance, where ARGs and metal resistant genes (MRGs) encode resistances to antibiotics and metals respectively but are physically located closely (generally in MGEs, plasmids for example), and (iii) co-regulatory resistance, where genetic transcription links metals and antibiotic resistance together, observed as unselective, active efflux of inhibitory toxicants, regardless of exposure to either metals or antibiotics. Among these resistance mechanisms, the co-selection of antibiotic and metal resistance was most commonly observed in bio-treatment systems, probably due to the high abundances of MGEs (Di

Cesare et al. 2016; Yu et al. 2016).

Li et al. (2017) have revealed that i) ARGs and MRGs are genetically associated with MGEs (signal of co-selection) in all environmental samples; ii) HGT of ARGs and MRGs does rarely result in same ARG-MRG couples being shared across different environments. These two findings suggest that co-selection of ARGs and MRGs is a pervasive mechanism in all types of environments on one hand, but also imply that environmental conditions, rather than MGEs, determine which ARG-MRG pair shall be coupled on the other. This is presumably because that, as a defensive function (similar to the AMR) whose acquisition costs extra energy and risks low reproduction rates of bacteria (**Fig. 1**), the metal resistance (mechanism) has evolved to benefit the resistance gene(s) carriers and/or the whole microbial community (Miao et al. 2015). Additionally, Li et al. (2017) have shown that the multidrug resistance type(s) are most frequently observed to co-occur with MRGs (25% of all samples). Metagenomic analyses have shown that efflux pumps, known as multi-resistance to metals and antibiotics, are the prevalent mechanism (50% - 80% of resistance types) in domestic waste impacted areas and bio-treatment systems (Chen et al. 2013; Christgen et al. 2015; Su et al. 2015). Thus, future studies aiming to contain the rapid dissemination of ARGs should pay attention to the distribution of multidrug resistance and co-conferring antibiotic resistances of MRGs.

What if bacteria are victims?

In the previous sections, we have discussed the dissemination of ARGs in domestic wastes and waste treatment systems from bacterial perspectives as beneficiaries that master the game-theory in gaining maximal energy-gains. The published studies have also shown that bacteria can actively compromise resistance sensitive species for the interest of whole community (Lee et al. 2014; Wu et al. 2017). However, bacteria are not the microbes at the end of multi-hierarchical “food chains”. They can be

“consumed” by bacteriophages (or phages). Compared to other life forms, phages are the most abundant entities on earth (Frost et al. 2005). Their high multiplication rates ($\sim 1 \times 10^{25}$ per second) and concomitant lysis rates of the bacterial hosts greatly facilitate the reproduction and release of genetic elements to other bacterial cells, namely, transduction (Canchaya et al. 2003).

The genome size of phages ranges from a few to several 100 kb (Wu and Liu 2009), which is equivalent to the size of plasmids. This indicates that the phage-head is spacious enough to accommodate ARGs (Colomer-Lluch et al. 2011). During transduction, DNA fragments from an infected donor (host) can be accidentally loaded into the phage and then transferred to a recipient cell (**Fig. 4**). The aggregated biomass in waste and waste treatment systems is densely populated with phages (Colomer-Lluch et al. 2014; Withey et al. 2005). Moreover, according to Shapiro and Kushmaro (2011), some phages favor the introduction of antibiotics or metals in that toxicants may facilitate lysis of bacterial populations in domestic wastes. Rather than establishing a mutualistic relationship with bacteria, phages sometimes function as bacterial population manipulators (Rodriguez-Valera et al. 2009; Thingstad 2000). Phages are capable of attenuating the proliferation of some ARB strains in activated sludge (Yu et al. 2017). Presently, we have very limited knowledge of the benefits for phages by integrating ARGs or attacking ARB. But, phages were commonly observed to infect the dominant bacterial strains in the waste treatment systems (Shapiro et al. 2010), where bacteria could benefit from the acquisition of ARGs and then become major species, the “winners of microbial competition” (**Fig. 1**).

However, phages may not be used for removal of AMR genes from bio-treatment systems. Calero-Caceres and Muniesa (2016) have shown that ARGs (*bla*_{TEM}, *bla*_{CTX-M} and *sul1*) could persist longer when they were in phages than when in the bacterial fraction. Importantly, the commonly used disinfection technologies,

including UV-irradiation and chlorination, have no significant effects on the inactivation of the phage-fraction ARGs (Brown-Jaque et al. 2015; Calero-Caceres and Muniesa 2016; Colomer-Lluch et al. 2014). This suggests that predation of ARG carrying bacteria by phages increase the retention time of ARGs in waste treatment systems (Chen et al. 2016). **Fig. 4** shows that these ARG-containing phages will be released into environment along with the treated wastewater (waste) being discharged. They might re-infect bacterial populations in receiving environments and transduce ARGs from phage head into the, possibly dominant (Thingstad 2000), bacteria carrying no ARGs. Therefore, the phage-mediated ARG transduction could also indirectly promote propagation of ARGs. To validate this assumption, future studies should focus on the direction of the flow of the phage-fraction ARGs, possibly via DNA isotopic or fluorescent labeling.

Conclusions

Domestic wastes are hotspots of antibiotics and ARGs. Their treatment systems conventionally utilizing dense microbial communities facilitate the dissemination of ARGs within these aggregates and exacerbate AMR issues in the environments receiving the wastes. Thus, to contain the propagation of ARGs, we need to consider the salient characteristics of ARGs from the perspective of the bacteria, and then determine the possibility to reduce ARGs' contents. Our literature review underlines that dissemination of ARGs is not a random, rapid process, but requires the presence of selective pressure (metals and antibiotics) and mobile genetic elements (e.g. plasmids), the two prerequisites that are provided in the bio-treatment system due to bio-adsorption. Here, we propose a theory that the propagation of ARGs is mediated by the bacteria at the microbial community level: the community controls which species shall carry ARGs to counter the pressure of antibiotics, metals or other toxicants, and thereby maximize its expansion rates and survival chances.

297 Furthermore, we have conceptually analyzed potentials of AMR dissemination by
298 bacterial phages, where a quantitative insight in the integration and release flux of the
299 bacteriophage-fraction ARGs is yet to be explored.

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308 **Ethical/COI Statements**

309 The authors declare that they have no conflict of interest.

310 The manuscript has not been published elsewhere and all authors have seen the
311 manuscript and approved to submit to your journal with mutual consent. There is no
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313 .

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LEGEND OF FIGURES

Fig. 1 HGT requires considerable extra energy (yellow stars) and increases metabolic burdens of the bacterial cells (bacteria in pink color). This will decrease the proportion of ARGs in environmental gene pools without the imposed stresses from antibiotics. . The dark yellow circles represent the bacterial plasmids; and the red rods (Abs) represent antibiotics in surrounding environment.

Fig. 2 Active sludge aggregated in conventional bio-treatment systems can rapidly adsorb organic matters from wastewater. The high contents of antibiotics and MGEs formed on the periphery of bio-flocs promoted the ARGs dissemination across the whole bio-treatment systems. The dark yellow circles represent the bacterial plasmids; and the red rods (Abs) represent antibiotics.

Fig. 3 AMR can be offered by one strain to protect whole microbial community. When exposed to different antibiotics (Abs-1 or Abs-2), the AMR and resistant species can switch to others. The dark yellow circles represent bacterial plasmids. And the red and red segments represent different types of ARGs.

Fig. 4 In bio-treatment systems, phages acting as population manipulators prefer to infect the dominant ARB (in the presence of antibiotics). The phage-fraction ARGs are more resistant to disinfection (e.g. UV and chlorination) than the bacteria-fraction. This facilitates the dissemination of ARGs in wastes receiving environments. Red rods represent antibiotics (Abs); and red and green segments represent two different ARGs.

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