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From chemical mixtures to antibiotic resistance

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ABSTRACT

In real environment, it is unlikely that contaminants exist singly; environmental contamination with chemical mixtures is a norm. However, the impacts of chemical mixtures on environmental quality and ecosystem health have been overlooked in the past. Among the complex interactions between different contaminants, their relationship with the rise of antibiotic resistance (AR) is an emerging environmental concern. In this paper, we review recent progresses on how chemicals or chemical mixtures promote AR. We propose that, through co-selection, agents causing stress to bacteria may induce AR. The mechanisms for chemical mixtures to promote AR are also discussed. We also propose that, mechanistic understanding of co-selection of chemical mixtures for AR should be a future research priority in environmental health research.

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Introduction

The environment contains a lot of natural and man-made chemicals and/or their degradation products (Backhaus and Faust, 2012). Environmental contamination with chemical mixtures is ubiquitous. For example, in pig manures and manured soils in China, metals such as copper, zinc, and arsenic, as well as numerous antibiotics, are found present simultaneously (Qiao et al., 2012; Zhu et al., 2013). Although most environmental studies focus on one type of pollutants at a time, understanding the toxicity of a chemical mixture is a major challenge in environmental health research (Braun et al., 2016). To address this challenge, in-depth studies of toxicity and risk assessment of chemical mixtures have been emerging, starting from mixtures of metals (Nys et al., 2017; Traudt et al., 2017).

Antibiotic resistance (AR) has become a worldwide concern for public health. Since the discovery of penicillin and other antibiotics, they have been used to treat infectious diseases and have saved millions of lives. Almost in every case, introduction of a new antibiotic was followed by resistance to the very antibiotic (Lobanovska and Pilla, 2017), leading to huge cost of health care and deaths. The genetic determinants of AR, antibiotic resistance genes (ARGs), are found in almost every ecosystem (Su et al., 2017), even in extreme environments. The spread of ARGs in the environment could potentially increase the opportunity that human pathogens acquire AR from environmental bacteria. Exposure to antibiotics also can lead to amplification of existing ARGs by processes such as gene duplication or increasing the copy number of plasmids carrying ARGs (Paul et al., 2017; Sandegren and Andersson, 2009).

Conventionally the emergence and spread of AR is believed to be the consequence of use and abuse of antibiotics. However, in natural environment, bacteria are exposed to miscellaneous potential hazards such as heavy metals, antibiotics, and solvents. Therefore, bacteria were under selective pressure to evolve and develop mechanisms to better tolerate not only single stressors but actually mixtures of contaminants. Resistance mechanisms for heavy metals, antibiotics, organic solvents and other substances have been described in great detail (Chandrangsu et al., 2017; Hughes and Andersson, 2017; Ramos et al., 2002). In contrast, not much is known on how different resistance determinants evolved together and how much these resistances are simultaneously regulated. The importance of chemicals other than antibiotics causing cross-resistance may be overlooked. It is clear that the presence of metals in the environment co-selects for resistance to antibiotics (Song et al., 2017; Wu et al., 2016; Pal et al., 2017). Here we review recent studies that suggest various chemicals or chemical mixtures promote AR. Research on this front is vastly needed and chemical mixtures' role in AR should be taken into account when the overall strategy to counter AR is considered.

1. Chemicals or chemical mixtures and antibiotic resistance

Among all chemicals, metals are probably the most thoroughly studied in relation to AR. The association of AR with metals has been reviewed extensively (Baker-Austin et al., 2006; Pal et al., 2017). Metal mixtures, along with antibiotics, are often used as feed supplements in animal farms. High throughput analysis of ARGs from pig farms and impacted soils reveals that ARGs can be enriched up to 28,000-fold (Zhu et al., 2013). Despite locations separated by over 1000 km, the diversity and abundance patterns of ARGs show similar profile of the same management types. More importantly, the abundance of ARGs is correlated with the concentrations of antibiotics, and metals such as copper, zinc, and arsenic, suggesting metals provide selection pressure for AR (Zhu et al., 2013).

In pure cultures, individual metals have been found to induce AR. In a bacterium LSJC7, arsenic, copper, and zinc enhanced the resistance towards tetracycline (Chen et al., 2015). This is further demonstrated by the surface-enhanced Raman scattering (SERS) technique (Cui et al., 2016), in which spectral changes representing phenotypic bacterial responses, in combination with multivariate analysis, indicated that arsenic enhanced the resistance to tetracycline.

In addition to the more thoroughly studied metals, other chemicals are increasingly found to be linked to the rise of AR. Halogenated nitrogenous disinfection byproducts (N-DBPs) are a group of unintended byproducts formed during chlorination or chloramination for treatment of drinking water. It has been found that exposure to bromoacetamide, trichloroacetonitrile or tribromonitromethane, three representatives of N-DBPs, increased the resistance of *Pseudomonas aeruginosa* PAO1 to both individual and multiple antibiotics (Lv et al., 2015). The same induction phenomena were also observed in *Escherichia coli*, raising concerns about the rise of AR in drinking water.

Triclosan is an antiseptic present in many health care and consumer products, such as soaps, lotions, toothpaste, and some commonly used household fabrics and plastics. It has been shown that a *P. aeruginosa* mutant, which is susceptible to triclosan due to the deletion of triclosan-resistant MexAB-OprM efflux system, selects multidrug-resistant bacteria at high frequencies when exposed to triclosan (Chuanchuen et al., 2001). The minimum inhibitory concentrations (MICs) of several antibiotics for some of the mutants were increased up to 500-fold.

In wastewater treatment plants, UV/H_2O_2 process is considered an effective method to control spread of antibiotic resistant bacteria. Although UV/H_2O_2 process is effective for bacterial inactivation, it's not effective in ARGs removal from water suspension (Ferro et al., 2016). Actually, an increase of antibiotic resistance gene bla_{TEM} was observed in total DNA after 240 min treatment. Chlorination is commonly used for treatment of wastewater and disinfection of drinking water. It has been shown that after chlorination, a higher proportion of the surviving bacteria is resistant to several antibiotics (Shi et al., 2013). Chlorination results in enrichment of some ARGs. Osmotic stress can also influence microbial susceptibility to antibiotics. For example, when isolates of *Listeria monocytogenes* are exposed to different concentrations of salt, their resistance to antibiotics increases as salt concentration increases (Al-Nabulsi et al., 2015).

Some herbal extracts, such as essentials oils, have been used in various consumer products. Pine oil, a disinfectant used in household products, has been found to select mutants

of *E. coli* for resistance to multiple antibiotics (Moken et al., 1997). The *Thymus maroccanus* essential oil and its major components, can select *E. coli* strains for resistance to a number of antibiotics (Fadli et al., 2014). Human pathogens exposed to sub-lethal concentrations of tea tree oil have displayed reduced susceptibility to clinically relevant antibiotics (McMahon et al., 2007). However, one study found no evidence to suggest that tea tree oil induces resistance to antibiotics (Thomsen et al., 2013). A clinically isolated *Salmonella* Senftenberg, when exposed to linalool, a component of basil oil, exhibited increased MICs to several antibiotics by 2- to 32-fold (Kalily et al., 2017).

When one *Staphylococcus aureus* reference strain and 14 clinical isolates were exposed to *Rhizoma coptidis* extract, 11 isolates were found to cross-resistant to at least one antibiotic, measured by MICs (Wu et al., 2016). Moreover, all isolates were cross-resistant to more than one other antibiotic when exposed to tetracycline.

The mechanisms of AR selected by these chemicals seem diverse, although mutations in resistance genes may play a critical role. In the case of N-DBPs, mutations are observed in ARGs, both in *P. aeruginosa* and *E. coli* (Lv et al., 2015). Triclosan selects multidrug-resistant bacteria with mutations in regulatory gene *nfxB*, hyperexpressing the MexCD-OprJ efflux system (Chuanchuen et al., 2001). The mycobacterial transcriptional regulator WhiB7 autoregulates its own promoter in response to both antibiotics and redox changes, suggesting a link among cell metabolism, redox homeostasis, and AR (Burian et al., 2012).

Besides by mutations, microbial resistance to antibiotics can occur by acquisition of resistance conferring genes *via* horizontal gene transfer (HGT). Evidences are emerging that some chemicals induce HGT. Six organic compounds, representative ones used in textile dyeing wastewater, are found to facilitate the transfer of resistance-carrying RP4 plasmid from *E. coli* HB101 to *E. coli* NK5449 (Jiao et al., 2017). The highest transfer frequencies range from 4- to 200-fold higher than those in the control group. Nanomaterials have been widely used nowadays. It has been found that nanoalumina can promote the horizontal conjugative transfer of multiresistance-genes-carrying RP4 plasmid across genera, from *E. coli* to *Salmonella* spp., by up to 200-fold compared with untreated cells (Qiu et al., 2012).

In agriculture, sewage sludge and manure are commonly used as soil amendments. Long-term application of sewage sludge and chicken manure to soil increases the abundance and diversity of ARGs significantly, with enrichment of 108 unique ARGs and mobile genetic elements (MGEs) up to 3845 folds (Chen et al., 2016). Not surprisingly, organically produced lettuce has 8 fold higher of ARGs, measured in absolute copy numbers, in its phyllosphere than conventionally produced lettuce (Zhu et al., 2017). As reviewed above, metals and antibiotics probably promote AR in sewage sludge and manure. However, given the complex nature of sewage sludge and manure, other chemicals may also play a role.

A brief summary of selected chemicals or chemical mixtures mentioned in this paper is shown in Table 1. A common feature of the agents promoting AR, reviewed here, is that they all put stress on bacteria to some extent. Thus we propose that other agents, as well as physical, chemical, or biological conditions stressing bacteria, have the potential to induce AR development.

2. How chemical mixtures select for antibiotic resistance?

2.1. Co-resistance

Evolution would favor microorganisms that can best adapt to mixed contaminants when these contaminants often occur together. For example, we previously determined that arsenic and copper resistance determinants often occur together on plasmids (Hao et al., 2017). We suggested copper and arsenic often occur together both in the environment and in protozoan predation (Hao et al., 2016, 2017). There are many instances where heavy metal resistance genes (HMRGs) and antibiotic resistance genes (ARGs) are located on the same plasmid and in close vicinity on the chromosome. This is co-resistance and the basis for many instances of co-selection (Li et al., 2017; Pal et al., 2017). For example, plasmid pAFS11, isolated from a bovine methicillin resistant *S. aureus* (MRSA), contains five different antimicrobial resistance genes, two heavy metal resistance gene operons and an *ica*-like gene cluster. The heavy metal resistance determinants confer resistance to copper and cadmium, the antimicrobial resistance determinants confer resistance to apramycin, macrolides, tetracycline, kanamycin and trimethoprim. The *ica*-like gene cluster is probably involved in biofilm formation (Fessler et al., 2017). In addition, plasmids in the HI incompatibility group (IncHI) occur widely in the Enterobacteriaceae and can be readily isolated from humans and chickens, and sporadically from swine. IncHI2 could frequently be isolated from *Salmonella enterica*, *Enterobacter cloacae*, *Klebsiella pneumonia* and *E. coli*. Due to use of copper and zinc as growth promoters in poultry and swine in addition

Table 1 – Chemicals or chemical mixtures promoting antibiotic resistance.

Chemicals/mixture (concentration)	Antibiotics involved	Microbes tested	References
As, Cu, Zn (2 mmol/L and up)	Tetracycline	Enterobacteriaceae LSJC7	Chen et al. (2015)
Linalool (up to 0.96 mol/L)	Multiple	*S.* Senftenberg	Kalily et al. (2017)
NaCl (6% and up)	Multiple	*L. monocytogenes*	Al-Nabulsi et al. (2015)
N-DBPs (1 ppm and up)	Multiple	*P. aeruginosa* PAO1, *E. coli*	Lv et al. (2015)
Pine oil (0.3% and up)	Multiple	*E. coli*	Moken et al. (1997)
Rhizoma coptidis extract (0.95 mg/mL and up)	Multiple	*S. aureus*	Wu et al. (2016)
Tea tree oil (0.075%)	Multiple	*S. aureus*	McMahon et al. (2007)
Thymus maroccanus essential oil (0.23 mL/L)	Multiple	*E. coli*	Fadli et al. (2014)
Triclosan (24 μg/mL and up)	Multiple	*P. aeruginosa*	Chuanchuen et al. (2001)
UV/H_2O_2 (20 mg/L)	β-Lactams	*E. coli*	Ferro et al. (2016)

to roxarsone (organic arsenic compound) and antibiotics, resistance determinants against many of these compounds can readily be found. A recent study isolated 25 *E. coli* strains carrying an IncHI2 plasmid conferring resistance to copper and silver as well as fluoroquinolones and ciproflaxin (Fang et al., 2016).

Co-resistance is best studied in mobile genetic elements, *e.g.*, plasmids that contain multiple resistance determinants. These could be genes or operons conferring resistance to antibiotics, solvents or heavy metals (Baker-Austin et al., 2006; Summers, 2006). Microbes can grow faster with less genetic information owing to reduced metabolic burden in DNA replication, transcription, and translation. Genome reduction is one of the drivers in bacterial evolution (Moran and Bennett, 2014). This also makes sense in the context of pollutants since they might only occur infrequently and the presence of these resistance determinants would slow down growth. Therefore, the microbial community could grow faster but would still be protected if a subset of the population contains resistance determinants on a mobile genetic element, *e.g.*, a plasmid as the presence of this genetic information will be selected for once at least one contaminant is challenging this microbial community. It has been pointed out before that a single challenge, for example mercury, will not only select for the presence of a mercury resistance determinant on a mobile element but also co-selects for the presence of all the other resistance determinants encoded on that mobile element (Summers, 2006). However, one should also ask whether the presence of the other resistance determinants is random. From an evolutionary standpoint, this would not make sense. The make-up of chromosomes and mobile genetic elements is based on the history of selection. In nature, microbes are often challenged by complex mixtures where the composition of these is not random. For example, copper, zinc and antibiotics such as tetracycline are often used in pig and poultry farming. Therefore, selection would favor the presence of copper, zinc and tetracycline resistance determinants on mobile genetic elements in exposed microbial communities (Zhu et al., 2013). Hot thermal vents are high in metals and metalloids but also in other antimicrobial compound therefore selecting for the presence of these determinants (Farias et al., 2015). However, it should be pointed out that the increased presence of tetracycline resistance determinant in environments where no tetracycline is added does not suggest tetracycline now poses a threat in these communities. It often means that multidrug efflux pumps are induced or their genetic information transmitted that confer resistance to a variety of often unrelated substances that might also include tetracycline.

2.2. Cross-resistance

Cross-resistance is a different answer to the same problem; how can microbes protect themselves from complex mixtures? Transporters could have a broad substrate spectrum and be involved with efflux of heavy metals, biocides and certain antibiotics. For example, resistance to solvents has been linked to the presence of efflux pumps; at the same time the presence of these efflux pumps such as resistance, nodulation, division (RND)-type transport systems confers resistance to certain antibiotics (Fernandes et al., 2003). This would be termed cross-resistance. RND-type transports systems are multicomponent, proton-gradient driven transport systems that transport a variety of substances mostly from the periplasm across the outer membrane into the extracellular space (Anes et al., 2015). Since they transport such a variety of mostly hydrophobic substrates they have been dubbed periplasmic vacuum cleaners (Lomovskaya and Totrov, 2005). The promiscuity of substrate binding and transport thus insures protection against a broad spectrum of substrates (Conroy et al., 2010).

2.3. Co-regulation

Another possibility of co-selection is co-regulation. For example, certain HMRGs and ARGs could be regulated in response to the same environmental cues such as species. In this context it is of interest that copper and zinc can be involved in inducing resistances to substrates such as the antibiotics novobiocin and deoxycholate (Nishino and Yamaguchi, 2001). It has been shown that a two-component system in *S. enterica*, BaeSR, responded to a wide variety of environmental cues including tannin, indole, and flavonoid but surprisingly also to high concentrations of copper and zinc (Wang and Fierke, 2013). BaeRS controls regulation of eight genes, *mdtA*, *mdtB*, *mdtC*, *mdtD*, *baeS*, *baeR*, *acrD* and *spy*. MdtA, MdtB and MdtC form a RND-type trans-envelope efflux multiplex with the outer membrane pore protein TolC in *E. coli*. AcrD is homologous to AcrB and forms the inner membrane component of a typical RND-type transport complex, MdtD is a member of the major facilitator superfamily where the physiological function is yet not known. MarR senses and binds Cu(II) which leads to derepression of multiple antibiotic resistance regulatory operon *marRAB* in *E. coli*, causing enhanced bacterial antibiotic resistance (Hao et al., 2014). SoxS was also shown to upregulate expression of *acrAB-tolC* in response to the presence of chromate and copper in *E. coli* resulting in increased resistance to a number of antibiotics. *Enterobacter* LSJC7 displays tetracycline resistance that is induced by arsenic, copper and zinc. Interestingly, LSJC7 also contains *baeRS, acrD, mdtABC, soxS*, *acrAB, tolC, marRAB* and could thus display a similarity to the co-regulation described in *E. coli* and *S. enterica* (Chen et al., 2015). The Cpx stress response in *E. coli* is induced by misfolded proteins but also copper (Mahoney and Silhavy, 2013; Raivio et al., 2013; Yamamoto and Ishihama, 2006). The upregulated genes code for proteins involved in protein degradation like DegP and aiding membrane integrity. In addition, the Cpx stress response influences antibiotic resistance and other functions involved in energetics (Raivio et al., 2013). It is evident that stress responses can be determinants that compromise bacterial antimicrobial activity (Poole, 2012).

In *P. aeruginosa* PAO1 the two components system CzcRS induces zinc-dependent expression of the RND-type transport system CzcCBA responsible for zinc and cadmium resistance. Resistance to imipenem is achieved by downregulation of the expression of *oprD* encoding the outermembrane protein OprD that is the entry point for imipenem. When there is

less OprD present, there would be reduced uptake of carbapenems (Perron et al., 2004).

Why should copper and zinc induce expression of genes that appear not to be connected to conferring copper and zinc resistance? One highly likely scenario would predict that exposure to these substrates occurs at the same time. It has recently been shown that amoeba, and macrophages as part of the innate immune system both use copper and zinc to kill bacteria or fungi (Hao et al., 2016). This selective pressure would select for the presence of copper and zinc resistance determinants as amoeba (Protista) are predators of microbes in many environments. In addition to metals, Protista and macrophages also use reactive oxygen and nitrogen species, acidic conditions, proteases and antimicrobial peptides as weapons to kill bacteria and other microbes (Akya et al., 2009; Zhang and Soldati, 2013).

Antimicrobial peptides (AMPs) have been studied in great detail as an important component of the immune system (Brogden, 2005). However, AMPs are also present as amoebapores and nemapores in the immediate predators of bacteria in the environment, protista and nematodes (Tarr, 2012). These AMPs contain a saposin domain and share similarity with protozoan amoebapores and mammalian NK-lysin and granulysin (Leippe, 1995). These AMPs were inserted into the membranes and form deleterious pores or channels. Therefore, bacteria had to develop resistance mechanisms against this deadly threat (Joo et al., 2016). AMPs have not been studied much in protozoans yet so at this point we know but a fraction of possible compounds employed as AMPs in protista. Antimicrobial peptides, rather than conventional antibiotics, are a natural component in the host environments. Thus, it is more likely that expression of the tripartite multidrug efflux systems actually is to facilitate bacterial resistance to antimicrobial peptides when bacteria are located in certain host niches. We propose that protection from AMPs by multidrug type efflux pumps is important for survival when escaping predation and the presence of multidrug efflux pumps is therefore selected for (Weatherspoon-Griffin et al., 2014). Since metals such as copper are also used by protists as a weapon to kill microbes it makes sense they are co-regulated. In support of this hypothesis, the copper and envelope-stress induced Cpx stress response was shown to activate *mar* transcription and thereby facilitating *E. coli* resistance to a model antimicrobial peptide *via* tripartite multidrug transporters. MarR as mentioned earlier is also directly derepressed by copper and directly involved in induction of increased expression of multidrug transporters and hence resistance against antimicrobial peptides.

While this is still hypothetical, it is necessary to understand the challenges microbes face in nature and how those challenges drive selection. In the case of AMPs and metals such as copper and zinc that challenge could be predation. Nevertheless, antibiotics can also play a role in the natural environment such as for microbes living in the vicinity of Actinomycetes or Streptomycetes. However, we believe predation would also pose a general and widespread challenge. It is important to realize that all described mechanisms, co-resistance, cross-resistance and co-regulation, lead to co-selection (Fig. 1).

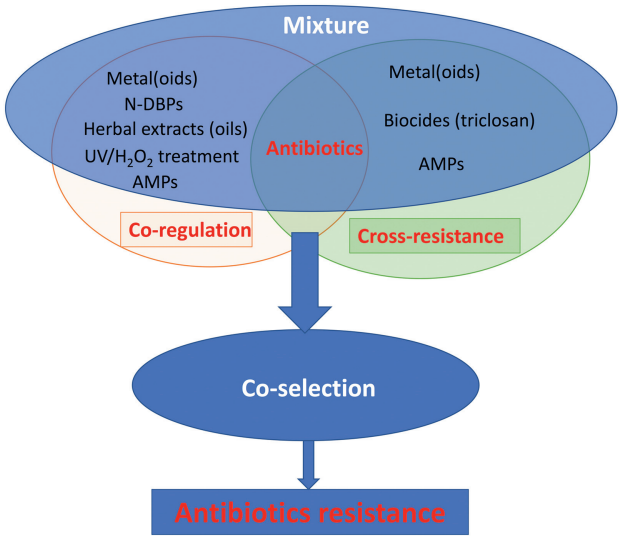

Fig. 1 – Mechanisms for co-selection of the resistance to antibiotics and chemical mixtures. N-DBPs, Halogenated nitrogenous disinfection byproducts. AMPs, Antimicrobial peptides.

3. Conclusions and future perspectives

There is a general knowledge gap regarding the impacts of chemical mixtures on ecosystem and human health. Interactions between chemical mixtures will involve both chemical and biological processes. Antibiotic resistance is not only a global environmental health problem, but also a topic closely related to environmental contamination of chemical mixtures. It is therefore suggested that antibiotic resistance is one of the priority areas for tackling chemical mixtures.

Future research should focus on: (1) what are the mode of actions that specific chemical mixtures induce antibiotic resistance? (2) how to unravel the quantitative relationship between chemical mixtures (both composition and concentration equivalence) and antibiotic resistance?

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