

==== REVIEW ====

Sources and mechanisms of combined heavy-metal and antibiotic resistance traits in bacteria

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SUMMARY: Nowadays, antibiotic resistance poses a great threat to the health of the individuals worldwide. In this context, scientific interest on how bacteria adapt in stress-related environmental conditions like those enriched in heavy metals and how the heavy-metal adaptive mechanism influence the antibiotic resistance is increasing. It was noted that the simultaneous use of heavy metals and antibiotics in agriculture and aquaculture might positively impact the dissemination of the antibiotic resistance genes in the environment. Current knowledge on the sources of simultaneous pollution with heavy metals and antibiotics, the co-occurrence of heavy-metal and antibiotic resistance traits in bacteria altogether with physiological mechanism underlying this phenomenon are overviewed.

Keywords: agriculture, antibiotic resistance, aquaculture, heavy metal resistance.

Introduction

Besides the wide use in the clinical field to treat a large variety of diseases caused by bacterial infections, antibiotics are also applied as growth promoters in agriculture and aquaculture. At the same time, high concentrations of heavy metals in soils and waters of agricultural interest might originate either from anthropogenic

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activities (such as mining, smelting, waste disposal, vehicle exhaust, sewage sludge, pesticides, fertilizers application) (Binggan and Linsheng, 2010) or natural causes (Tchounwou *et al.*, 2012). Co-existence of heavy metals and antibiotics occurs in various environments such as human and mammalian guts, livestock and animal manure, poultry and fish farms (aquaculture) etc. (Fig. 1) (Heuer *et al.*, 2009; Chen *et al.*, 2015).

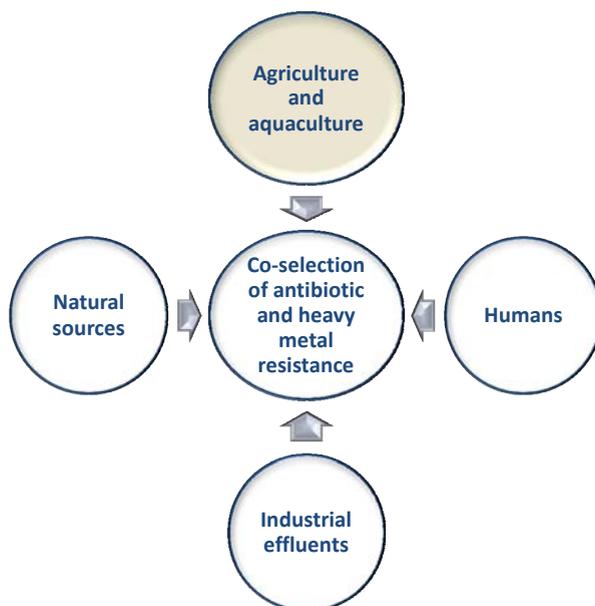


Figure 1. The sources of combined antibiotic and heavy metal contamination in natural environments. The agriculture and aquaculture (emphasized in gray background) are most frequent sources for simultaneous antibiotic and metal pollution.

Besides their various toxic effects on human health (Tchounwou *et al.*, 2012), presence of elevated levels of heavy metals (mostly divalent Cu^{2+} , Zn^{2+} , Pb^{2+} , Cd^{2+} but also zerovalent Hg^0 and trivalent/hexavalent $\text{Cr}^{3+/6+}$) in the environment was shown to stimulate the enrichment of indigenous organisms (mainly *Bacteria*) which bear antibiotic resistance genes (Baker-Austin *et al.*, 2006). Stressful conditions created by the co-existence of heavy metals and antibiotics triggers the onset of adaptive mechanisms that include, for example, extracellular immobilization or efflux pumps helping the affected microbial cells to entrap or expel toxic compounds. Additionally, manifold increase of antibiotic resistance genes was correlated with the high concentration of the heavy metals in the environment (Chen *et al.*, 2015).

Understanding the mechanisms underlying co-selection of heavy-metal and antibiotic resistance capabilities in microorganisms of medical and environmental relevance is crucial for understanding the patterns of their dispersal and persistence in various natural or antropoc environments (Resende *et al.*, 2012). Moreover, such knowledge would allow i) designing of strategies to monitor and/or prevent spreading of toxic antibiotics and heavy metals; ii) bioprospecting of microbes to aid in xenobiotics and metal bioremediation.

The present review discusses recent advances on the links between heavy metal and antibiotic resistance and the bacterial mechanisms underlying the simultaneous adaptation to both types of pollutants.

The use of heavy metals in agriculture and aquaculture

Heavy metals are extensively and widely used in agriculture due to their capacity to improve the efficiency of animal growth, quality of the meat production and the fitness of the animals providing a healthier trait (Zhang *et al.*, 2012). Copper (Cu^{2+}) used as copper sulfate (CuSO_4) is well known as biocidal compound with bactericidal and fungicidal effects. Therefore, it is used in foot-bath in milking yards to treat digital dermatitis that can lead to lameness, unless untreated, in dairy cattle (Thomsen *et al.*, 2015). Copper sulfate is also used in piggery and poultry units as growth promoter (Zhang *et al.*, 2012; Yazdankhah *et al.*, 2014). Furthermore, it is used on laying hen for lowering the cholesterol content in eggs (Karimi *et al.*, 2011). Zinc (Zn^{2+}) used as zinc white (ZnO) is added in pig feed (24 - 33 mg/ kg) diet for preventing and curing parakeratosis and stimulate growth (Hill *et al.*, 1986; Forrest, 2012). For poultry units zinc acts like a growth promoting factor influencing skeletal and feather development and also the reproduction system (Forrest, 2012). It was recognized that feeding 20-35 mg Zn/kg will meet the daily diet requirement of the chicks (Underwood and Suttle, 1999). Arsenic (As^{3+}), although well known for its toxicity, is used in animal feed to help in gaining weight thus enhancing the feed efficiency (Zhang *et al.*, 2012). Arsenic derivatives are also used as medicine against external parasites and protozoa (Banu *et al.*, 1982). Arsenic confers pigmentation of the animals improving their aspect (Li and Chen, 2005; Jackson *et al.*, 2003). Although cadmium (Cd^{2+}) is not added in animal feed, it is spread as residuals in agriculture mineral supplements (e.g., in phosphates, zinc sulfate, zinc oxide etc.) because of close chemical similarity with Zn^{2+} (Plum *et al.*, 2010).

The rapid growth of population in the last century resulted in sharp increase in the demand of seafood products (fish and edible invertebrates). Intensive seafood farming (or *aquaculture*) uses formulated feeds containing antibiotics, antifungals and other pharmaceuticals in addition to application of pesticides and disinfectants. Moreover, human and animal faeces are often used as source of food in aquaculture (Sapkota *et al.*, 2008). As a consequence of their addition in the mineral supplements to stimulate growth, heavy metals such as Zn, Cu, Fe, Mn, Pb, Cd, etc. accumulate in tissues (liver, muscle, shell) of farmed animals (Wu and Yang, 2011).

Pollution and accumulation of heavy metals in the marine environment

The main sources of coastal heavy metal pollution may include ship dismantling (Hossain and Islam, 2006), mining, industrial and urban discharge through polluted rivers (Morillo *et al.*, 2004; Gao and Chen, 2012). Heavy metals tend to enrich in the coastal and deep sea sediments, subsequently entering the food chain through bioaccumulation and biomagnification, and thus affecting the marine ecosystems (Gao and Chen, 2012; Kacar and Kocyigit, 2013). The presence of heavy metals in wild seafood (shrimps, shellfish and finfish) raises great health concern for the consumers. Even the fishes in cage aquaculture contain high levels of heavy metals. When exposed for 28 days with daily feed of 0.01 mg Cu/L or 0.016 mg Pb/L, carp (*Cyprinus carpio*) accumulate up to 21.53 mg Cu/kg and 7 mg Pb/kg, respectively (Salami *et al.*, 2008).

Microbial diversity of heavy metal contaminated aquatic sediments

Diversity of microbial communities associated with (usually mixed) heavy metal (and other pollutants)-contaminated marine sediments was explored revealing that most frequently retrieved *Bacteria* belonged to Proteobacteria (γ -, δ - and ϵ -Proteobacteria), Firmicutes and Bacteroidetes phyla (Gillan *et al.*, 2005; Zhang *et al.*, 2008; Quero *et al.*, 2015), whereas archaeal communities were dominated by Methanobacteria and Methanomicrobia (Quero *et al.*, 2015). Moreover, increase in the diversity and abundance of genes involved in heavy metal tolerance were reported in metal-contaminated aquatic (riverine) sediments by GeoChip 5.0 functional gene array (Jie *et al.*, 2016). Among all heavy metals, cadmium was found to be most toxic, whereas lead was generally best tolerated in representatives of bacterial communities investigated from metal-contaminated samples tested for metal susceptibility (Table 1).

Table 1.

Toxic metal susceptibility of bacterial representatives of microbial communities associated with sediments contaminated with heavy metals

Source	Metal sensibility ranking	Reference
Golden Horn Estuary	Cu>Mn>Ni>Zn>Pb>Cd>Fe	Altug and Balkis, 2009
Iskenderun Bay	Cd>Cu>Cr>Pb>Mn	Matyar <i>et al.</i> , 2008
Eastern Aegean Sea	Hg>Cd>Cr>Zn>Cu>Co>Ni>As>Pb	Kacar and Kocyigit, 2013
The Eastern Harbour, Egypt	Cd>Co>Zn>Ni>Hg>As>Cu>Pb	Sabry <i>et al.</i> , 1996
Equatorial Indian Ocean	Cd>Zn>Cu>Pb	Devika <i>et al.</i> , 2013

Antibiotics and their use in agriculture and aquaculture

Antibiotics have been largely used in agriculture and aquaculture for decades. They were seen as very effective especially by protecting livestock from pathogens and, thus, usually administered as preventive measure (Jassim and Limoges, 2014). Classes of antimicrobials like tetracyclines, macrolides, beta-lactams, streptogramins, and sulfonamides may be used at different times during the life cycle of poultry, cattle, and swine (Sarmah *et al.*, 2006). For example, by 2012, 16% of all lactating dairy cows in the U.S. received antibiotic therapy for clinical mastitis each year, but nearly all dairy cows received intramammary infusions of prophylactic doses of antibiotics following each lactation to prevent and control mastitis, mostly with penicillins, cephalosporins, or other beta-lactam drugs. Also, beef calves that enter feedlots were treated with antibiotics for clinical respiratory disease. Moreover, therapeutic antibiotic doses have been administered to healthy calves to prevent outbreaks. To ensure growth, calves are fed a nourishment supplemented with tylosin, a macrolide drug, to prevent liver abscesses. Growing swine were commonly treated with tetracyclines or tylosin for growth promotion and disease prevention (Landers *et al.*, 2012).

Large amounts of antibiotics were used in fish feed for prophylactic and therapeutic purposes before being replaced by vaccines (Seiler and Berendonk, 2012). Oxytetracycline and chloramphenicol were two of the most used antibiotics in aquaculture (Sapkota *et al.*, 2008). Extensive use of multiple antibiotics in aquaculture led to the rise of cross-resistance to a certain type of antibiotic and to multi-drug resistance. For example, in farmed shrimp, infections with vibrios multiresistant to penicillin, tetracycline, and ampicillin were reported. In addition, the most common cross-resistance was observed in penicillin-cephalothin relation (Costa *et al.*, 2015).

Factors that drive the accumulation and spread of antibiotic and heavy-metal resistant bacteria

Both heavy metal- and antibiotic-resistant bacterial strains are disseminated into the environment through animal manure (Chen *et al.*, 2015). Natural fertilizers from farms used for agricultural purposes may contribute to the enrichment of the genes responsible of antibiotic resistance (or 'resistome') in soil (Forsberg *et al.*, 2012) and also to the dissemination of resistance genes in the environment. The *resistome* was defined as the entire pool of antibiotic determinants and their precursors in a microbial community (Wright, 2007). Humans also play an important role in the dissemination of the antibiotic resistance genes by consuming antibiotics to treat bacterial infections. The extensive use of antibiotics by humans led to an increased resistance in the human-associated bacterial resistome. Moreover, hospitals contribute to

the uncontrolled dispersal of antibiotics by discharging insufficiently treated wastewater into rivers or lakes (Matyar *et al.*, 2010; Amador *et al.*, 2015, Țugui *et al.*, 2015; Szekeres *et al.*, 2017). As a consequence, the emergence of bacteria with multiple antibiotic resistance and increased virulence became a global issue (Walker *et al.*, 2009; Guidos, 2011).

By co-existing in the same environment, heavy metals can influence the resistance to antibiotics (Chen *et al.*, 2015) and are able to co-select for different types of antibiotic resistance, thus influencing survival of certain bacteria in a more polluted environment. For instance it has been noticed that resistance to Zn/Cd co-occurs with aminoglycoside/macrolide resistance. Also copper in the form of CuSO₄ is related to higher ampicillin resistance in soil samples (Berg *et al.*, 2005). It has been stated that metals like copper, zinc, antimony, cobalt, nickel, cadmium, iron, and mercury are all co-selectors for strain resistance. Antibiotics like tetracycline, chloramphenicol, ampicillin, and gentamicin have a strong relation with various heavy metal resistance (Table 2). On the other hand, three types of resistance genes for arsenic, copper and silver cluster separately from antibiotic resistance genes on plasmids making it possible to select among each other. This fact suggests that these MRGs are less likely to select for antibiotics (Pal *et al.*, 2015).

Mechanisms of metal tolerance and its influence on the antibiotic resistance

The proliferation of antibiotic resistance can be determined by heavy metal contamination of the environment, as there are clear indications on the coupling of resistance mechanisms against antibiotics and heavy metals. The co-selection mechanisms include *cross-resistance* and *co-resistance*.

Cross-resistance occurs on mobile elements (plasmids, transposons) and inquires genes encoding for generic detoxifying mechanism like efflux pumps which non-specifically reduce intracellular concentrations of both metals and antibiotics. On the other hand, **co-resistance** involves separate genes which are integrated on the same genetic element (Knapp *et al.*, 2011).

Microbial strategies to overcome toxic levels of heavy metals

Through evolution, bacteria has developed different adaptive strategies to tolerate elevated metal levels. Generally, three mechanism of heavy metal resistance are known in prokaryotes (*Bacteria* and *Archaea*) (Silver, 1996; Nies, 2003; Voica *et al.*, 2016):

- passive extracellular or intracellular **sequestration** of toxic metals either by extracellular polymeric substances or intracellular compounds such as polyphosphates;

- **detoxification** through chemical neutralization of intracellular ions by enzymes (for example by the activity of mercury reductase encoded by *merA* gene, Seiler and Berendok, 2012);

- active **extrusion** of toxic ions by efflux systems (or ‘efflux pumps’). An example in this case is Czc which is a cation/proton antiporter mediating resistance to divalent metal ions like Cd^{2+} , Zn^{2+} , and Co^{2+} by expelling metals from the cytoplasm through the inner and outer membrane to the surrounding environment (Silver, 1996; Nies, 2003; Baker-Austin *et al.*, 2006; Seiler and Berendok, 2012).

Table 2.

Co-occurrence of heavy metals and antibiotic resistance in various bacterial strains

Heavy metal	Antibiotic categories /Generic names	Source
Cu	tetracycline	soil bacteria Scotland(a), Fecal <i>Enterococci</i> (b)
	carbapenem	<i>Pseudomonas aeruginosa</i> (c)
	vancomycin	<i>Enterococcus faecium</i> (d)
	chloramphenicol	bacteria isolated from ship (e)
	erythromycin	soil bacteria Scotland (a), <i>Enterococcus faecium</i> (d)
Hg	sulfonamide	<i>Salmonella enterica</i> (f), Fecal Gram-negative bacteria (g)
	chloramphenicol	<i>Salmonella enterica</i> (f), Fecal Gram-negative bacteria(g)
	ampicillin	soil bacteria Kenya (h), <i>Salmonella enterica</i> (f), Fecal Gram-negative bacteria (g)
	streptomycin	<i>Salmonella enterica</i> (f), Fecal Gram-negative bacteria (g)
	augmentin	soil bacteria Kenya (h)
Zn	norfloxacin	soil bacteria Kenya(h)
	augmentin	soil bacteria Kenya (h)
	gentamicin	soil bacteria Kenya h)
	ampicillin	soil bacteria Kenya (h)
	carbapenem	<i>Pseudomonas aeruginosa</i> (c)
Cr	tetracycline	soil bacteria Scotland (a)
	carbapenem	soil bacteria Scotland (a)
Fe	tetracycline	soil bacteria Scotland (a)
	ciprofloxacin	<i>Escherichia coli</i> (i)
Ni	tetracycline	soil bacteria Scotland (a)
Cd	augmentin	soil bacteria Kenya (h)
	ampicillin	soil bacteria Kenya (h), bacteria isolated from ship (e)

(a)-Knapp *et al.*, 2011(b)-Amachawadi *et al.*, 2013(c)-Li *et al.*, 2012(d)-Wales *et al.*, 2015

(e)-Kacar and Kocyigit, 2013

(f)-Baker-Austin *et al.*, 2006(g)-Wireman *et al.*, 1997

(h)-Budambula and Kinyua, 2013

(i)-Mehi *et al.*, 2014

Main mechanisms of antibiotic resistance in bacteria

Antibiotics may be combated by bacteria and archaea through three mechanisms (Kohanski *et al.*, 2010; D'Costa *et al.*, 2011; Wright and Poinar, 2012):

- by changing the target of the antibiotic compound as in Gram-positive and some Gram-negative bacteria, where antibiotics like aminoglycosides usually interact with the 30S ribosomal subunit blocking the protein synthesis and affecting the translation of mRNA (Tsai *et al.*, 2013);
- by inactivating the antibiotics using enzymes; for example AmpC beta – lactamases (a hydrolase) conferring resistance for different classes of beta-lactams;
- by active export of antibiotics through the efflux multidrug-resistance (MDR) pumps (i.e. MexAB, AcrAB and TolC are among the most thoroughly investigated efflux systems). Gram-negative bacteria that have higher prevalence of efflux pumps are resistant to many antibiotics like beta-lactams, cephalosporins, fluoroquinolones and gentamicin (Fair and Tor, 2014).

Common antibiotic and metal resistance systems

Strategies to respond the toxic effect of both heavy metals and antibiotics present in the environment were demonstrated in bacteria. Antibiotic and metal resistance systems were found to work in similar manner. For example, the passive mechanisms may involve the lowering of membrane permeability or sequestration. It was observed that the mutants of *Serratia marcescens* deficient in porins (outer membrane proteins, Omp's) acquired resistance for antibiotics like ciprofloxacin, tetracycline, chloramphenicol, beta-lactams (Ruiz, 2003). The sequestration of both drugs and heavy metals represents another common resistance system. Bacteria are able to sequester metal ions either by extracellular polymeric substances or intracellular polyphosphates (Voica *et al.*, 2016). Similarly, *E. coli* was capable to sequester antibiotics with the help of polypeptides (del Castillo *et al.*, 1991).

The active mechanisms for AB-HM detoxification may include secondary and primary efflux systems. For example, the efflux of drugs and metals has been hypothesized to co-work for pollutants like Cu, Cd, Zn, Co, tetracycline, chloramphenicol (Nies, 2003; Levy, 2002). ATPases (like the CadA or ArsA cadmium or arsenic resistance ATPases) may also play significant roles in combined antibiotic-metal resistance (Silver, 1996).

In some cases, single enzymes function as efflux pumps for multiple metals and antibiotics (Stepanuskas *et al.*, 2006). For example, DsbA-DsbB of *Burkholderia cepacia* KF1 is an efflux system formed of membrane-bound oxidoreductase (DsbB) and a periplasmic disulfide oxidoreductase subunit DsbA. This MDR system confers resistance to Cd and Zn and to a variety of beta-lactam antibiotics like kanamycin, erythromycin, novobiocin and ofloxacin (Hayashi *et al.*, 2000).

Plasmid vs. chromosome - different types of resistance genes and their localization

In a recent work, Pal *et al.* (2015) stated that co-selection of antibiotic resistant bacteria occurs when antibiotics and heavy metals are simultaneously present in the surroundings of bacteria possessing determinants for both types of resistance or tolerance. Genetic determinants for these capabilities were found to be both chromosome- and plasmid-borne. Resistance to sulfonamides, beta-lactams, aminoglycosides, and tetracyclines are coded by antibiotic resistance genes (ARGs) found both on plasmids and chromosomes. In addition chromosomal ARGs are involved in macrolide and chloramphenicol resistance. Metal resistance genes (MRGs) code for detoxification of mercury, cadmium, arsenic, and copper, whereas chromosomal MRGs are mostly responsible of arsenic, copper and chromium resistance (Pal *et al.*, 2015). Interestingly, MRGs were found to belong to a larger family of genes responsible for both biocide (e.g. disinfectants such as quaternary ammonium compounds, peroxides, acriflavines, etc.) and biocide/metal resistance genes (BMRGs). However it was shown that, when MRG's and ARG's co-occur on plasmids, the first tend to promote horizontal gene transfer (HGT) of antibiotic resistance genes through co-selection (Pal *et al.*, 2015).

In polluted environments bacterial isolates carried notably higher proportion of plasmids with genes and conjugative systems (Pal *et al.*, 2015).

Strategies for dissemination of metal/antibiotic resistance bacteria

Large bacterial dissemination is not possible without mechanisms to ensure the survival of descendant cells. One mechanism is called 'toxin-antitoxin system' and has a huge impact on the dissemination of bacteria containing MRGs. This system stabilizes plasmids in their host by killing daughter cells that do not inherit the plasmids (Gerdes *et al.*, 1986). This strategy helps bacteria to grow resistance faster. Instead of letting the environmental pressure to select fit individuals, bacteria might be able to regulate its fitness beyond environmental control.

An interesting evolutionary curiosity is the size of the plasmid and its potential to be transferred by HGT. Considering the fact that not all plasmids can be transferred to other bacteria, conjugative plasmids are larger than non-conjugative ones. In the study by Pal *et al.* (2015) it has been discovered that in a colony, 57% of plasmids carrying ARGs and BMRGs (were conjugative and their size was under 20 kb. Smaller plasmid (<10 kb) were able to contain only ARGs whereas plasmids over 250 kb contain BMRGs.

Conclusions

Human activities, especially through the land, animal or fish farming (i.e. agriculture and aquaculture-related activities) enrich and promote the heavy metal and antibiotic resistance in bacteria. In addition, industrial areas and other areas subjected to a high anthropic impact are supplementary sources for heavy metal pollution thus potentiating the metal and antibiotic co- and cross-resistance in natural environments. Heavy metals might favor the spread of the antibiotic resistance genes, making the bacteria fitter to survive in an antibiotic and heavy metal-polluted environment.

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