# Introduction to Microbial Ecotoxicology

The combined effects of mixtures of toxins and multiple stressors has long been a source of fascination for the field of academic ecotoxicology (Bliss, 1939). The environment today is awash with anthropogenic stressors, including potentially harmful chemicals (over 20,000 registered at last count (ECHA, 2018a)), habitat loss, climate change, and competition with invasive species.

Despite this level of interest, advances in knowledge have not translated to corresponding legislative changes, with existing mixture toxicity regulations limited and distinctly human-focused (European Commission, 2012). Consensus on experimental and statistical design has also been lacking, with studies applying a variety of potentially inappropriate models (Piggott, Townsend and Matthaei, 2015; Schäfer and Piggott, 2018), and a longstanding criticism from within the field of the difficulty of integrating ecotoxicological results into larger-scale understanding of how ecosystems respond to stress (Chapman, 2002; Gessner and Tlili, 2016).

A particular complication to more advanced ecosystem-level understanding of stressors is found at the highly consequential microbial layer. A great deal of research has been conducted into the bioremediation potential of individual bacterial isolates (Mary Kensa, 2011), the size, diversity and abundance of microbial life has limited both the commissioning and consistency of research on microbial ecotoxicology (Ghiglione, Martin-Laurent and Pesce, 2016) compared to more traditional clades of study, such as aquatic invertebrates and vertebrates.

Traditional null models of stressor interaction typically assume an additive interaction between stressors, whereby the combined effect of two or more stressors is equal to the sum of its parts (Piggott, Townsend and Matthaei, 2015). However, where stressors act by similar mechanisms or metabolic pathways, it is possible to hypothesise on their interactions.

## Chemical Stressors

Despite the high-profile banning of certain chemical pollutants – DDT, early organochlorides – a bewildering and unpredictable diversity of chemical stressors remain present in the environment. With a wide range of sources, structures and effects, these stressors can nonetheless be categorised into several broad families with similar modes of action or applications.

*Heavy Metals*

Heavy metals refer to a somewhat nebulous family of high-density metallic elements that are frequently toxic, sometimes essential nutrients, and produced as waste products by a wide range of industrial and agricultural processes (Tchounwou *et al.*, 2012). Heavy metals are regulated under the EU’s Water Framework Directive (WFD) (EU, 2000), which provides a relatively rich dataset of concentrations in UK tap and surface water (Drinking Water Inspectorate, 2010; Water Framework Directive implementation in England and Wales: new and updated standards to protect the water environment, 2014), while the UK soil and herbage pollutant survey (Environment Agency, 2007) provides relatively up-to-date data on soil heavy metal concentrations. Two heavy metals were selected for bacterial exposures, based on environmental impact and prevalence, mode of action, and lab safety:

Nickel (28Ni) is a common heavy metal and EU priority pollutant (EC, 2008), carcinogen (Shen and Zhang, 1994) a common alloy ingredient, and occasional essential nutrient in bacteria and fungi (Zamble, 2015). Defence systems against nickel are common in bacteria (Mighanetara *et al.*, 2009), but bacterial responses to environmental nickel vary from greedy consumption (Zamble, 2015) to enzymatic inhibition and disruption of iron and zinc homeostasis (Samland and Sprenger, 2006; Wang, Wu and Outten, 2011) necessitating a delicate balance of active efflux (Macomber and Hausinger, 2016) and sequestration (Nishimura, Igarashi and Kakinuma, 1998) with osmotic influx in order to maintain internal nickel at an optimum concentration. Nickel can also cause DNA damage via the generation of reactive oxygen species (ROS), although it is believed to be a minor source of oxidative stress compared to copper (Bal and Kasprzak, 2002).

Copper (29Cu) is another common heavy metal, used in alloys, electronics, anti-microbial surfaces and a wide range of sundry applications. Copper is an essential nutrient due to its presence in cytochrome-c-oxidase (Babcock and Wikström, 1992), a practically omnipresent respiratory enzyme. Bacteria have been shown to be extremely sensitive to copper, due to its disruption of vital biosynthesis enzymes, and effect resistance against it through active efflux, chelation, and rapid repair of damaged enzymes (Macomber and Imlay, 2009). Copper is also a prolific producer of harmful ROS (Bal and Kasprzak, 2002), although chronic copper toxicity is not typically of concern to humans and copper is not considered a carcinogen (ECHA, 2018b). While little mechanistic information is available, it appears that copper and nickel affect and are resisted by similar cell elements and pathways (Mykytczuk *et al.*, 2011).

*Antibacterials*

Antibacterials or antibiotics refers to a range of bactericidal agents that are used for the therapeutic treatment of infections thanks to their relative selectivity. The issue of environmental antibiotic pollution has attracted a great deal of scientific and legislative attention in recent years due to the threat of antimicrobial resistance (Holmes *et al.*, 2016; European Commission, 2017), but current understanding of how antibiotics will affect wild communities of bacteria, and the indirect effects on their ecosystems, is far more limited (Roose-Amsaleg and Laverman, 2016).

Despite this increasing focus on antibacterials as an emerging pollutant, their status in EU legislation remains in limbo, with plans to require environmental risk assessments on future pharmaceuticals dropped (Neslen, 2018), and no current legal requirement under the WFD to test surface, ground or drinking water for antimicrobial concentrations. Environmental concentrations are generally only available where specific studies have been conducted, and vary considerably with distance from point pollution sources. Antibacterials enter the environment from a variety of sources, including hospital wastewater, human excrement, and agricultural run-off.

Two common antibiotics were selected as stressors, the first being chloramphenicol, a broad-spectrum bacteriostatic first isolated from the bacterium *Streptomyces venezuelae* in 1947 and synthesised in 1949 (Rebstock *et al.*). Chloramphenicol has historically proved effective against a broad range of bacteria including *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Escherichia coli*, but in addition to its ineffectivity against *Pseudomonas aeruginosa* (Toku-E, 2018b), resistance is common both in the wild and in areas exposed to antibacterial pollution (Allen *et al.*, 2010). Resistance to chloramphenicol takes a variety of forms, including multidrug-resisting reduced membrane permeability (Cohen *et al.*, 1989), mutant ribosomes (Weisblum, 1995) or chloramphenicol-degrading enzymes (Shaw *et al.*, 1979). It has been suggested that many of the genes that provide resistance to common antibiotics including chloramphenicol also provide tolerance to environmental stress in non-pathogenic species (Groh *et al.*, 2007). Research has also found that exposure to ROS from heavy metals, including copper, can co-select for chloramphenicol resistance (Harrison *et al.*, 2009).

The second antibiotic of choice is amoxicillin or amoxycillin, a broad-spectrum antibacterial from the penicillin family that acts by interrupting bacterial wall synthesis, resulting in cell lysis (Pubchem, 2018). Amoxicillin is a broad-spectrum antibiotic that is effective against *Streptococcus*, some *Bacillus*, *Enterococcus*, *Haemophilus*, *Helicobacter*, but is resisted by *Citrobacter*, *Klebsiella* and *P.* *aeruginosa* (Toku-E, 2018a). Resistance to amoxicillin is widespread in both pathogens and the environment due to amoxicillin’s long life of use – since 1972 – in medical and veterinary applications (Boxall *et al.*, 2003; Palmer *et al.*, 2008). This resistance is most commonly effected through the synthesis of beta-lactamase, an enzyme that breaks down penicillin-family antibiotics, but can in other cases arise from active efflux. Initial *in-vitro* evidence suggests that in the human body amoxicillin and chloramphenicol may interfere, reducing their effectiveness (Nasir, Das and Hasan, 2016), although Olajuyigbe, Coopoosamy and Afolayan (2007) showed some additive but largely synergistic effects between the two across a diverse panel of bacteria.

*Chemical Pesticides*

Covering a broad range of biocidal agents designed or selected to target undesired pest species, pesticides have retained significant non-target effects despite advances in selectivity. While many more indiscriminate pesticides have been banned (EPA, 2017), many pesticides still in use today have been shown to have non-target effects on aquatic (Harrison *et al.*, 2009) and soil bacteria (Imfeld and Vuilleumier, 2012).

Two of the most environmentally prevalent and well-studied pesticides were selected: Atrazine, a triazine herbicide, has been banned in the EU since 2004 (EU, 2004), but has remained the most commonly used herbicide in the US, where over 42 million kg were used in 2016 (USGS, 2017). Atrazine, which acts on plants by disrupting photosynthesis (Shimabukuro and Swanson, 1969) has been shown to be both a food source (Wackett *et al.*, 2002) and ROS stressor (Zhang *et al.*, 2012) to various species of bacteria.

Metaldehyde, both the UK’s and the world’s most heavily used molluscicide is a highly mobile agent that frequently affects untreated terrain and waterways due to its propensity to run-off in surface water (Castle *et al.*, 2017). Metaldehyde is rapidly converted within the body of molluscs to aldehyde, which damages mucus producing cells, causing excessive mucus production, dehydration, and eventual death (Triebskorn, Christensen and Heim, 1998). Metaldehyde has occasionally caused deaths in humans at high concentrations (Thompson, Casey and Vale, 1995), but information on its effects on bacteria is scant: to date, one study has examined interactions between bacteria and metaldehyde (Thomas *et al.*, 2017), showing only that *Variovorax* and *Aceinetobacter* strains can be isolated from metaldehyde-treated soil and can degrade the molluscicide.

*Aromatic Hydrocarbons*

Aromatic hydrocarbons, characterised by the presence of one or more (polycyclic) aromatic rings, cover a range of carcinogenic agents (ATSDR, 2007) generally found in crude oil products or produced as a result of incomplete combustion.

Benzene, consisting of a single 6-carbon aromatic ring is frequently used as a chemical feedstock, solvent and formerly as fuel additive. Benzene is relatively unstable in the environment (Po Yung Lu and Metcalf, 1975), with a reported half-life of 17 days in water (Hustert *et al.*, 1981), although it is more frequently biodegraded. Benzene is a potential food source to some bacteria but a stressor to all, causing solvent stress to cell membranes, and has been shown to cause shifts in community abundance to gram-positive bacteria in a lab setting (Fahy *et al.*, 2008).

Benzo[a]pyrene is a high molecular weight polycyclic hydrocarbon consisting of six conjoined aromatic rings, principally produced by incomplete combustion of vehicle fuel or wood. Benzo[a]pyrene is highly toxic and carcinogenic to humans (Haritash and Kaushik, 2009), but may be less toxic to bacteria (Sverdrup *et al.*, 2007), many of which are capable of breaking it down for use as a food source (Meulenberg *et al.*, 1997).