Environmental Risk Assessment of Antibiotics: Investigations into Cyanobacteria Interspecies Sensitivities and Establishing Appropriate Protection Limits

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Abstract

Antibiotics have been described as a ‘wonder drug’ that have transformed medicine since their discovery at the beginning of the 20th century and are used globally in safeguarding human and animal health. Environmental risk assessment (ERA) aims to ensure their environmental safety by setting protection limits that seek to prevent adverse effects upon populations and ecosystem function. In the case of antibiotics however, there is concern that ERA may not be fully protective of bacterial populations.

This thesis examines the ERA of antibiotics and highlights that protection limits may in some cases be under-protective or over-protective for bacteria populations (including cyanobacteria), depending on the antibiotic mode of action and the species on which the protection limit is based. The first section of the thesis contains a systematic review including a meta-analysis of all publically available aquatic ecotoxicity data. The results illustrate that generally bacteria are the most sensitive taxa to antibiotics compared with eukaryotes but that interspecies variability in sensitivity among bacteria can range by up to five orders of magnitude. This far exceeds the assessment factor of 10 used to account for such uncertainty in protection limits. It also shows that the costly testing on fish may not be required and in accordance with the principle of the 3Rs could be excluded from the ERA of antibiotics, as they are not likely to drive the protection limit. Further, it demonstrates that protection limits established for antimicrobial resistance (AMR) may not always be protective of environmental health and that both protection limits should be determined in ERA.

Next, the thesis reports on the development and validation of a microplate assay for the rapid screening of chemical effects (here antibiotics) on cyanobacteria. The microplate assay is optimised to allow for the direct comparison of species sensitivity, ensuring consistent test conditions and thus limiting differences in antibiotic behaviour between assays. Reference toxicity testing with potassium dichromate demonstrates reproducibility over time and comparability with the standard shake flask test used in ERA.

The microplate assay is then used to experimentally confirm the findings of the meta-analysis. In this work, interspecies sensitivity across eight cyanobacteria species was demonstrated to vary by up to 70 fold following exposure to β-lactam antibiotics but only by an order of magnitude for macrolides. Cyanobacteria were not sensitive to sulfonamides and thus are not likely to be suitable for the setting of protection limits for this antibiotic class.

Finally in this thesis, species sensitivity distributions were created to examine how effective the protection limit currently derived in ERA is for antibiotics. For cephalosporins, there was a higher probability of under-protection whilst the protection limits were over-protective for classes of antibiotics with less interspecies differences in sensitivity, such as macrolides. Further, a probabilistic ecological risk assessment suggested that 60 to 100% of cyanobacteria species might be adversely affected at the higher measured environmental concentrations in the literature, while no significant risk was found at average concentrations.

The findings from this thesis illustrate that protection limits, as currently determined in ERA for antibiotics, may not be suitable for the adequate protection of cyanobacteria populations and most likely other bacterial taxa. The thesis proposes several approaches for improving ERA, including incorporating greater bacterial diversity in ecotoxicity testing, the inclusion of functional and/or community testing and the use of probabilistic methods to derive protection limits.

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Acknowledgements

That's it! 36,648 hours later, I have finally completed it. I have a thesis. Hopefully the findings will help, in some small way, to make the world a little bit better than it was.

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Chapter 1

General Introduction

General background

Microorganism communities in the environment

Microorganisms and their communities are vital components of all ecosystems, performing many services on which the normal functioning of the ecosystem relies. These functions include, but are not restricted to, biomass production, primary productivity, regulation of nutrient cycles and the biodegradation of pollutants. Furthermore, recent genomic and bioinformatic analyses have started to demonstrate the importance of microorganism abundance, metabolism and community structure in a multitude of ecological relationships related to animal health. For example, the interrelationships between the microbiota of a gut and the digestive, immune and mental well being of the animal host (Knip and Siljander 2016; Rieder *et al*. 2017; Rogers *et al*. 2016; Rooks and Garrett 2016), and for ecosystem health, from the creation of soils that suppress pathogens and disease outbreaks (Bardgett and van der Putten 2014; Panke-Buisse *et al*. 2014; Wagg *et al*. 2014) to biofilms that play vital roles in water purification (Besemer 2015) and wastewater treatment systems (Shu *et al*. 2015). Indeed, the importance of healthy, diverse and balanced microorganism communities to the functioning of all life on the planet is becoming ever more apparent as more research has been directed to this field of study.

Ecosystem services in freshwater and the functional role of bacteria

Freshwater microbial communities can be broadly categorised into three groups: i) free-moving planktonic cells, ii) biofilms upon the surface of substrates, plants and animals and iii) below the surface/benthic sediment communities. All three communities perform a wide range of ecosystem services, some of which are now described briefly below.

Primary productivity, biomass production and supporting the food web

Microorganisms in aquatic ecosystems are an essential component in the food webs of aquatic ecosystems. Some are autotrophs, for example cyanobacteria, which photosynthesise to produce energy that supports the ecosystem. Other bacteria, archaea and fungi found in biofilms and sediments, and to a lesser extent in the water column, constitute a large biomass that incorporate nutrients harnessed from the decomposition of organic material which are then released following their death (Fischer and Pusch 2001; Hoppe *et al*. 2002; Lee and Bong 2008). Cole *et al*. (1988) estimated that bacterial production constituted 30% of primary production in the water column (data largely based upon lakes and coastal waters) and that the levels of production in the sediments of lakes depended on whether the lake was eutrophic or oligotrophic.

Nitrogen cycle

Microorganisms are vital in the biogeochemical cycle of nitrogen, which is required for primary productivity and involves a diverse range of bacteria, including cyanobacteria (Falkowski 1997; Kuypers *et al*. 2003). Briefly, nitrogen fixing bacteria convert nitrogen into ammonium, which is then converted to nitrite and then nitrate by nitrifying bacteria. Bacteria are also responsible for denitrification, the reduction of nitrate ions to nitrogen gas (N_2), which prevents the build up of nitrates in the environment that may otherwise lead to acidification of aquatic environments, eutrophication and have direct toxicity on wildlife (Camargo and Alonso 2006).

Decomposition of organic material

Heterotrophic bacteria decompose organic matter and release dissolved organic matter into the water reincorporating it into the food web (Bertilsson and Jones 2003; Griffiths *et al*. 2009). The decomposition of organic material involves many different and diverse bacterial groups. For example, decomposition of sea grass in estuarine system was shown to be dominated initially by heterotrophic bacteria, with those attached to the substrate having a greater role than free bacteria, these were then succeeded by flagellates (Anesio *et al*. 2003). Bacteria are thus are vital for the decomposition of organic matter and the recycling of nutrients into biomass.

Water purification

Microorganisms have long been used in the treatment of waste water (Wagner *et al*. 2002) and they play key roles in the purification of freshwaters (Edwards and Kjellerup 2013; Sabater *et al*. 2002). Indeed, considerable effort has recently gone into understanding the roles that biofilms in particular play in this regard and how to maximise their water purification and ecosystem remediation potential. It has been shown that microorganisms are responsible for the degradation of pharmaceuticals in polluted waters (Cai *et al*. 2016; Zuehlke *et al*. 2007) and that pharmaceutical pollution in freshwater systems may influence the spatial variation in biofilm community structure in rivers (Cai *et al*. 2016).

The full extent of the relationship between microbial diversity and function are still unclear (Antwis *et al*. 2017; Burgin *et al*. 2011). The functions microbial communities provide can be affected by a range of environmental, chemical and biological factors such as light, temperature, substrate composition and topography, water current, nutrient availability, competition and predation (Ponsatí *et al*. 2016; Sabater *et al*. 2002). How anthropogenic stressors, including chemical contaminates such as antibiotics, interact with these processes as well as the diversity and function relationship is generally poorly understood.

Cyanobacteria

Cyanobacteria are a phylum of bacteria that produce their own energy through photosynthesis and are considered to have originated at least 2.6 billion years ago playing a major role in the formation of oxygen on the planet and reduction of atmospheric carbon dioxide (Altermann and Kazmierczak 2003; Graham *et al*. 2009; Mulkidjanian *et al*. 2006; Riding 2006). Cyanobacteria have evolved into a wide range of morphological forms (unicellular, multicellular to colonial) and became highly diversified more than 2 billion years ago and before green algae even existed, towards the end of the proterozoic era (Knoll 2008). They are ubiquitous in both aquatic and terrestrial environments and have adapted to perform a range of ecological functions that are crucial for the regulation of our aquatic ecosystems, of which nitrogen fixation and primary production are of particular importance (Falkowski 1997).

Antibiotics

Antibiotics kick started the age of “wonder drugs” in the early 20th century following the discovery of penicillin by Alexander Fleming in 1929, although arsphenamine, a sulphonamide, was in fact discovered earlier in 1910 (Zaffiri *et al*. 2012). In the following 100 years many classes of antibiotics with several modes of action have been discovered to treat a wide range of diseases, but this progress has stalled in the last 10 to 15 years with very few new antibiotics being clinically trialled (Review on Antimicrobial Resistance 2015).

Antibiotics have been transformative in modern medicine and are essential for the treatment of bacterial infection and in surgical interventions in humans. They are also vital for animal welfare in the farming of livestock as well as in veterinary medicine. Additionally, they are also used as growth promoters in some livestock farming and in the fish farming industry, although in the light of concerns relating to the development of antimicrobial resistance (AMR) this practice is in decline or has been stopped in some countries (Cully 2014).

Antibiotics are a specific class of antimicrobials that target bacteria (and fungi) in humans and animals, although they are also used as anticancer drugs, growth promoters in aquaculture and as pesticides (Grenni *et al*. 2018).

Current use and sales of antibiotics

Several investigators have attempted to assess the sales and distribution of antibiotics globally in an attempt to help identify trends and associated potential risks (Klein *et al*. 2018; Kümmerer 2009; Van Boeckel *et al*. 2015; Van Boeckel *et al*. 2014). These studies are limited generally due to the fact that different methods apply for the reporting such information across the globe as well as, in many instances, the lack of any records at all. Additionally, there is limited scope to combine the sales and distribution of antibiotics across industries (e.g. for human and veterinary health). Moreover, the trends in antibiotic use appear to change over the space of several years, meaning that the data collected soon becomes out of date. This is something that might be expected to occur more rapidly in the future as policy to address AMR drives changes in their use even more quickly. However, most investigations conclude that macrolides, floroquinolones and sulfonamides are of relatively high risk to bacterial populations (Kümmerer 2009; Välitalo *et al*. 2017) and due to their persistent

nature may remain so for the foreseeable future. Indeed, the macrolides, clarithromycin, erythromycin and azithromycin are all included in the European Unions watch list of priority substances and the US Environmental protection agencies (Carvalho *et al*. 2015; US EPA 2009).

There was an estimated 36% increase in global antibiotic consumption for human healthcare between the years 2000 and 2010 (Van Boeckel *et al*. 2014) and an increase of 65% between 2000 and 2015 (based on defined daily doses), much of which was driven by use in the low- to middle- income countries (Klein *et al*. 2018). Currently, broad-spectrum penicillin's are the most widely used class of antibiotics (39% increase in defined daily doses between 2000 and 2015), followed by cephalosporins, macrolides and fluoroquinolones; with cephalosporins seeing the greatest rise in global sales (Hamad 2010; Klein *et al*. 2018). Contrasting with this the macrolide class of antibiotics has seen a decrease in the sales (5% in 5 years) due to patent expiry.

There is a clear overlap between the antibiotics most commonly used for human medicine with those favoured for veterinary usage, with Charuaud *et al*. (2019) reporting that tetracyclines, sulfonamides, penicillins and macrolides are the most frequently used antibiotics worldwide for veterinary purposes, although many sulfonamides are not used in the treatment of human medicine (sulfamethoxazole is used in treatment of human health). Van Boeckel *et al*. (2015) estimated that 63,151 tonnes of antibiotics were used in the treatment of livestock in 2010 and predicted it would increase by 67% by 2030, most of which will be in the BRICS countries (Brazil, Russia, India, China, and South Africa; countries where there was a predicted doubling in antibiotic use for livestock).

In total, the antibiotic industry is valued at 40 billion USD a year. Most of this (88%) coming from off-patent molecules (Review on Antimicrobial Resistance 2016) reflecting the limited development of new antibiotics and the likely continued use and release of legacy antibiotics into the environment.

Antibiotics therefore have a clear and large social and economic importance. However, the release of antibiotics into the environment can pose a significant risk to; 1) bacterial communities and the ecosystem functions that they provide and on which the normal functioning and resilience of whole ecosystems rely

upon; and 2) human health through the development of AMR in human (or animal) pathogens.

How antibiotics work – the modes of action and susceptible taxonomic groups

For an antibiotic to be deemed suitable for effective use against pathogenic bacteria they need to be able to kill or selectively inhibit their growth whilst also having limited or no detrimental effects upon humans (or in the case of veterinary medicine; fish, reptiles, birds and other mammals). As such, antibiotic drug targets are those that are generally specific to bacteria and can be grouped into three broad modes of action (MoA); i) those that target the cell wall and/or its synthesis, ii) those that target nucleic acid synthesis, and iii) those that target the ribosome and/or protein synthesis. Some investigators split nucleic acid synthesis inhibitors into two groups, RNA synthesis inhibitors and DNA synthesis inhibitors but for the purposes of this thesis these are categorised jointly as nucleic acid synthesis inhibitors.

Cell wall synthesis inhibitors

The bacterial cell envelope is an antibiotic target that, for the most part, enables the discrimination between bacteria and eukaryotic cells. This is because eukaryotes have a phospholipid plasma membrane rather than the lipopolysaccharide and/or peptidoglycan membranes found in bacteria.

The cell envelopes of bacteria can be quite variable in structure but they are broadly divided into one of two types; i) the first, found in Gram-negative bacteria, is where there is an outer membrane made of lipopolysaccharide, a peptidoglycan layer and an inner/cytoplasmic membrane and ii) in the second, found in Gram-positive bacteria, the outer membrane is composed of a much thicker peptidoglycan layer but has no lipopolysaccharide layer (Silhavy *et al*. 2010). There are variations on these cell envelope structures in the different taxonomic classes of bacteria. For example, cyanobacteria have a cell envelope that is structured like that of the Gram-negative bacteria with three layers but the peptidoglycan layer in cyanobacteria is much thicker, resembling the envelope of Gram-positive cells (Hoiczyk and Hansel 2000). Other bacterial classes that do not necessarily follow the normal Gram-negative or Gram-positive cell envelope structure are the spirochaetes that have a fluid outer

membrane and a flagella in the periplasm (Haake 2000) and the planctomycetes that have a similar cell envelope structure to Gram-negative bacteria but with an enlarged periplasm forming cavities into the cytoplasm (Boedeker *et al*. 2017).

The vast majority of antibiotics that inhibit the synthesis of the bacterial cell wall target the synthesis of the peptidoglycan layer, which maintains structural integrity and the shape of the bacterial cell. Some inhibit the synthesis of peptidoglycan, for example fosfomycin (Silver 2017), whilst glycopeptides (e.g. vancomycin) prevent the polymerisation of the peptidoglycan chain (Allen and Nicas 2003). Most cell wall synthesis inhibitors however are β-lactams and these target the penicillin binding proteins that are responsible for the formation of the side-chains between peptidoglycan polymers.

Protein synthesis inhibitors

The bacterial ribosome is the main target for antibiotics that inhibit protein synthesis. The bacterial ribosome (70S, 2300 kd) is smaller than that of the eukaryotic ribosome (80S, 4300 kd) and is made up of a 30S and 50S subunits compared with the eukaryotic ribosome that is comprised of 60S and 40S subunits (Melnikov *et al*. 2012). The bacterial and eukaryotic ribosomes are thus sufficiently different from each other such that antibiotics can be used to selectively target the bacterial ribosome whilst having less affinity for that of the eukaryotes.

Antibiotics can be divided into those that target the 30S subunit of the ribosome (aminoglycosides and tetracyclines) and those that target the 50S subunit (oxazolidinones, amphenicols, pleuromutilins and the MLS antibiotics; namely macrolides, lincosamides and streptogramins grouped together based on their similar MoA). The smaller, 30S subunit is where the mRNA enters the ribosome and pairs with tRNA. Aminoglycosides target this subunit to cause mistranslation of the mRNA and tetracyclines target it to prevent the tRNA associating with the ribosome (Chopra and Roberts 2001; Kotra *et al*. 2000; Melnikov *et al*. 2012). The surface side of the 50S subunit which constitutes the interface between 30S and 50S subunits is where the peptide bonds are catalysed and sent through an exit tunnel in the 50S subunit (Melnikov *et al*. 2012). Antibiotics target and bind to various parts of the ribosome and in doing

so prevent it functioning properly and/or inhibit the correct translation of mRNA into a peptide sequence.

Although the ribosome is evolutionarily well conserved, there is variability in composition and structure between species in each domain (bacterial and eukaryotic), which may lead to differences in species sensitivity to the effects of antibiotics. Some off-target effects are sometimes observed in mitochondria and chloroplasts due to their endosymbiotic origin from α-proteobacteria and cyanobacteria respectively and this in turn can lead to affects in eukaryotes (Chopra and Roberts 2001; Wang *et al*. 2015).

Nucleic acid synthesis inhibitors

There are several drug targets in bacteria through which the synthesis of nucleic acids can be inhibited. The first are the bacterial type II topoisomerases, DNA gyrase and topoisomerase IV, which are enzymes required in the management of DNA cleavage and ligation (re-joining DNA strands) and are essential for DNA replication, transcription and recombination. These enzymes are specifically targeted by quinolones which bind to them and inhibit their function causing permanent DNA breakages and initiation of the SOS response (Aldred *et al*. 2014; Hooper 1999). DNA gyrase is evolutionarily well conserved across bacteria and is even found in some archea, into which they were likely introduced from bacteria via horizontal gene transfer (Forterre *et al*. 2007; Sioud *et al*. 1988). Additionally, gyrase has been identified in the mitochondria and chloroplasts of plants and algae and is phylogenetically closely linked with cyanobacteria, likely due to their endosymbiotic origin (Falcon *et al*. 2010; Forterre *et al*. 2007; Moriyama and Sato 2014; Wall *et al*. 2004). Indeed, toxic effects of DNA gyrase inhibitors have been observed in plants (Brain *et al*. 2008a; Brain *et al*. 2004; Ebert *et al*. 2011; Evans-Roberts *et al*. 2016)

A second target for antibiotics that inhibit the synthesis of nucleic acids is RNA polymerase. This is targeted by the antibiotic class rifamycins. Rifamycins inhibit the initiation of RNA synthesis and show similar comparative affinity for both Gram-positive and Gram-negative bacterial enzymes, although it is thought that the more protective cell envelope of the Gram-negative bacteria explains their lower sensitivity overall (Floss and Yu 2005). Rifamycins are also able to inhibit eukaryotic RNA polymerase but they have a much higher affinity for

prokaryotic RNA polymerase (prokaryotes are between 1000 to 100000 time more sensitive) (Floss and Yu 2005).

Both DNA and RNA synthesis can also be inhibited by antifolates that prevent the synthesis of folic acid, which is required in the production of nucleic acids. The folic acid synthesis pathway includes specific catalysing enzymes that are the target for some antibiotics. Examples of these include sulfonamides that target dihydropteroate synthase and trimethoprim that targets dihydrofolate reductase. As observed for DNA gyrase, plants and algae have been shown to possess the same folate synthesis pathway as bacteria and therefore they also show sensitivity to these antibiotics (Basset *et al*. 2005; Brain *et al*. 2008b; Zhang *et al*. 2012). Some bacteria, including cyanobacteria, are not sensitive to sulphonamides, likely due to the presence of a folate transporter protein in the membrane that allows them to survive on environmental folates thus avoiding the dependence on the folate synthesis pathway on which these antibiotics target (de Crécy-Lagard *et al*. 2007).

In chapters 5 and 6 of this thesis the effects of three classes of antibiotics upon cyanobacteria are experimentally considered; β-lactam (cell wall synthesis inhibitors); macrolides (protein synthesis inhibitors); and sulphonamides (DNA synthesis inhibitors). The sections below give a brief outline of the MoA of these classes of antibiotics.

β-lactams

β-lactam antibiotics largely consist of the classes of penicillin, cephalosporin and carbapenem, with a few other smaller classes including penems and monobactams. These antibiotics are bactericidal, working by targeting the penicillin binding proteins and preventing the synthesis peptide cross chains in the peptidoglycan layer of the cell envelope which in turn leads to cell lysis (Figure 1).

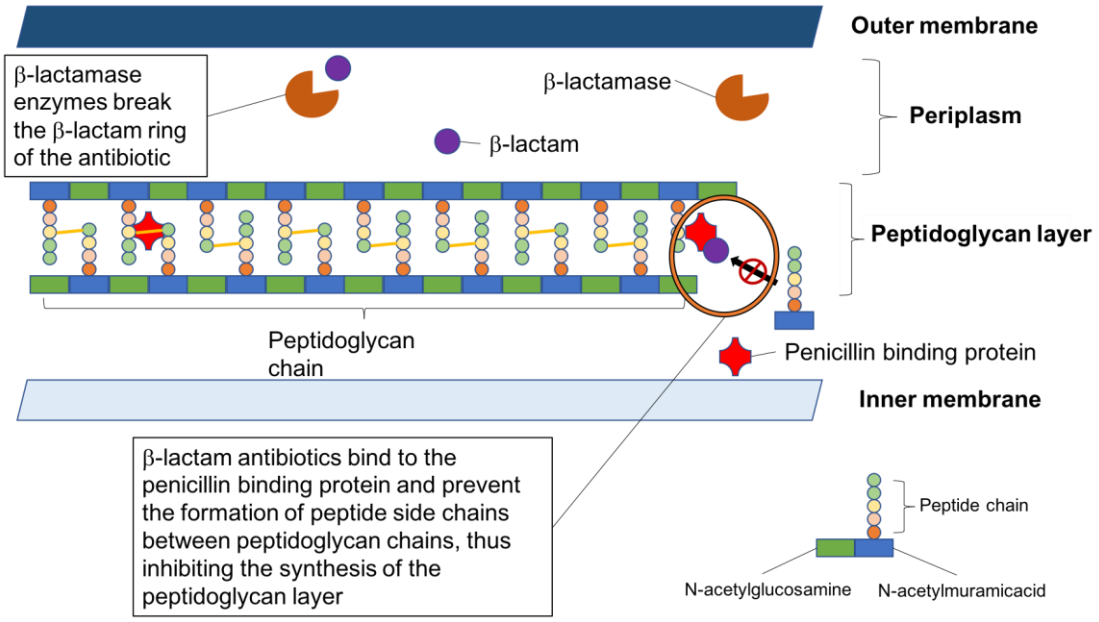

Figure 1: How β-lactam antibiotics inhibit the synthesis of the bacterial cell wall. This diagram depicts the structure of a cyanobacteria or Gram-negative bacteria with an outer membrane and periplasm (not present in a Gram-positive bacteria). It also shows the presence of β-lactamase enzymes that hydrolyse the β-lactam antibiotics as a resistance mechanism.

Macrolides

Macrolides are antibiotics that prevent the synthesis of proteins by binding to the exit tunnel of the 50S subunit of the ribosome. Once bound, the macrolide prevents the ribosome from catalysing the bonding between amino acid when a specific sequence of codons in the mRNA, called an macrolide arresting motif, enters the peptidyl transferase centre of the ribosome (Vázquez-Laslop and Mankin 2018).

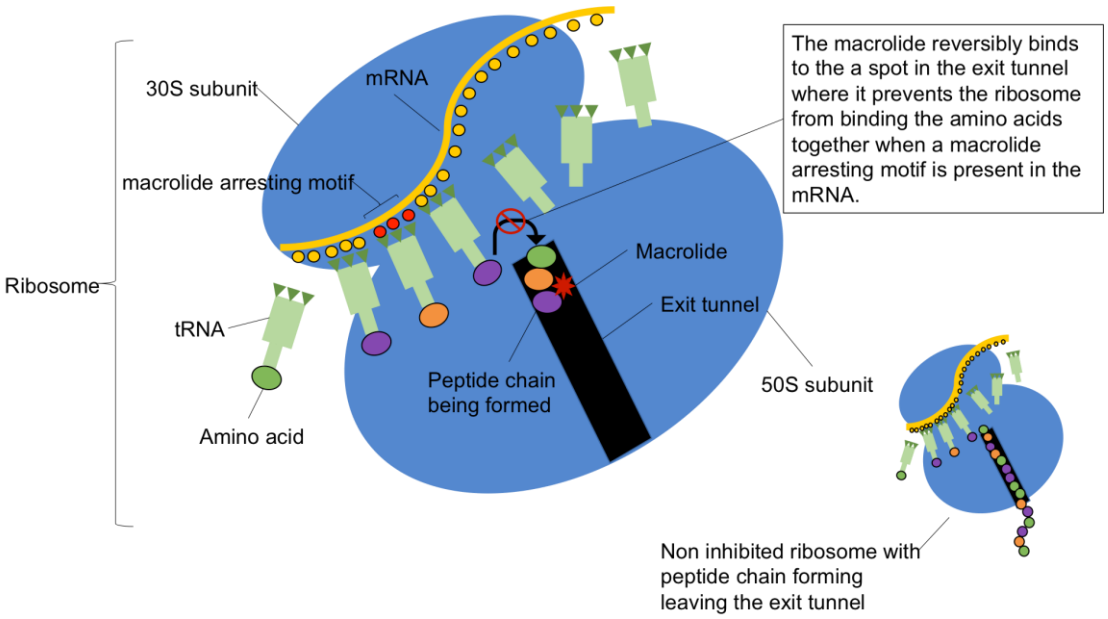

Figure 2: How macrolide antibiotics inhibit protein synthesis.

Sulfonamides

Sulfonamide antibiotics inhibit the production of folic acid, which is a precursor in the DNA synthesis pathway. Specifically, they competitively inhibit dihydropteroate synthase, an enzyme that catalyses the reaction between para-aminobenzoic acid (of which sulfonamides are structural analogues) and dihydropteroate diphosphate to create dihydropteroic acid (Figure 3) (Bermingham and Derrick 2002).

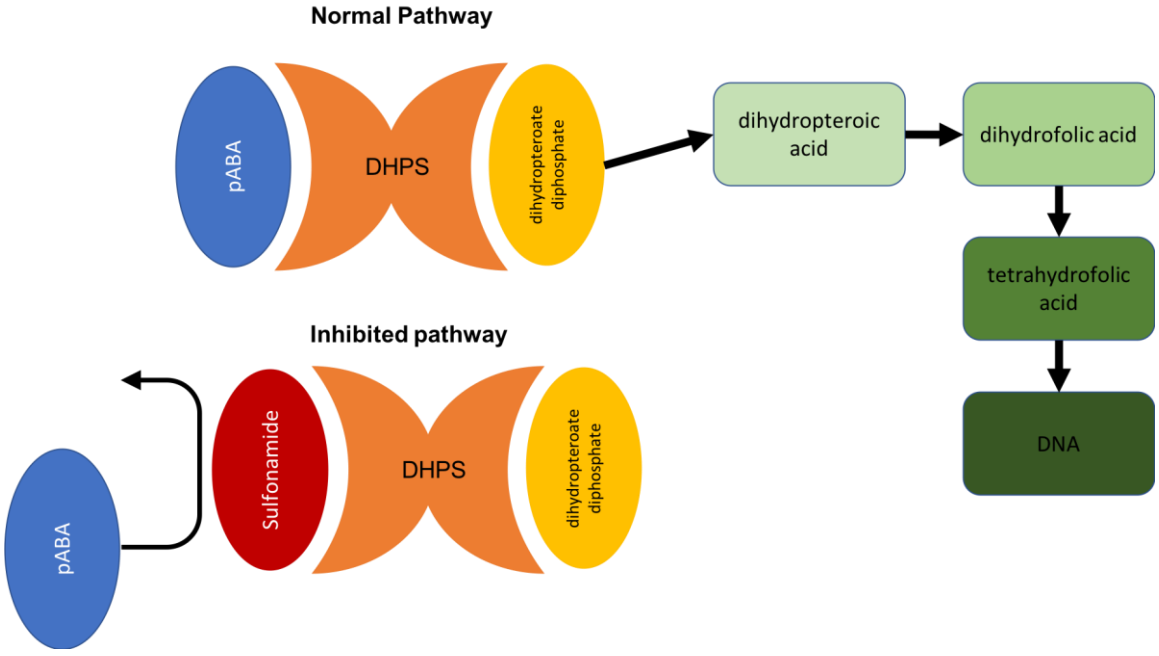

Figure 3: How sulphonamide antibiotics inhibit the synthesis of DNA.

Release of antibiotics into the environment

There are several ways that antibiotics may enter aquatic and terrestrial environments due to their use in both human and animal healthcare. They may be discharged into aquatic systems via waste/contamination from antibiotic manufacturing plants, effluent from WWTPs and STPs, hospital effluents, as leachate from landfill into groundwater or from use in aquaculture. Terrestrial soils may be contaminated directly from livestock urine/faeces, from the spreading of sewage sludge/biosolids on agricultural land as fertiliser and leaching from land fill into surrounding soils, all of which may leach out and run-off into the groundwater and surface waters (Batt *et al*. 2007; Brown *et al*. 2006; Carvalho and Santos 2016; Kümmerer 2009; Larsson 2014; Larsson *et al*. 2007; Michael *et al*. 2013). Antibiotics are also found in the marine environment (Gaw *et al*. 2014) and in sediments (Lalumera *et al*. 2004; Pei *et al*. 2006).

Following human use, antibiotics and their transformation products are generally excreted unmetabolised and processed in WWTPs where, depending on the type of treatment facilities, they are broken down in to transformation products or passed into the environment (He *et al*. 2015; Watkinson *et al*. 2007; Xu *et al*. 2015). Investigators have found that removal efficiencies are highly dependent on the antibiotic (He *et al*. 2015; Gulkowska *et al.* 2008; Xu *et al.* 2007). For example, one study found this was 49% for olfloxacin, 64% for molifloxacin and 74% for ciprofloxacin in WWTPs in USA (He *et al*. 2015). Removal efficiencies are also dependent on the type of treatment technology implemented; such as conventional activated sludge sewage plants compared to more advanced microfiltration/reverse osmosis plants (Batt *et al*. 2007; Watkinson *et al*. 2007). Furthermore, since the removal efficiency is highly associated with the type of sewage treatment process, there tends to be a disparity between countries based on their wealth, with lower income countries having less advanced waste water treatment (if any) and less stringent regulations (if any) which results generally in higher concentrations of antibiotics entering the environment compared to higher income countries (Kookana *et al*. 2014; Review on Antimicrobial Resistance 2016; Segura *et al*. 2009; Segura *et al*. 2015). It is important to recognise however, that robust data sets that can reliably inform upon global patterns of the occurrence of antibiotics in the environment (and antibiotic resistance) are skewed, with large data sets

available for regions that are better funded for research (e.g. Europe, USA, east China) and often, little or no information is available for other countries (Kookana *et al*. 2014; Schafhauser *et al*. 2018; Segura *et al*. 2009).

As for other contaminants, antibiotics that are not easily transformed or degraded can persist in the environment. Persistent antibiotics, such as the macrolide erythromycin, may not be effectively broken down in the WWTP into 'safe' (i.e. non-active) transformation products and may therefore reach the environment as the parent compound where they can accumulate and cause adverse effects on the microbiota. Some antibiotics are pseudo-persistent, such as occurs for some β-lactams, where partial degradation occurs in WWTPs but because of a constant discharge (e.g. from urban centres, hospital effluents, manufacturing discharges) levels persist or can even accumulate in the receiving environment. These environments can sometime be hotspots for the accumulation of antibiotics and AMR.

Antibiotics are regularly found in the ng/L range in the effluents of WWTPs, although some antibiotics have been recorded at levels reaching into the μg/L range (Batt *et al*. 2007; Brown *et al*. 2006; Monteiro and Boxall 2010; Watkinson *et al*. 2007; Watkinson *et al*. 2009). Hospital effluents however, have been identified as a particularly important source of antibiotics where concentrations can reach high μg/L or even mg/L levels (Brown *et al*. 2006; Kümmerer 2001). Rodriguez-Mozaz *et al*. (2015) found that of nine antibiotics they measured (notably ciprofloxacin, ofloxacin, cefazolin, cefotaxime, azythromycin, clarithromycin, sulfamethoxazole, trimethoprim and metronidazole) eight were recorded at higher concentrations in the hospital effluent than in the WWTP effluent. Of particular concern were the fluoroquinolones, ciprofloxacin and ofloxacin, which were regularly found in hospital effluents at concentrations of 8.3 to 13.8, and 4.8 to 14.4 μg/L, respectively. Downstream of the WWTP the highest recorded concentrations of ciprofloxacin and ofloxacin were 0.72 and 0.138 μg/L, respectively. Antibiotic manufacturing plant effluents have also been identified as a particular concern in the release of antibiotics into the aquatic environment with recorded levels also in the mg/L range (Larsson 2014; Larsson *et al*. 2007). Worryingly, there are currently no regulations that govern the safe discharge of antibiotics from manufacturing plants but attempts have

been made to address this via the AMR industry alliance (AMR Industry alliance 2018).

Effects observed upon bacteria and resistance selection in aquatic environments are generally observed in the low μg/L concentrations (see chapter 2) and therefore where environmental concentrations reach these levels there is clearly a potential risk to the bacterial communities and the functions that they perform. For example, in China, the production and consumption of antibiotics are some of the highest globally and some of the highest recorded environmental concentrations are found there. Indeed, in a review of antibiotics in the lakes of China (Liu *et al*. 2018) found 12 antibiotics occurring at concentrations at levels giving a risk quotient (RQ) of ≥1 and 4 with RQs of ≥10, indicating a high risk to the environment.

The spreading of biosolids on agricultural land is another important source of antibiotic contamination in the environment (Kinney *et al*. 2006; Walters *et al*. 2010). This thesis work is focused on the effects and environmental risk assessment (ERA) of antibiotics in aquatic ecosystems however and the effects of antibiotics on the terrestrial environment are therefore not explored further here.

Antimicrobial resistance

Ever since the discovery of penicillin, the first antibiotic used in the treatment of human infection, the emergence of resistance to every newly introduced antibiotic has quickly followed, reducing their efficacy (Davies and Davies 2010). If antibiotics are rendered ineffective in the control and treatment of pathogenic bacteria, medical advances in treating infectious diseases, surgical interventions and cancer treatment will be affected hugely (Review on Antimicrobial Resistance 2016). This is in addition to the potential damage it would do to food security. AMR is a crisis that the World Health Organisation has identified as “a major threat to public health” (World Health Organization 2014). Indeed, a series of reports (Review on Antimicrobial Resistance 2016) has predicted that AMR will be responsible for 10 million deaths a year by 2050 (currently estimated to be approximately 700,000) and cost up to $40 billion over the next ten years to address.

AMR genes are selected for where the benefit of possessing and expressing the resistance gene outweighs the fitness costs of carriage. Thus when antibiotic contaminants are present in the environment resistance genes in the bacterial populations/communities could be selected for and enriched, potentially leading to their transfer into human pathogens (Ashbolt *et al*., 2013).

There are four broad resistance mechanisms employed by bacteria: i) modification of the drug target to reduce or eliminate the binding affinity of the antibiotic; ii) reducing the bioavailable concentration of the antibiotic in the cell by reducing the permeability of the membrane or increasing efflux of the antibiotic; iii) production of cellular molecules (e.g. enzymes) to metabolise or degrade the antibiotic molecules; and iv) use of an alternative biochemical pathway to bypass the antibiotic mode of action (Fernandes *et al*. 2013; Walsh 2000).

The role cyanobacteria have in harbouring AMR genes and if they are able to transfer these genes to other bacteria, including pathogenic bacteria, is poorly understood. But recent evidence suggests that they may have the potential to hold and transfer resistance genes. *Microcystis aeruginosa* was found to possess the gene for thymidylate synthase (an alternate enzyme in the folic synthesis pathway that reduced susceptibility to trimethoprim), some β-lactum resistance genes, the *mfd* gene, which is involved in quinolone resistance and also some tetracycline resistance genes (Barata 2017). Although transference of AMR genes in cyanobacteria has not yet been observed directly, cyanobacteria have been demonstrated to undergo horizontal gene transfer (Humbert *et al*. 2013; Shi and Falkowski 2008) with some species demonstrating the ability to transfer large DNA sequences. As an example Humbert *et al*. (2013) found that *Microcystis aeruginosa* was able to transfer gene clusters of up to 19kb.

One of the many key questions being addressed by researchers regarding AMR is what role does the environmental compartment play in the harbouring and selection of AMR genes and how does it contribute to the potential transfer of these resistance determinants into pathogens. Furthermore, since the environmental compartment has been identified as a potential source for the harbouring, selection and dissemination of AMR determinants (Laxminarayan *et al*. 2013; Martínez 2008; Segawa *et al*. 2013; Taylor *et al*. 2011), there is an

urgent need to better understand the risk posed from the release of antibiotics into the environment. As such, it has been suggested that AMR could be considered within human and environmental risk assessment frameworks and a protection limit established to mitigate the risk posed from antibiotics in the environment to the selection of AMR (Agerstrand *et al*. 2015; Ashbolt *et al*. 2013). However, further research is required to identify the most appropriate methodologies for investigating AMR and what should be measured (e.g. population growth or AMR gene abundance) to obtain endpoints from which to base protection limits. Figure 4 illustrates the number of publications relating to AMR and highlights that environmental AMR risk assessment has received relatively little study compared with AMR research more generally, (only 2.5% of academic publications on AMR include “risk assessment”, and when the term “environment” is included this decreases to 1.6%).

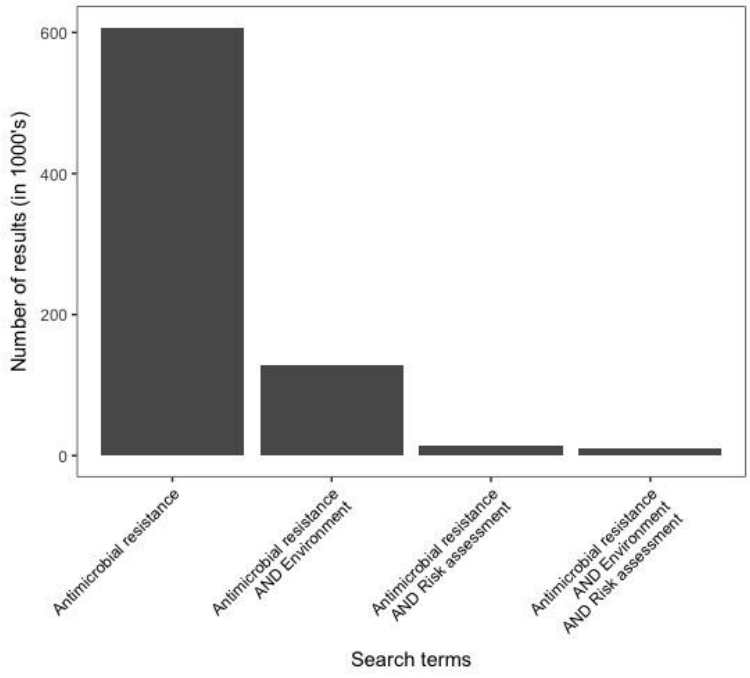

Figure 4: Google Scholar search for publications relating to AMR, using key words relating to antimicrobial resistance, environment and risk assessment. Search’s were conducted on 19 September 2018 and exclude citations and patents.

The minimum inhibitory concentration (MIC), which is the concentration at which there is complete inhibition of growth in the strain of bacteria being tested, is commonly used in microbiology when determining the effects of an antibiotic on

bacteria. MICs are monitored and collected for clinically relevant bacteria to ensure clinical efficacy and are recorded in the European Committee on Antimicrobial Susceptibility Testing database (http:// www.eucast.org). However, it has been established that resistance determinants are selected for at sub-lethal concentrations, well below the MIC and in some cases by up to two orders of magnitude below sub-lethal concentrations (Gullberg *et al*. 2011; Hughes and Andersson 2012; Lundström *et al*. 2016). The minimum selective concentration (MSC) is the concentration at which there is a net fitness advantage for the cells that carry and express the resistance gene compared to those in the same species/strain that do not (Gullberg *et al*. 2011). This may, however, still be somewhat too simplistic, as it does not take into account the added complexities added by the horizontal gene transfer of resistance genes, community interactions, the presence of extracellular resistance molecules (e.g β-lactams) that benefit non-resistance carrying cells, nor does it consider the biological and/or exposure effects due to growth in biofilms (Greenfield *et al*. 2017; Murray *et al*. 2018).

Nonetheless, the experimental determination of the MSC for use in risk assessment is an approach several investigators are exploring. Greenfield *et al*. (2017) propose a theoretical method using the parameters of modelled dose response relationships of susceptible and resistant bacterial strains. A recent experimental approach has been proposed using a complex community (raw untreated wastewater) and measuring the selection coefficients of resistance genes by qPCR (Murray *et al*. 2018). Using this method they identified positive selection for the resistance gene bla-CTX-M (and others) at 0.4 μg/L in the presence of cefotaxime and a significant change in community structure, increasing the abundance of some pathogenic bacteria, although in reality this may occur at lower than 0.4 μg/L due to degradation. Another more theoretical approach to establish a protection limit for AMR was proposed by Bengtsson-Palme and Larsson (2016) using the MIC data in the EUCAST database to predict the upper boundaries for resistance.

This thesis primarily considers the ecotoxicity of antibiotics in the environment and with a primary focus on cyanobacteria as the prokaryotic representative in ERA. However, AMR is intrinsically involved within the debate on antibiotics in the environment and it is impossible to fully consider the ecotoxicological effects

of antibiotics and their risk assessment without its consideration. As such, although this thesis does not provide an in depth discussion of AMR nor its threat to human health, chapters 2, 3, 6 and 7 do reflect on the relationship between protection limits for ecotoxicity and AMR and the implications for ERA.

Environmental risk assessment of antibiotics

Current EU regulations

In the European Union pharmaceuticals are regulated according to Directive 2001/83/EC and Regulation (EC) No 726/2004. To comply with these, an ERA is required performed according to the guideline EMEA/CHMP/SWP/4447/00 corr 2 (European Medicenes Agency (EMA) 2006), which consists of a two phases. Phase I requires the estimation of the environmental exposure, termed a predicted environmental concentration (PEC). If the PEC is greater than a threshold of 0.01µg/L a second phase of testing is required. This is split into two tiers. Phase II Tier A requires a base set of ecotoxicity studies upon a green algae, invertebrate and fish species and the performance of an activated sludge respiration inhibition test (ASRIT). In the case of antibiotics the green algae is replaced with a test on a cyanobacteria species. From these studies a no observed effect concentration (NOEC) is determined and an assessment factor of 10 applied (NOEC/10) to the most sensitive NOEC to establish a predicted no effect concentration (PNEC). Depending on the ratio between the PEC and the PNECs further testing may be required to refine the risk assessment. Table 1 summarises the phase II ecotoxicity testing according to (EMA 2006). There is currently no legislation or guidance to cover/include the risk assessment of AMR.

Data on the ecotoxicological effects of some pharmaceuticals, including antibiotics, upon the environment is scarce, partly due to the lack of regulatory requirement previous to 2006 before which the vast majority of antibiotics were registered (Le Page *et al*. 2017; Välitalo *et al*. 2017). Indeed, following our systematic review of data for the meta-analysis in chapter 2, we found data for a complete phase II, ERA dataset for only 7 out of 79 antibiotics (9%). Many antibiotics were patented before the introduction of environmental regulation and because pharmaceuticals have a maximum patent period of 20 years starting from when an active pharmaceutical ingredient (API) is discovered, any

manufacturer can now generically produce many antibiotics. These antibiotics are therefore sometimes termed as ‘legacy compounds’ and are of concern because they are untested (environmentally speaking) and there is no longer any responsibility over their environmental profile given that anyone is able to manufacture them. There have thus been calls for approaches to prioritise legacy compounds in the environment in order to identify important substances that may pose a risk and require additional research (Brooks *et al*. 2009; Burns *et al*. 2018; Roos *et al*. 2012). In support of this, chapter 4 details the development and validation of a microplate assay that establishes the effects of antibiotics (or any other soluble compound) on the growth inhibition of eight species of cyanobacteria.

There is a high dropout of potential APIs in the process of bringing a new drug to market and thus ERA, which is expensive to perform, is not carried out until just before the submission of a regulatory dossier in order to prevent the wasting of investment if the molecule fails late stage clinical trials. This results in the environmental profile of a molecule only being discovered at the end of the process of bringing a new drug to market and the need to implement environmental management for any higher risk APIs. Since many hundreds of molecules may be developed at the very beginning of drug discovery, it is possible that more ‘green’ (and thus lower risk) molecules were excluded in the discovery process that may have the same or similar clinical efficacy. It has therefore been suggested that obtaining environmental data earlier on in the drug discovery process could help support decision making, lead to greener drug design and inform ERA when it is carried out. The micro plate assay developed in chapter 4 is also designed in support of this objective.

Threshold for ecotoxicity testing	Testing required	Further testing threshold	Testing required	Further testing threshold	Testing required
Phase II Tier A testing required if PEC > 0.01µg/L or logK_{ow} > 4.5	ASRIT, Chronic testing with green algae (or cyanobacteria if antibiotic), Reproduction test with Daphnia magna and Fish early life cycle toxicity test	If PEC_{SW}:$PNEC_{W}$ < 1	No further testing		
		Phase II Tier B testing if PEC_{SW}:$PNEC_{W}$ > 1	Evaluation on fate of API and/or metabolites in aquatic environments		
		Phase II Tier B testing if PEC_{SW}:$PNEC_{M}$ > 0.1	Determine PEC_{AT} using exposure concentration in the aeration tank of the SimpleTreat model	if PEC_{AT}:$PNEC_{M}$ > 1	Evaluation on fate of API and/or metabolites on single microbial species (e.g. *Pseudomonas putida*)
		Phase II Tier B testing if PEC_{GW}:$PNEC_{GW}$ > 1	Evaluation on fate of API and/or metabolites in aquatic environments		
		Phase II Tier B testing if K_{OW} >1000	Bio-concentration study required		
		Phase II Tier B testing if K_{OC} > 10000 L/kg	Terrestrial risk assessment		
		Phase II Tier B testing if > 10 % API bound to sediment in OECD 308 biodegredation study	Evaluation effects of API on sediment organism (e.g. *Chironomus riparius*)		

Table 1: European Medicines Agency environmental risk assessment ecotoxicity Phase II testing. Phase I, the estimation of exposure, is not included. PEC = Predicted environmental concentration; PNEC = Predicted no effect concentration; PNECW = PNEC in water (based on most sensitive NOEC of algae/cyanobacteria, invertebrate and fish test); PEC_{SW} = refined PEC in surface water; $PNEC_{M}$ = PNEC of microorganisms (from ASRIT); PEC_{AT} = PEC in aeration tank; PEC_{GR} = PEC in groundwater; $PNEC_{GR}$ = PNEC in groundwater; K_{OW} = n-octanol/water partition coefficient; K_{OC} = adsorption coefficient; API = active pharmaceutical ingredient

Current methodologies for the testing of antibiotics in ERA

For antibiotics, the ERA framework includes just two ecotoxicity tests upon prokaryotic organisms, the ASRIT and a growth inhibition test upon a single species of cyanobacteria. The ASRIT has been demonstrated to not be sensitive to antibiotics and is thus not suitable for establishing protection limits that are protective of bacteria populations (Kümmerer *et al*. 2004; Le Page *et al*. 2017). Furthermore, since antibiotics are designed or selected to have MoAs that are bactericidal or bacteriostatic whilst having limited effects upon humans (or other mammals in the case of veterinary medicine), there is unlikely to be affects upon fish or invertebrates as they don't possess the drug targets (Le Page *et al*. 2017). Although, as discussed previously, some effects have been observed in some algae, macrophytes and terrestrial plants due to the prokaryotic origin of the chloroplasts and mitochondria.

Thus, out of four ecotoxicity tests in ERA, all prokaryotic diversity is effectively represented by a single species cyanobacteria growth inhibition study. This bias towards tests that are inherently not sensitive to antibiotic MoAs has been identified by several investigators, whom have raised concerns regarding the capability of ERA to establish suitable protection limits for the protection of bacteria populations, communities and the ecosystem functions that they perform (Agerstrand *et al*. 2015; Brandt *et al*. 2015; Le Page *et al*. 2017). Furthermore, within species that possess the drug target, interspecies sensitivity differences can still be highly variable, as demonstrated by species sensitivity distributions of clinically relevant bacteria exposed to ciprofloxacin, erythromycin and tetracycline that each have sensitivity ranges of up to or more than two orders of magnitudes (Tello *et al*. 2012). Indeed, a single bacterial species is highly unlikely to be representative of the whole assemblage. This has also been shown for algae exposed to herbicides (Nagai *et al*. 2016; Van den Brink *et al*. 2006) and accordingly, the relevant ERA regulations require multiple algae species to be tested in ERA (EFSA 2013; US EPA 2017).

There is a clear need for a critical assessment of antibiotic ERA to establish more suitable protection limits that are protective of bacterial diversity and ecosystem function. There are however, only limited experimental data available for MoA relevant species for the majority of antibiotics on which to base a critical assessment (Le Page *et al*. 2017). Thus a test method that

allows the rapid assessment of the effects of antibiotics to several bacterial species and allows for the direct comparison of sensitivity would be valuable in order to determine if testing upon a single species of cyanobacteria can be protective of a cyanobacteria diversity more broadly. Furthermore, it is vital to ascertain whether cyanobacteria are a suitable representative of all other bacterial clades and if not, identify what other bacteria should be represented.

Shake flask for the growth inhibition of cyanobacteria

Traditionally the shake flask growth inhibition test has been used to determine the growth inhibition of green algae and cyanobacteria following chemical exposure and there are several internationally recognised test guidelines, including (ISO 2004; OECD 2011; US EPA 1996).

Briefly, the test consists of exposing an anexic culture of cells in balanced (exponential) growth to a range of test chemical concentrations and measuring the cell density at the beginning of the test and every 24 hours for 3 to 4 days. The exposures are generally performed in 100mL of culture medium in 250mL conical flasks, being shaken and under continuous lighting and temperature conditions. The cell density at each concentration at the start and end of the test is used to calculate the growth rate, yield and occasionally the area-under-the-curve to determine the inhibitory effects of the chemical on growth. There are several benefits to this test methodology including limited experimental error due to the large volumes, stable growing conditions (due to large volumes) and large historical use and thus published data for comparison, analysis and validation.

There are however also several drawbacks to this methodology, mainly based around the large size, resource requirement and cost. The shake flask test requires several litres of test media and enough API, which may be expensive (especially if early on in drug design process), to make up the test concentration range. It also takes up considerable space and requires a lot of operator time, all restricting the throughput. Finally, there is little scope for automation of this test methodology to convert it into a medium/high-throughput test.

Miniaturised assays and surrogates for cell density determination

The use of miniaturised assays, often performed in 24 or 96 well microplates, have been frequently proposed to address the limitations associated with the shake flask test design (Eisentraeger *et al*. 2003; Nagai *et al*. 2013; Skjelbred *et al*. 2012) and are employed in the Environment Canada testing guidelines (Environment Canada 2007). A microplate test design has the advantages over the shake flask test in that it requires fewer resources in terms of consumables and operator time, requires less space allowing many tests to be performed in the same space as a single shake flask test, more easily allows for the rapid assessment of fluorescence/optical density (OD) in plate readers (as surrogates for cell density) and has higher potential for automation. Thus, the use of microplate assays enables screening of several test compounds at the same time, several species to the same test compound or indeed both. Given the current limitations in ERA previously identified, a microplate test that allows the rapid screening of several species of cyanobacteria would be of great value in both early drug discovery and prioritising legacy compounds. Indeed, similar assays have been developed for the screening of chemical toxicity and produce comparable results to the shake flask test, although the vast majority focus on green algae rather than cyanobacteria (Eisentraeger *et al*. 2003; Guo *et al*. 2016; Nagai *et al*. 2013; Nagai *et al*. 2016; Paixão *et al*. 2008; Skjelbred *et al*. 2012). There are however, several drawbacks from the use of microplate assays that need consideration in test design, such as the potential for higher variability in growth rates due to the smaller volumes used and potential underestimation of toxicity for volatile or hydrophobic compounds (Riedl and Altenburger 2007); although, these can be mitigated with a suitable analytical testing regime and the use of glass-coated microplates.

In order to maximise the throughput of microplate assays and minimise the operator time required, most studies use an automated surrogate for cell density determination. Various surrogates have been proposed including fluorescence and OD that is also known as absorbance. In addition to the ability for precise, rapid and automated measurement, fluorescence and OD have the additional benefit that they are non-destructive sampling techniques and allow for repeated measurement of the same replicate without risk of contamination or

the reduction in replicate volume (Berden-Zrimec *et al*. 2007; MacIntyre and Cullen 2005).

Two types of fluorescence have been used as a surrogate for biomass in algae toxicity studies; prompt fluorescence (often simply termed fluorescence) and delayed fluorescence. Prompt fluorescence is the emission of light from pigment molecules (e.g. in chlorophyll), excited by a specific wavelength and which occurs rapidly before charge separation in photosynthesis (the transfer of an electron from the primary election donor (Cardona *et al*. 2012)). Delayed fluorescence however, is a longer lasting but weaker emission of light that occurs following charge separation (Berden-Zrimec *et al*. 2010). Delayed fluorescence has the advantage of only occurring from actively photosynthesising cells and thus reflects living cells only, whilst prompt fluorescence may also occur from excited pigments in dead cells. Delayed fluorescence has been successfully used for algae toxicity testing (Berden-Zrimec *et al*. 2007; Katsumata *et al*. 2006) but since its measurement was not possible in the experimental work of this thesis it will not be discussed further.

Prompt fluorescence (just referred to as fluorescence from herein), is more regularly used than delayed fluorescence. Many studies use fluorescence *in situ* for monitoring cell density of phytoplankton communities in the field, especially in regards to harmful algal blooms (Bowling *et al*. 2016; Zamyadi *et al*. 2016), but most laboratory studies have focused on green algae and thus use chlorophyll fluorescence with excitations and emissions at approximately 420-430nm and 670-685 respectively. For example, Eisentraeger *et al*. (2003) demonstrated that for the growth inhibition of the green algae, *Raphidocelis subcapitata*, both a 96 well and 24 well microplate test using chlorophyll fluorescence were equally as sensitive as a shake flask test.

Cyanobacteria have phycobiliproteins in addition to chlorophyll that most algae do not possess. Phycobiliproteins include phycocyanin, allophycocyanin and phycoerythrin and they absorb light at different wavelengths than for chlorophyll, passing the energy harvested from one to the other and finally to chlorophyll. Because they are at higher levels than chlorophyll in cyanobacteria cells they have been suggested to be a better surrogate for cell density. Nagai *et al*. (2013) used phycocyanin fluorescence to measure the growth inhibition in

the cyanobacteria *Pseudanabaena galeata* obtaining comparable results to a shake flask test design that used direct counting to determine cell density.

OD is the absorbance of a specific wavelength of light in a suspension of the cells. Paixão *et al*. (2008) tested five compounds and six wastewater effluents on the growth of *R. subcapitata* in a microplate using OD at 450nm as a surrogate for cell density and found the results comparable to the shake flask test. The selection of a wavelength for OD is of particular importance in order to reduce error in biomass determination because cellular pigment concentration may vary considerably both under different conditions and in different parts of the growth curve (Griffiths *et al*. 2011; Hecht *et al*. 2016). An OD wavelength of >700nm, which outside of the range absorbed by cellular pigments (e.g. chlorophyll) or fluorescent proteins has thus been shown to be most suitable (Griffiths *et al*. 2011; Hecht *et al*. 2016).

Using precise techniques such as OD and fluorescence also has the advantages of allowing the detection of low cell numbers with less experimental error compared with other techniques such as coulter counting or direct counting with a haemocytometer. Low cell densities provide higher environmental relevance, assay reproducibility and don't affect the physio-chemical properties in the test (e.g. pH) nor bioavailability as greatly as higher cell densities (Berden-Zrimec *et al*. 2007; Franklin *et al*. 2002). Fluorescence has however, been shown to be more sensitive at lower cell densities than OD (Eisentraeger *et al*. 2003) and this has been confirmed in cyanobacteria in chapter 4 of this thesis.

Which surrogate, fluorescence or OD, is best for determining cell density depends on several factors in the test design, but generally fluorescence appears to have more advantages. Firstly, fluorescence is preferable for lower cell densities than OD, providing several benefits as described above, but if the assay intends or requires testing at higher cell densities neither technique outperforms the other. Additionally, OD will likely be affected if the sample has other particulate matter other than the cells to be measured, whereas fluorescence is targeted to a fluorescent pigment with specific excitation and emission wavelengths making interference less likely. Finally, OD is also affected more than fluorescence by changes in cell morphology that change the optical properties of the suspension (Griffiths *et al*. 2011), although this is

unlikely to have severe detrimental affects in its application for single species assays given that cells are generally uniform in nature, unless the toxicity of the test compound changes the morphology of the cells.

One key consideration in all algae and cyanobacteria growth inhibition assays is to ensure that, as best as possible, the test is run when the population is in balanced growth. Balanced growth, sometimes called exponential growth, is where a population is replicating at the maximum growth rate possible under the specific environmental conditions they are in and without nutrient limitations. Thus, reproducing cells are equally distributed across all stages of the cell cycle and at any given time interval the cell density increases at the same rate as well as the cellular components (ribosomes, proteins etc) (Campbell 1957; Schaechter 2015). Populations in other phases of the growth cycle (e.g. lag and stationary phases) have cells that vary in metabolic states and replication rates, leading to variability in cellular component ratios (Chang *et al*. 2012). This is of particular importance for obtaining reproducible results over time in growth inhibition studies (Schaechter 2015) and especially when measuring cell density with a surrogate such as phycocyanin fluorescence. For example, cells in the lag phase are preparing themselves for exponential growth by synthesising all the cellular components required (RNA, proteins, light harvesting complex's, enzymes etc) (Rolfe *et al*. 2012). The lag phase will thus differ depending on the previous environmental conditions and their metabolic state on inoculation, as well as the amount of light harvesting pigments such as phycocyanin, which will in turn affect the fluorescence, growth rates and therefore the reproducibility of the assay (Chang *et al*. 2012; MacIntyre and Cullen 2005; Van Wagenen *et al*. 2014). Furthermore, Chang *et al*. (2012) show that phycocyanin levels per cell change throughout a populations growth curve and that that there is considerable interspecies variability in this respect.

In order to develop a test method that could provide data in early drug discovery and/or for the prioritising of legacy antibiotics, a microplate-based assay that allows for the testing of several cyanobacteria could be of great value. It will however need to ensure, as best as possible, that the test is performed on populations that are in balanced growth and careful consideration of the individual growth conditions for each species will thus be required. In addition, for a fair comparison of cyanobacteria sensitivity and for the data to be

environmentally relevant, the exposure conditions should be the same across species to ensure the behaviour of the antibiotic is controlled (e.g. differences in light intensity and temperature may affect antibiotic degradation across assays). Chapter 4 sets out to develop, optimise and validate such an assay for eight species of cyanobacteria. Chapters 5 and 6 in turn use this assay in order to consider cyanobacteria sensitivity to antibiotics and the implications this may have for protection limits derived in ERA.

Aims of the thesis

There are growing concerns regarding the suitability of current ERA to identify the risk antibiotics pose to bacterial communities and the essential ecosystem functions that they provide. Furthermore, ERA does not address for the selection of AMR in the environment. The primary aim of this thesis was therefore to determine if ERA is able to establish adequate limits for the protection of environmental bacteria populations. I have addressed this issue by performing both meta-analytical and experimental techniques whilst ensuring that the methods, taxa and focus remain relevant to traditional ERA.

Specifically, the aims of this thesis were:

- Evaluate current antibiotic ERA through the collation and analysis of publically available data on organisms regularly used in ecotoxicology testing (*Chapter 2*)
- Compare the protection limits established traditionally for the environment in ERA with those proposed for the protection of human health via AMR selection (*Chapter 2*)
- Provide a considered response to a series of criticisms made by Bengtsson-Palme and Larsson (2018) on the work I conducted and subsequently published in chapter 2 (*Chapter 3*)
- To develop and validate a microplate assay suitable for a range of cyanobacteria species to determine growth inhibition following chemical exposure (*Chapter 4*)
- Using the microplate assay developed in chapter 4, establish differences in cyanobacteria species sensitivity across a range of antibiotics (*Chapter 5*)
- To compare current protection limits derived for ERA with the probabilistic species sensitivity distribution approach using data obtained from the microplate assays conducted in chapter 5 (*Chapter 6*)

- To establish the expected total risk antibiotics may pose to freshwater cyanobacteria based upon species sensitivity distributions and measured environmental concentrations data (*Chapter 6*)

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Chapter 2

Integrating human and environmental health in antibiotic risk assessment: a critical analysis of protection goals, species sensitivity and antimicrobial resistance

Gareth Le Page, Lina Gunnarsson, Jason Snape, Charles R. Tyler;
published in *Environmental International* (2017) 109: 155-169.

This is a systematic review and meta-analysis of the publically available aquatic ecotoxicity data and minimum inhibitory concentrations of clinically relevant bacteria. It includes a comparative analysis of species sensitivity and the protection goals derived for environmental health and for human health (in regards to antimicrobial resistance).

I performed all data collection, analysis and first drafting of the paper. All authors were actively involved in discussions on the work and in the preparation of the manuscript for submission. This paper is included in the thesis appendix in its published format. The data tables referred to in the text as supplemental material table 1 and 2 are too large to be included within this thesis, but can be viewed online with the article.

https://www.sciencedirect.com/science/article/pii/S0160412017309005?via%3Dihub

Since publishing this article the conclusions and recommendations have been employed by the AMR Industry Alliance in their proposals for manufacturing discharge targets and by the European Medicines Agency in the recent update to Environmental Risk Assessment Guidelines going out for consultation in 2019.

Link for AMR Industry Alliance:

https://www.amrindustryalliance.org/shared-goals/common-antibiotic-manufacturing-framework/

Integrating human and environmental health in antibiotic risk assessment: a critical analysis of protection goals, species sensitivity and antimicrobial resistance

Abstract

Antibiotics are vital in the treatment of bacterial infectious diseases but when released into the environment they may impact non-target organisms that perform vital ecosystem services and enhance antimicrobial resistance development with significant consequences for human health. We evaluate whether the current environmental risk assessment regulatory guidance is protective of antibiotic impacts on the environment, protective of antimicrobial resistance, and propose science-based protection goals for antibiotic manufacturing discharges. A review and meta-analysis was conducted of aquatic ecotoxicity data for antibiotics and for minimum selective concentration data derived from clinically relevant bacteria. Relative species sensitivity was investigated applying general linear models, and predicted no effect concentrations were generated for toxicity to aquatic organisms and compared with predicted no effect concentrations for resistance development. Prokaryotes were most sensitive to antibiotics but the range of sensitivities spanned up to several orders of magnitude. We show reliance on one species of (cyano)bacteria and the 'activated sludge respiration inhibition test' is not sufficient to set protection levels for the environment. Individually, neither traditional aquatic predicted no effect concentrations nor predicted no effect concentrations suggested to safeguard for antimicrobial resistance, protect against environmental or human health effects (via antimicrobial resistance development). Including data from clinically relevant bacteria and also more species of environmentally relevant bacteria in the regulatory framework would

help in defining safe protection limits for the protection of environmental and human health

Introduction:

Antibiotics are crucial in human healthcare. They are used in the treatment of bacterial infectious diseases, supporting surgical interventions, and in cancer and prophylactic treatment. Antibiotics are also used widely in livestock and domestic animal veterinary treatments and as growth promoters in aquaculture. Global production of antibiotics for human use is valued at $40 billion a year (O'Neill 2015) illustrating their societal and economic importance. Antibiotic consumption is on the rise and between the years 2000 and 2010 there was an estimated 36% increase in use globally for human healthcare (Van Boeckel *et al*. 2014).

Antibiotics, as other pharmaceuticals, enter the environment via patient and animal use, through manufacturing plants and/or improper disposal. Common points of entry into the environment from human therapeutic use are via effluents from hospitals, domestic sewerage treatment plants, as well as via leachates from landfill sites. Antibiotics can enter into surface waters from sewerage treatment plants directly or they can be transferred via surface run off. Ground waters can be exposed from agricultural land treated with sewage sludge biosolids as a source of fertiliser (Kümmerer 2009). Veterinary antibiotics enter the aquatic environment either directly, and may leach into or run off surface water or via groundwater from the manure of treated livestock (Davies 2012; Kümmerer 2009; Sarmah *et al.* 2006). Antibiotics in surface waters and sewerage treatment plant effluents/wastewaters are generally measured at concentrations ranging between 0.01 and 1.0 μg/L (Batt *et al*. 2007; Miao *et al*. 2004; Monteiro and Boxall 2010; Watkinson *et al*. 2009). The highest levels of antibiotic residues in effluents - in the milligram per litre range, with records in excess of 1000 mg/L - are reported from manufacturing plants in China and India (Larsson 2014; Larsson *et al*. 2007; Li *et al*. 2008; O'Neill 2015). Hospital effluents too can contain antibiotic residues in the mg/L concentration range (Brown *et al*. 2006; Watkinson *et al*. 2009).

Antibiotics affect prokaryotic cells via a number of distinct mechanisms of action, including the inhibition of cell envelope synthesis, inhibition of protein

synthesis or inhibition of nucleic acid (DNA/RNA) synthesis. Antibiotics are designed for use in the treatment of bacterial infection in humans and livestock and are thus developed to avoid, or limit, effects on mammalian cells. It is, therefore, reasonable to assume that environmental bacteria are more likely to be adversely affected as a result of non-therapeutic exposure compared with aquatic vertebrates, such as fish.

Within Europe, an environmental risk assessment (ERA) is required for a medicine if the predicted environmental concentration exceeds 0.01 μg/l (EMA 2006). In the USA effect studies are triggered if the expected environmental concentration exceeds 0.1 μg/L (US Food and Drug Administration 1998). The ERA aims to establish the safe concentrations for the protection of wildlife populations, ecosystem structure and function and includes the calculation of three predicted no effect concentrations (PNEC) for aquatic organisms, namely $PNEC_{surfacewater}$ ($PNEC_{SW}$), $PNEC_{microorganism,}$ and $PNEC_{groundwater}$ (EMA 2006). These are determined by establishing a no observed effect concentration (NOEC, the test concentration at which there is no statistically significant effect in the response being tested, such as on growth rate or reproduction) for a range of aquatic taxa and applying an assessment factor of ten to account for variability in species sensitivity and extrapolation from laboratory data to the field. $PNEC_{microorganism}$ is based on the 'activated sludge respiration inhibition test' (ASRIT, (OECD 2010)) and is primarily used to establish risk to microorganisms in (and the function of) sewerage treatment plants. The $PNEC_{groundwater}$ is based on a chronic test with *Daphnia magna* (e.g. OECD 211 test guideline, (OECD 2012) and $PNEC_{SW}$ is calculated from the toxicity to three eukaryotic species – a green algae, invertebrate and fish. For antibiotics, in Europe the ERA guidance encourages ecotoxicity testing with prokaryotes rather than a green algae "*as they are [a] more sensitive indicator organisms than green algae*" (EMA 2006), and this is conducted in one species of cyanobacteria only.

There is concern that the ERA for antibiotics is biased towards testing on metazoan species (invertebrates and fish in this instance), and does not consider fully the possible impacts of antibiotics on microbial community structure, function and resilience (Agerstrand *et al*. 2015; Brandt *et al*. 2015). This is a major shortfall considering the fundamental ecosystem services

microbial communities provide (e.g. primary production, nutrient cycling, metabolism and degradation of organic, inorganic and synthetic compounds). A major aim of this meta-analysis therefore was to test if current ERA is protective of vulnerable populations in the environment.

Microorganisms exposed to antibiotics at low, sub-lethal or sub-inhibitory exposure concentrations can develop, or acquire, antimicrobial resistance (AMR) and this has been identified as a major threat to public health (Smith and Coast 2002; World Health Organization 2014). AMR is likely to persist and disseminate in diverse environments, including in aquatic ecosystems (Laxminarayan *et al*. 2013; Taylor *et al*. 2011). Where the benefit of possessing and expressing the resistance gene outweighs the fitness costs of carriage, antibiotics in the environment may select for and enrich resistance genes in bacterial populations/communities which can then harbour these resistance determinants and transfer them to human pathogens (Ashbolt *et al*. 2013).

To ensure clinical efficacy and protection of human health, minimum inhibitory (growth) concentrations (MICs, the lowest concentration at which there is no observable growth) are monitored in clinically relevant bacteria (CRB) and recorded in the European Committee on Antimicrobial Susceptibility Testing database (http://www.eucast.org). In addition to monitoring MICs in clinically relevant species, studies with clinical isolates have also identified the lowest concentration that will select for AMR, called minimum selective concentrations (MSCs). MSCs are the minimum concentration at which the presence and expression of resistance gene(s) give bacteria a fitness advantage over non-resistant cells of the same species/strain. This can occur at concentrations considerably below the MIC of the non-resistant cells (Gullberg *et al*. 2011). Indeed, selection may occur at exposures up to two orders of magnitude lower than the MIC for growth (Gullberg *et al*. 2011; Hughes and Andersson 2012; Lundström *et al*. 2016).

From both human and environmental health perspectives, it is important that risk assessment frameworks incorporate the risk of AMR selection. An approach to establish a surrogate PNEC for AMR ($PNEC_R$) has been suggested adopting MICs from CRB, which are available through the European Committee on Antimicrobial Susceptibility Testing database (Bengtsson-Palme and Larsson 2016). This is the most comprehensive dataset available where

theoretical PNECs ($PNEC_{R(T)}$) have been calculated for 111 antibiotics. This approach uses growth (via the MIC) to predict upper boundaries for resistance, although there has been no verification of an increase in resistance determinants. The approach also assumes that the CRB are representative of the diversity of bacteria in nature. Furthermore, whilst AMR maybe enriched at concentrations well below the MIC of clinical bacteria, the AMR enrichment could potentially occur at concentrations below the effects determined in traditional ERA ecotoxicity growth tests on cyanobacteria. This meta-analysis therefore also sought to determine the relationship between protection goals proposed to protect against resistance development and the traditional aquatic protection goals; i.e. establish if the proposed methods used to derive a PNEC for AMR development ($PNEC_R$) are protective of those currently used for aquatic ecosystem function ($PNEC_{sw}$) and *vice versa*.

Recognising that antibiotic releases from drug production and formulation facilities represent 'hot spots' for the development of AMR it is critical that these discharges are minimised and managed effectively across the whole supply chain. To address this concern, the pharmaceutical industry recently established an AMR Road map which included a commitment to "establish science-driven, risk-based targets for discharge concentrations for antibiotics and good practice methods to reduce environmental impact of manufacturing discharges, by 2020" (IFPMA 2016).

To improve the testing paradigm for antibiotics for use in prospective regulatory frameworks and to establish safe discharge concentrations for antibiotic production, we conducted a meta-analysis based on a systematic review of the publically available aquatic ecotoxicity data and clinically relevant MICs for antibiotics. Specifically we; 1) assess the relative sensitivity of commonly used taxa in aquatic ecotoxicity, with a MOA perspective, to evaluate the reliability of the current ERA of antibiotics to identify risk to vulnerable populations; 2) assess the value of extending the toxicity testing for bacteria through an assessment on the relative sensitivity of several cyanobacterial species, the marine bacteria *Vibrio fischeri* and the CRB MICs; 3) critically evaluate the current proposed approaches for determining the risk of AMR and its incorporation into risk assessment for the protection of human health; i.e. whether a $PNEC_R$ is more or less protective than $PNEC_{SW}$ calculated using

traditional ecotoxicity testing; 4) test the assumption that CRB adequately represent environmental bacteria and evaluate the use of pre-clinical MIC data for the protection of other bacterial species through a comparison of the NOECs for cyanobacteria with the adjusted MIC, calculated by Bengtsson-Palme and Larsson (2016) from CRB and; 5) use the empirical data collected in these analysis to help establish science-driven, risk-based targets for manufacturing discharge concentrations for antibiotics.

Methods

Data search strategy

A comprehensive literature search was carried out to identify studies reporting toxicological effects of antibiotics on aquatic taxa commonly used in ERA. These taxa included cyanobacteria, green algae, macrophytes (the latter currently used in ERA for agrochemicals, but not pharmaceuticals), invertebrates and fish. Data were also collected for the effects of antibiotics on *Vibro fischeri*, for the ASRIT test and *Pseudomonas putida* (where available). Data were used in our analyses only if they met the following criteria: 1) the endpoint calculated was a NOEC, 50% effective concentration (EC_{50}) or 50% inhibition concentration (IC50), the concentration at which 50% of the population are effected or inhibited respectively; 2) the methodology adopted was according to (or with minor deviations from) currently accepted regulatory protocols (e.g. Organisation for Economic Co-operation and Development (OECD) or International Organisation for Standardisation (ISO) test guidelines); 3) the aquatic species belong to the taxa described above; 4) exposures were for single species not multiple species/community exposures (with exception of the ASRIT which is a community based exposure) and; 5) organisms were exposed to a single antibiotic (not a chemical mixture).

The aim of this paper was to conduct a meta-analysis of available data in the context of current regulatory guidance that uses population-relevant endpoints to establish PNECs. Therefore NOECs and EC/IC50s for growth, reproduction or mortality only (or accepted surrogates e.g luminescence in *V. fischeri* or respiration in the ASRIT) were collected and analysed. Moreover, interpretation of biomarker endpoints in relation to population-based NOECs and EC/IC50s are not well established.

Searches and data collections were conducted for the following public databases and literature:

- Environmental data on antibiotics from the trade organisation for the research-based pharmaceutical industry in Sweden (LIF), obtained from the Swedish fass.se database (www.fass.se accessed Jan 2016).
- Environmental data for antibiotics from the 'European public assessment report' database (www.ema.europa.eu, accessed Jan 2016).
- All published data in the Wikipharma database (http://www.wikipharma.org, accessed Jan 2016).
- All relevant data in the study by Vestel *et al*. (2015) which included the antibiotics azithromycin, bedaquiline, ceftobiprole, doripenem, linezolid, meropenem, sulfamethoxazole and trimethoprim.
- Data for sulfadiazine, neomycin and gentamycin, kindly provided by Merck Sharp & Dohme (MSD) through the 'Innovative Medicines Initiative' iPIE project (https://www.imi.europa.eu/content/ipie).
- A GoogleScholar search focused on cyanobacteria with the following search criteria for the 111 antibiotics listed in the paper by (Bengtsson-Palme and Larsson 2016): *Antibiotic* cyanobacteria "OECD 201" OR "ISO8962" OR "ISO 8962" OR "850.4500" OR "E1440-91"
- The theoretical $PNEC_R$ ($PNEC_{R(T)}$) and the size-adjusted MIC (MIC_{aj}) for antibiotics were collected from Bengtsson-Palme and Larsson (2016). For antibiotics where less than 40 species have been tested in the European Committee on Antimicrobial Susceptibility Testing database, Bengtsson-Palme and Larsson (2016) calculated a size-adjusted MIC. This is a theoretical adjustment to the MIC to include 99% of CRB. The number derived from that calculation was rounded down to the nearest concentration in the range operated in the European Committee on Antimicrobial Susceptibility Testing protocol. $PNEC_{R(T)s}$ were calculated by applying an assessment factor of 10 to account for differences between inhibitory concentrations and selective concentrations of the

antibiotics. Experimentally derived MSCs were identified from literature following a GoogleScholar search with search criteria: “Minimum selective concentration” MSC AND “antibiotic resistance”. We highlight here that currently there is no internationally standardised test method for MSC and that extrapolation to the environment is poorly understood due to the complex nature of resistance enrichment, the complex nature of communities and a range of environmental factors that may influence the MSC (Khan *et al*. 2017; Quinlan *et al*. 2011).

- Antifungal and antiviral drugs obtained through our search criteria were excluded from this assessment.

All data derived from these searches are provided in the supplemental material, Table S1 and a flowchart to illustrate the data collection and statistical processes for these analyses is provided in figure S1.

Assessment of data reliability

Assessments on data reliability were undertaken using the ‘Criteria for reporting and evaluating ecotoxicity data’ (CRED) system that is specifically designed for the evaluation of ecotoxicity data for regulatory use (Moermond *et al*. 2016). In this system reliability is defined as “the inherent quality of a test report or publication relating to (preferably) standardized methodology and the way the experimental procedure and results are described to give evidence of the clarity and plausibility of the findings”. The CRED system categorises the reliability of studies into one of four scores: R1 (reliable without constraints), R2 (reliable with constraints), R3 (unreliable) or R4 (not assignable). Studies identified as R3 are considered unsuitable for use in regulatory decision-making; whereas caution needs to be applied on a study-by-study basis for studies categorised as R2 or R4. The CRED evaluation method also provides guidance on the evaluation of the relevance of data (Moermond *et al*. 2016). This, however, was not applied as the data were considered relevant for this meta-analysis having fulfilled the selection criteria outlined in section 2.1. The CRED reliability score for each study is given in Table S1.

Relative taxa sensitivity data

The lowest 'reliable' NOEC and EC_{50} for each taxa were identified for each antibiotic. Data from studies that had CRED reliability scores of R1 and R2 were prioritised, without bias between R1 and R2, over those in the categories of R3 or R4. R4 data were selected over R3 data as the majority of R4 studies were assigned R4 due to unpublished/missing information in an otherwise (apparently) reliable study compared with R3, which were assigned unreliable for defined reason. The lowest 'reliable' NOEC and EC_{50} were applied in the analysis of relative taxa sensitivity and are presented in the Table S2. This conservative approach was deemed more appropriate rather than taking an average of all available data that has imbalanced taxa representation and varying data reliability.

An analysis of the relative sensitivity of cyanobacterial species adopted the same CRED criteria as described above to establish the lowest 'reliable' EC_{50}. EC_{50}s were used rather than NOECs as there was a larger dataset for cyanobacterial EC_{50}s. These data are presented in Table S3.

Censored data

For some antibiotics the data was either left or right censored, meaning that the value was not a precise number and was given as greater than (>) or less than (<) the value reported (i.e. no effect at the highest test concentration or an observed effect at the lowest tested concentration, respectively). Censored data values were used when no other data were available (< than numbers would represent conservative values and > numbers were included only when they represented the lowest 'reliable' data value). Where data were censored, this is indicated in Table S1.

Establishing relative taxa sensitivity to antibiotics

A sensitivity ratio (SR) was calculated between the different taxa and cyanobacteria for each antibiotic, where data were available. The SR was calculated using the lowest NOEC (or NOEC and MIC_{aj} in the case of CRB) or EC_{50} using the following equation:

$$Log_{10}SR = logE_{cyanobacteria} - logE_{taxa}$$

where E is the endpoint (NOEC, EC_{50} or MIC_{aj}).

A SR >0 indicates that the cyanobacteria are more sensitive than the other taxa and less sensitive when SR <0. Each unit of SR is equivalent to an order of magnitude difference in sensitivity.

The difference between a SR calculated from NOECs compared with those calculated from EC_{50}s was examined to identify how the endpoint used might impact the sensitivity ratio. Briefly, a generalised linear model (GLM) (Gaussian error family with identity link function) was constructed using the 'lmer' package with the restricted maximum likelihood method (Bates *et al*. 2015) in R (version 3.3.0; R Project for Statistical Computing, Vienna, Austria). The model residuals were normally distributed and significant differences identified using the "lmerTest" package in R (Kuznetsova *et al*. 2013). SRs were used only where a NOEC and EC_{50} were from the same species and publication in order to exclude effects of different methodologies. The SRs calculated from EC_{50}s were significantly higher by 0.5 (p = 0.05) than those calculated from NOECs i.e. cyanobacteria were less sensitive as measured by EC_{50}s. As such, SRs calculated from EC_{50}s were only included in subsequent analyses comparing taxa sensitivities where NOEC SRs were not available. We acknowledge that this will have a small effect on the output of the models. However, because of the sparse dataset and the relatively small difference in SR between EC_{50}s and NOECs compared with the differences between taxa, the inclusion of the EC_{50} SRs where NOEC SRs are not available increases the number of SRs for comparison and robustness of the models.

We established a GLM in R (version 3.3.0; R Project for Statistical Computing, Vienna, Austria) to determine the effects of exposure duration on the EC_{50} for *V. fischeri,* as EC_{50} are often reported for 5, 15 and 30 minutes and for 24 hours. Censored data were removed and the remaining EC_{50}s were $\log_{10}$ transformed before use in the GLM (Gaussian error family with inverse link function) that was constructed as described for comparing NOEC and EC_{50} SRs above. Significant differences were identified by applying a TukeyHSD post hoc test. Twenty four hour EC_{50}s were significantly lower (p = <0.001) than those following shorter exposure periods and data for this time point only were therefore used in subsequent analyses on relative taxa sensitivities.

Differences in SR across all taxa for all antibiotics were analysed using a GLM. The aim of the analysis was to compare the sensitivity of all taxa to

cyanobacteria. Cyanobacteria were chosen as the comparator because they are assumed to be the most mode-of-action relevant taxa (therefore, most sensitive species) in current ERA, and thus expected to drive the $PNEC_{SW}$. Briefly, to assess for statistical differences in SR the GLM was constructed forcing the intercept through 0 (the SR value of cyanobacteria). Therefore, the statistical differences identified by "lmerTest" (Bates *et al*. 2015) represent the statistical difference from 0 and thus the statistical difference between the taxa and cyanobacteria. This allowed for the exclusion of cyanobacterial SRs in the GLM as the sensitivity of cyanobacteria were already accounted for in the calculation of the SRs. TukeyHSD post hoc tests were applied to identify any further differences between the taxa groups. Details on model construction and validation are provided in the Supplemental Material. Adopting the same process and validation steps, further GLMs were established for analyses of antibiotics with different mechanisms of actions and, where sufficient data were available, for antibiotic classes (a more detailed methodology for this is presented in Supplementary Material).

Antibiotics were classified into three groups based on their broad mode of action, specifically, cell envelope inhibitors (Anatomical Therapeutic Chemical (ATC) classification system codes J01C and J01D), Nucleic acid synthesis inhibitors (ATC codes J01E and J01M) and protein synthesis inhibitors (ATC codes J01A, J01B, J01F, J01G, J01XC, J01XX08, J01XX11 and QJ01XQ).

It is important to note that in addition to comparing different endpoints and methodologies, representation of antibiotics - in both potency and number of antibiotics with data - varied between and within taxa and antibiotic classes. We acknowledge this may introduce some uncertainty and potential bias in our analysis and have thus avoided the use of more complex model designs that might otherwise have introduced random factors and interactions. However, the biases mentioned above are unlikely to have an impact on the overall conclusions drawn from these analyses.

Calculation of PNECs

Where a full set of ecotoxicity data for an European Medicines Agency Phase 2 ERA was available (cyanobacteria, invertebrate and fish tests) a $PNEC_{SW}$ was calculated by taking the lowest NOEC of the three studies and applying an

assessment factor of 10, as described in the regulatory guidance (EMA 2006). A theoretical $PNEC_R$ ($PNEC_{R(T)}$) was taken directly from Bengtsson-Palme and Larsson (2016). An experimental $PNEC_R$ ($PNEC_{R(Exp)}$) was calculated from the lowest experimental selective concentration and applying an assessment factor of 10.

There was not enough data to conduct species sensitivity distribution analysis and calculate 95% percentile protective limits, as this requires a minimum of 10 species and preferably more than 15 (ECHA 2008).

5th percentile determination

The calculated 5th percentiles for the NOEC and MIC data subsets were not normally distributed or fitting to other known distributions (e.g. gamma and weibull) before or following transformations (log, log_{10} or boxcox). The 5th percentile therefore was established using the non-parametric Harrell-Davis quantile estimator method. Analysis was conducted in R (version 3.3.0; R Project for Statistical Computing, Vienna, Austria) using the hdquantile function in the 'Hmisc' package (Harrell Jr 2016).

Results

Ecotoxicity data were collected for 79 antibiotics (Table S1) representing 48% of the 164 approved antibiotics identified in www.drugbank.ca and (Santos *et al*. 2017). Information on the ecotoxicity in cyanobacteria was available for 41 of these 79 antibiotics, but with NOECs for only 27 (16%). Antibiotics with NOECs for cyanobacteria were well distributed across all ATC sub-classes under J01, with exception of J01XX ('other antibacterials'; Figure S2).

A complete Phase 2, ERA dataset that included the full range of taxa for calculating a $PNEC_{SW}$ (EMA 2006) was available for only seven of these antibiotics. This may reflect the lack of pharmaceutical ERA datasets placed in the public domain and/or that few antibiotics have been approved since the existing European Medicines Agency guideline came into force in 2006 requiring full chronic toxicity testing on cyanobacteria/microalgae, invertebrates and fish and consequently lack a full ecotoxicity data set.

Relative species sensitivities

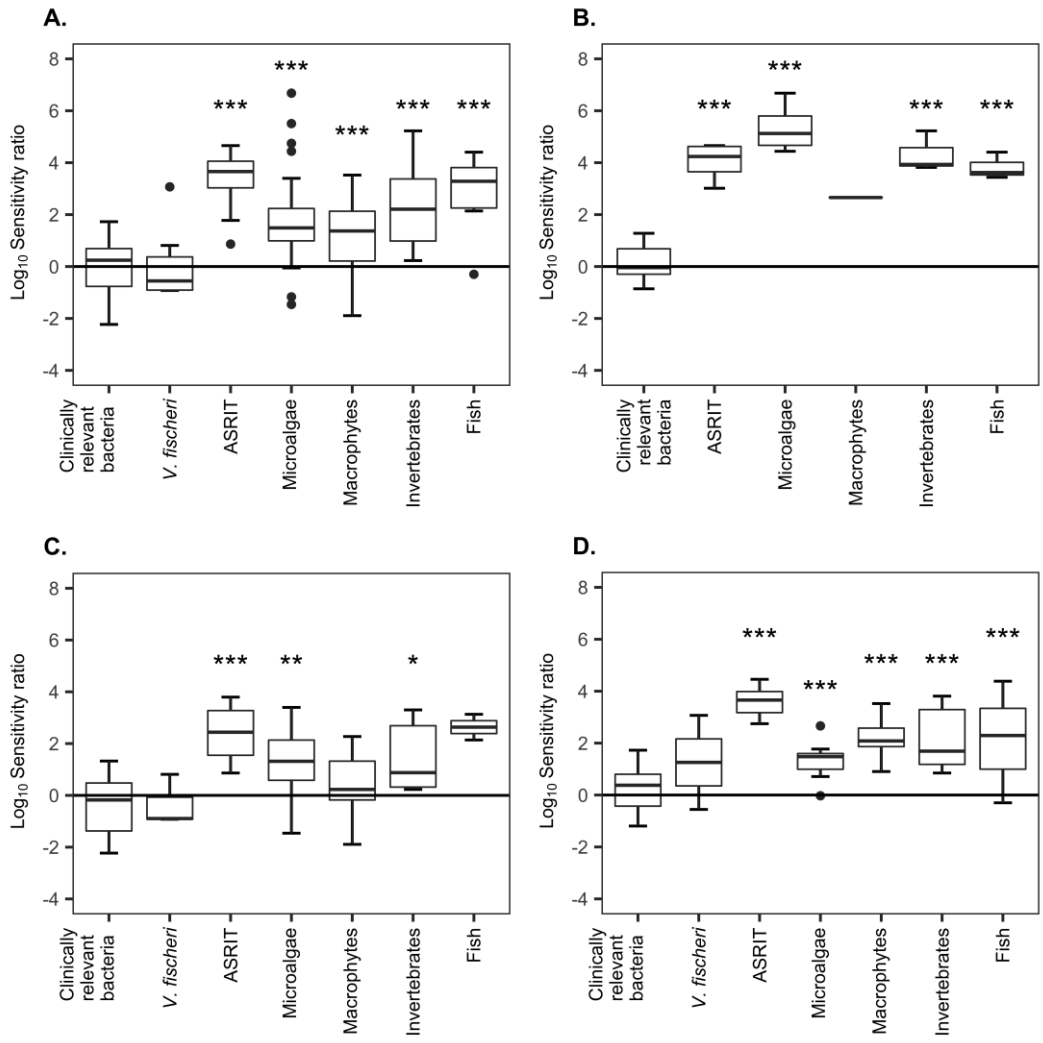

Figure 1. Boxplots of Log_{10} sensitivity ratio (SR) between cyanobacteria and other species/phyla for A) all antibiotics (n=37), B) cell envelope inhibitors (n=8), C) Nucleic acid synthesis inhibitors (n=12) and D) protein synthesis inhibitors (n=16). Log_{10}SR calculated based on log_{10}cyanobacteria NOEC or EC_{50} – log_{10}taxa NOEC or EC_{50}. Where SR = 0 the sensitivity of the taxa is equal to cyanobacteria, represented by horizontal line, where SR >0 taxa had a lower sensitivity and <0 indicates higher comparative taxa sensitivity. Significant differences of SR from cyanobacteria in the generalised linear mixed models are indicated by: * p<0.05; ** p<0.01; *** *p*<0.001. Statistical tests were not performed on macrophytes in cell envelope inhibitors as there was only one antibiotic tested in macrophytes.

Overall, cyanobacteria were the most sensitive taxa of those currently recommended in the ERA of human pharmaceuticals (EMA 2006; US Food and Drug Administration 1998) (p = <0.001, Figure 1A) and they were equally sensitive as other bacteria (CRB and *V. fischeri*) and more sensitive than macrophytes (that are not currently required in ERA of pharmaceuticals; p = <0.001).

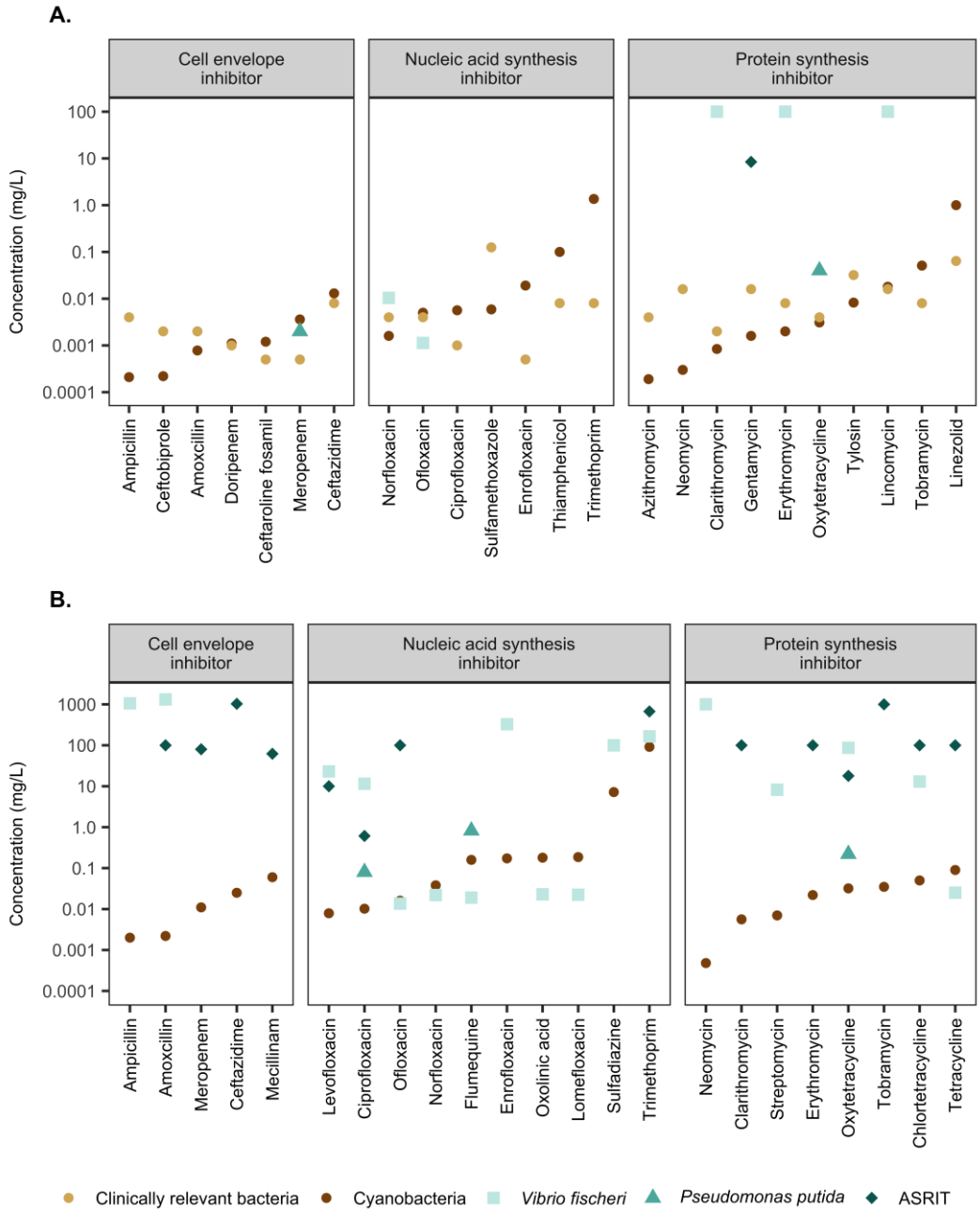

Figure 2. Chronic exposure effects of antibiotics on A) environmental bacteria and clinically relevant bacteria (no observed effect concentrations (NOEC) and adjusted minimum inhibitory concentrations respectively) and B) environmental bacteria EC_{50}s.

The sensitivity of cyanobacteria and CRB were not significantly different for any of the three broad antibiotic mechanisms of actions (Figures 1B-D); NOECs in cyanobacteria were lower than CRB MIC_{aj} for half (12 out of 24 antibiotics; Figure 2A). If we were to adopt the lowest MIC, instead of the modelled $MIC_{aj,}$ in this meta-analysis there would be more cases (18, rather than 12, out of 24) where the cyanobacteria were the most sensitive. Although there was no clear relationship between the CRB MIC_{aj} and cyanobacterial NOECs the difference in sensitivity was up to two orders of magnitude for specific individual antibiotics (Figure 2A and 6C).

There were no significant differences in sensitivity to DNA or protein synthesis inhibiting antibiotics between *V. fischeri* and cyanobacteria (Figure 1; there were no data for cell-envelope inhibiting antibiotics). Of the seven antibiotics where SRs could be determined five were for quinolones giving an antibiotic class bias for the *V. fischeri* data. EC_{50}s for *V. fischeri* were lower than those for the cyanobacteria on six occasions (Figure 2B), three of these were almost an order of magnitude lower (flumequine, lomefloxacin and oxolinic acid). *V. fischeri* was also the most sensitive organism to olfoxacin, with a NOEC one order of magnitude lower than the CRB MIC_{aj} (Figure 2A) and an EC_{50} half that for the cyanobacteria (Figure S3).

Pseudomonas putida, a model (soil) Gram-negative bacteria used in standard growth inhibition test guideline (ISO 1995) was more sensitive than cyanobacteria for one out of five antibiotics (meropenem; Figure 2A and B).

The ASRIT (OECD 2010) was consistently between two and four orders of magnitude less sensitive than cyanobacteria, with the exception of trimethoprim (Figures 1 and 2 p = <0.001).

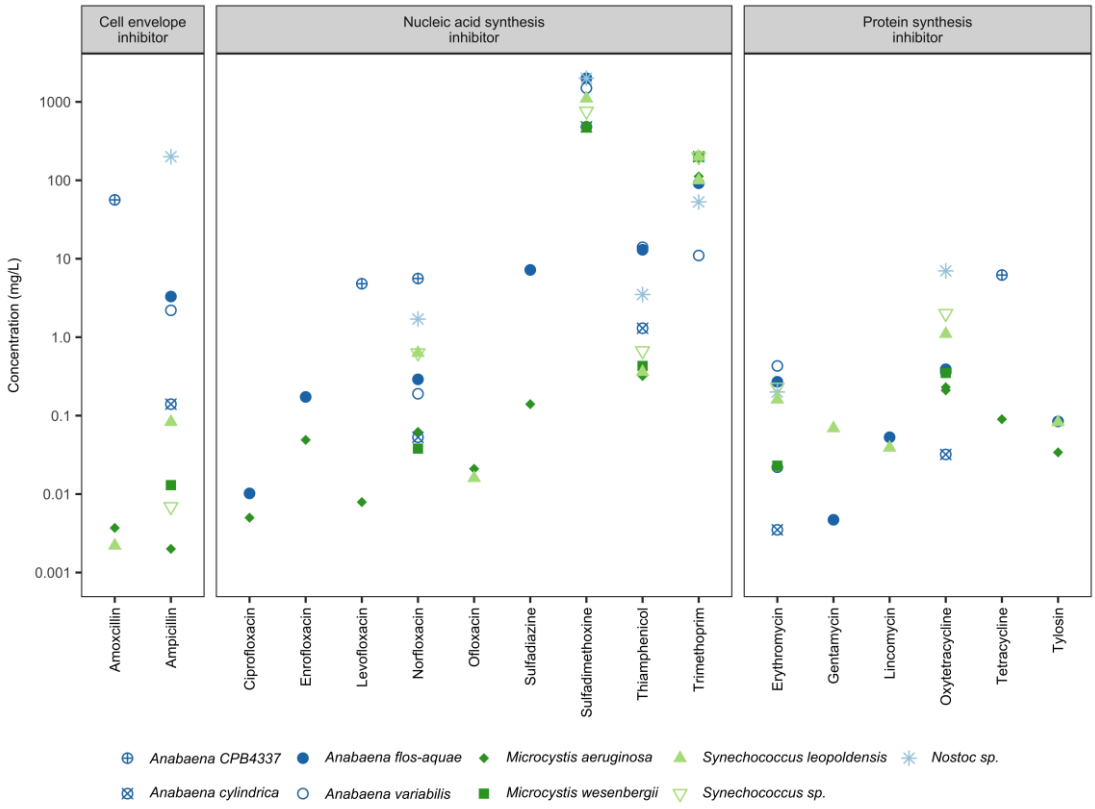

Figure 3. Chronic exposure effects (EC_{50}s) of antibiotics on different cyanobacteria species.

There were large differences in sensitivity between cyanobacterial genera and species, with between two and three orders of magnitude difference in EC_{50}s for 10 out of the 16 antibiotics, and approximately five orders of magnitude difference in response to the β-lactams amoxicillin and ampicillin (Figure 3). Overall, *Microcystis aeruginosa* was the most sensitive species (in half of the 16 antibiotics). *Anabaena cylindrical*, *Synechococcus leopoliensis* and *Microcystis wesenbergii* were each the most sensitive cyanobacterium for 2 of 16 antibiotics for which there were data on multiple species. *A. flos-aquae,* one of the cyanobacterial species recommended for testing in the OECD 201 test guideline, was the most sensitive species for only 1 of the 13 antibiotics in which it was tested. When considering antibiotic sensitivity based on their mechanisms of action, *Microcystis* species appeared to be more sensitive to nucleic acid synthesis inhibitors (7 out of 9 antibiotics). *Microcystis* and *Synechococcus* species were the most sensitive to cell envelope inhibiting antibiotics. *Anabaena* genera were the most sensitive to the protein synthesis inhibitors (3 out of 6) and in two cases by more than an order of magnitude.

Overall, macrophytes were generally less sensitive to antibiotics compared with cyanobacteria with a wide range of SRs (Figure 1, p = <0.001). However, they showed equal sensitivity with cyanobacteria to nucleic acid synthesis inhibitors (average SR = 0.42; p = 0.3). The NOECs for trimethoprim and sulfadimethoxine were lower for macrophytes than for cyanobacteria (Figure 4A). A comparison of macrophyte and environmental bacteria EC_{50}s is provided in Figure S3.

Microalgae were also generally less sensitive to antibiotics than cyanobacteria (Figure 1, p = <0.001). However, for sulfadiazine and sulfadimethoxine the NOECs in microalgae (0.135 and 0.529 mg/L, respectively) were over an order of magnitude lower than for the lowest in the cyanobacteria (Figure 4A). We interpret these data with caution, however, as the results for the cyanobacteria were derived from a study based on nominal (i.e. not measured) test exposure concentrations (Ando *et al*. 2007). A comparison of the EC_{50}s for microalgae with environmental bacteria is shown in Figure S3.

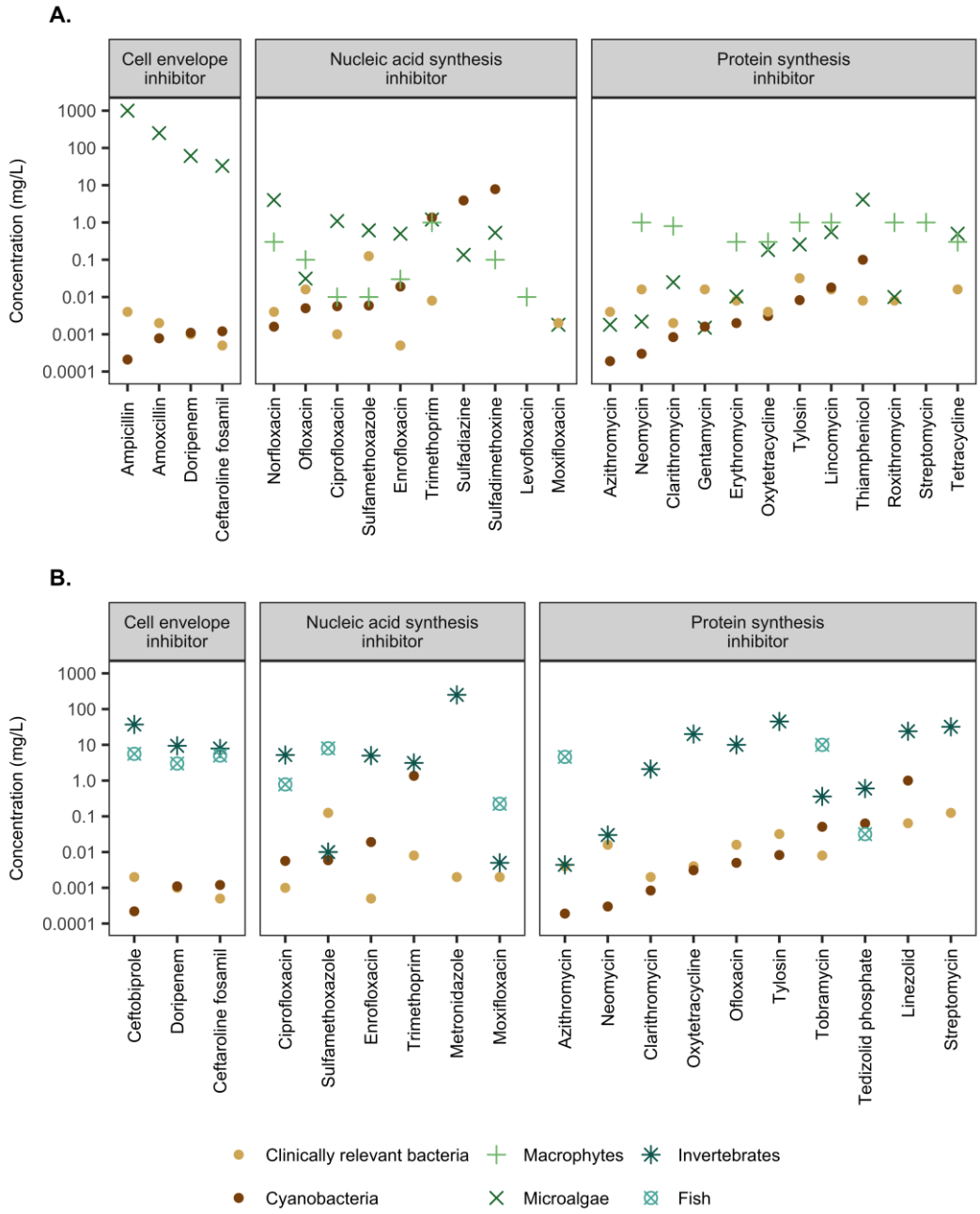

Figure 4. Chronic exposure effects of antibiotics on cyanobacteria and clinically relevant bacteria (no observed effect concentrations (NOEC) and adjusted minimum inhibitory concentrations respectively) compared with A) NOECs for microalgae and macrophytes and B) NOECs in invertebrates and fish.

Metazoans (fish and invertebrates) were significantly less sensitive across all antibiotics compared with cyanobacteria and often by between two and four orders of magnitude (with exception of tedlizolid phosphate, Figure 1 and 4, p = < 0.001, for both fish and invertebrates). There was substantial variation in SR between cyanobacteria and the metazoan taxa (as illustrated by the standard errors in the data; Figure 1). In the case of tedlizoid phosphate, a pro-drug, fish appeared more sensitive than cyanobacteria (NOECs of 0.032 versus 0.063 mg/L, respectively; Figure 4B). A MIC_{aj} for tedozolid (the active pharmaceutical ingredient) was not available from the (Bengtsson-Palme and Larsson 2016)) study, but a MIC of 0.016 mg/L (based on 12 species), corresponding to a MIC_{aj} <0.008 mg/L was recently (January 2017) reported the European Committee on Antimicrobial Susceptibility Testing database. This suggests that CRB are substantially more sensitive to tedozolid compared with fish and cyanobacteria. The fact that tedizolid phosphate (pro-drug) requires activation by phosphatases in the blood to convert it into the active ingredient (tedizolid), and the ecotoxicity assessments in cyanobacteria appear to be based on the pro-drug only, may explain why cyanobacteria were relatively insensitive. In no cases were the chronic NOECs for invertebrates lower than the NOECs for cyanobacteria (Figure 4). The daphnid EC_{50} for the antifolate trimethoprim, however, was lower than the EC_{50} for cyanobacteria (8.21 and 91.68 mg/L, respectively. Figure S3). This was not the case for the NOECs for the same compound, indicating differences in the shape of the dose-response curve. Importantly, in this case cyanobacteria would still drive the $PNEC_{SW}$.

PNEC comparisons

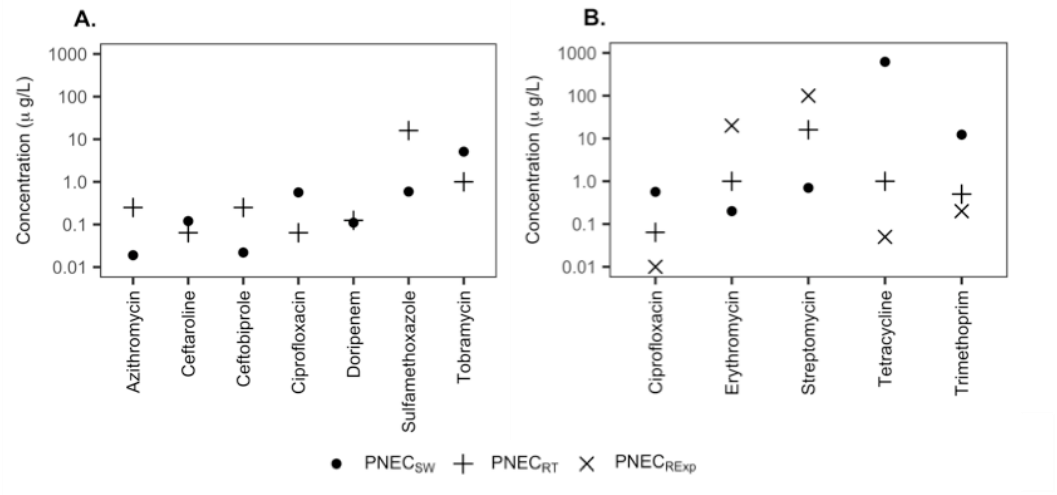

Figure 5. Comparisons of predicted no effect concentrations (PNEC) for antimicrobial resistance and ecotoxicity for aquatic taxa in surface water. A) Comparison of theoretically derived PNEC for resistance development ($PNEC_{R(T)}$) based on clinically relevant bacteria (Bengtsson-Palme and Larsson 2016) and PNEC for ecotoxicity in surface water ($PNEC_{SW}$). (B) Comparison of $PNEC_{R(T)}$, $PNEC_{R}$ based on experimentally derived minimum selective concentrations ($PNEC_{R(EXP)}$) and $PNEC_{SW}$. In A) data are presented for antibiotics only where a full data set including cyanobacteria, invertebrate and fish tests were available and calculated from no observed effect concentrations as described in (EMA 2006). $PNEC_{SW}$ in B) are calculated from cyanobacteria NOECs regardless of a complete ecotoxicity data set where a $PNEC_{R(EXP)}$ was available. $PNEC_{R(EXP)}$ is a less than (<) value in erythromycin and trimethoprim. $PNEC_{R(EXP)}$ based on strain specific MSC in ciprofloxacin, erythromycin, streptomycin and trimethoprim. $PNEC_{R(EXP)}$ based on community based MSC in tetracycline. EC_{50} for cyanobacteria was used because NOEC were not available for $PNEC_{SW}$ in streptomycin and tetracycline therefore NOEC may be up to an order of magnitude lower.

For the limited number of antibiotics where a definitive $PNEC_{SW}$ could be calculated (n=7) an analysis of the relationship between traditional ERA PNECs and those for AMR was conducted. Within this meta-analysis the theoretically determined PNEC for resistance development $PNEC_{R(T)}$) obtained from Bengtsson-Palme and Larsson (2016) for the different antibiotics was not always protective of (lower than) the $PNEC_{SW}$ (Figure 5A). The $PNEC_{R(T)}$ was lower than $PNEC_{SW}$ for ceftaroline, ciprofloxacin and tobramycin. However, the $PNEC_{SW}$ was approximately ten-fold lower than $PNEC_{R(T)}$ for ceftobiprole, sulfamethoxazole and azithromycin.

Where experimentally derived MSCs existed, the $PNEC_{R(Exp)}$ was lower than $PNEC_{R(T)}$ for three out of five antibiotics with available data (Figure 5B). However, $PNEC_{R(T)}$ overestimated the risk of resistance development for streptomycin by an order of magnitude. $PNEC_{R(T)}$ and $PNEC_{R(Exp)}$ were similar for trimethoprim (Figure 5B; trimethoprim $PNEC_{R(Exp)}$ was <0.2 μg/L). The $PNEC_{SW}$ for erythromycin and streptomycin were lower than their $PNEC_{R(T)}$ and $PNEC_{R(Exp)}$ (Figure 5B). The $PNEC_{R(Exp)}$ for erythromycin however, did not have a definitive value, (i.e. <0.2mg/L) and as such we assign caution to this comparison.

We determined the 5th percentile for growth inhibition data for cyanobacteria and environmental bacteria and MICs for CRB (See table S4). The rationale for this was to establish an environmental protection goal for antibiotic production discharges that would be protective of bacterial NOECs with 95% confidence. The 5th percentiles ranged from 225 to 2028 ng/L, depending on the bacteria and endpoints used. The lowest NOECs for environmentally relevant bacteria (cyanobacteria, *P. putida* and *V. fischeri*) gave the lowest value (225 ± 71 ng/L, Figure 6A).

Establishing 5th percentiles

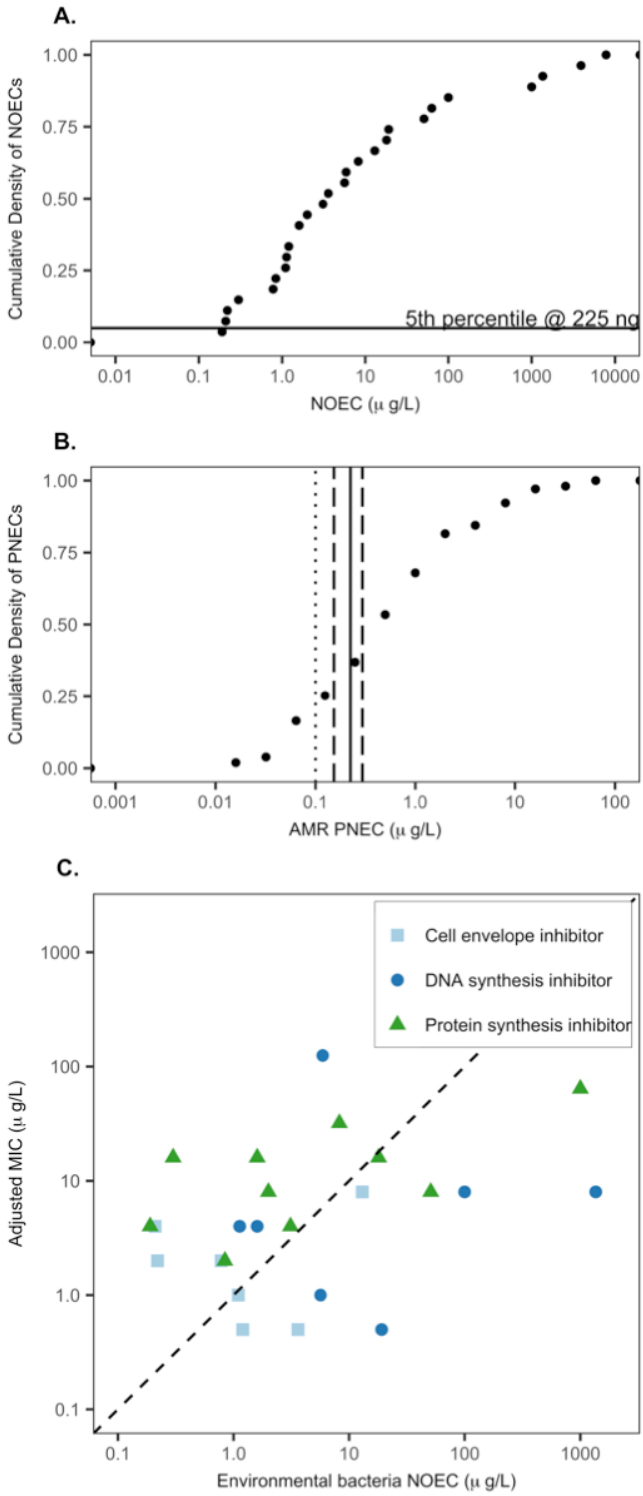

Figure 6. A) Cumulative density plot of the NOECs for environmental bacteria for 27 antibiotics, showing the 5th percentile. B) Cumulative density plot of PNECs for AMR for 103 antibiotics, as calculated by Bengtsson-Palme and Larsson (2016). The vertical solid line represents the 5th percentile of the bacteria NOECs, dashed lines represent the standard error and dotted line indicates the proposed discharge limit. Note each point can represent up to 17 antibiotics. C) Comparison of NOECs for environmental bacteria and clinically relevant bacteria minimum inhibitory concentrations.

Discussion

In our evaluation of the current regulatory ERA guidance we show that of the taxa tested, as expected based on the mechanisms of action, prokaryotes were most sensitive to antibiotics. However, we also show that reliance on one species of cyanobacteria to set protection levels (e.g. PNECs), as operates currently, is unlikely to be protective of environmental and human health (through AMR). Individually, neither traditional aquatic PNECs nor the AMR based PNECs protect fully against the effects of antibiotics. We thus recommend the inclusion of both clinically important bacteria and a wider range of species of environmentally relevant bacteria to improve the prospective regulatory framework for human and ERA. This approach will help also in defining more appropriate safe discharge concentrations for antibiotic production, and help to exclude unnecessary ERA testing on metazoan species.

Species relative sensitivity: the need for more bacteria

During their development, the efficacy and safety of new antibiotics are assessed in preclinical and clinical studies before market approval. It is therefore unlikely that toxic effects will occur in an aquatic vertebrate (such as fish) at water concentrations lower than those affecting prokaryotic species (target or non-target). As expected, in our analyses, those species evolutionarily more distant to pathogenic bacteria were generally less sensitive to antibiotics compared with clinically relevant and environmental bacteria. Our results also indicate that neither cyanobacteria, CRB nor other environmental bacteria (*V. fischeri* and *P. putida*) provide a single organism/test that is fully protective of the diversity of bacteria in the environment. Thus, a $PNEC_{SW}$ determined according to the current ERA guidance (EMA 2006; US Food and Drug Administration 1998) will not always be protective of the environment.

Sensitivity to any one antibiotic differed by up to five orders of magnitude across different species of cyanobacteria. Patterns of sensitivity for the different genera were observed across the different antibiotic mechanisms of actions, but no one species was consistently the most sensitive. Cyanobacteria are one of the most diverse phyla on the planet (Shih *et al*. 2013; Whitton 2012) and this large range in sensitivity to antibiotics might therefore be expected. In ERA *A. flos-aquae* is the most regularly used of the two OECD test guideline recommended

cyanobacterial species (the other being *S. leopoliensis;* (OECD 2011)) but *A. flos-aquae* was the most sensitive cyanobacteria for only one of the 13 antibiotics for which data were available for multiple genera and species. In the cases of ampicillin, erythromycin, norfloxacin, oxytetracycline, sulfdiazine and trimethoprim (35% of antibiotics with multiple cyanobacterial EC_{50}s) the difference in sensitivity between *A. flos-aquae* and the most sensitive taxon was greater than the assessment factor (x10) used to generate a PNEC for the risk assessment. For ampicillin, reliance on *A. flos-aquae* could underestimate the $PNEC_{SW}$ by more than three orders of magnitude. This questions the current over reliance on a single cyanobacteria test species within ERA frameworks and we propose at least three cyanobacteria genera should be included within these risk assessment frameworks. The case above for ampicillin highlights a further important issue relating to the relevance of high sensitivity for some cyanobacteria. Ampicillin is not persistent in the environment and undergoes partial degradation by bacteria; indeed, primary degradation is the resistance mechanism. If degradation were factored in, from an ecotoxicological point of view, exposure and environmental effects would be low, although community structure changes could impact resilience. Furthermore, since the resistance mechanism partially degrades the antibiotic resulting in a lower concentration of ampicillin in the environment care needs to be taken not to assume a low measured concentration of ampicillin necessarily equates with an absence of selection for AMR development and human health risk.

The cyanobacteria adopted for toxicity testing has been based largely on experimental convenience (e.g. the ability to grow them and measure cell density in the laboratory) with little knowledge on how representative they are of other cyanobacteria. No consideration has been given to how they grow and function in non-pelagic habitats, e.g. biofilms. From our analyses, *M. aeruginosa* would potentially provide a relatively high sensitivity to most antibiotics. This species however, has a slower growth rate and the current test with this species may therefore have to be extended to make the test comparable in terms of the growth and replication dynamics with that for *A. flos-aquae* and *S. leopoliensis*. We highlight that the requirement for optimised conditions for culturing a species and variation in life history components across species (e.g. growth rates and lag time) create further challenges for interspecies substance effects analyses. For example, exposure time can have a direct impact on the

perceived sensitivity. In this meta-analysis we have used data that are based on regulatory approved guidelines in which exposure time and exposure conditions have been optimized for the different organisms to ensure that growth in the controls do not reach the plateau phase, thus maximizing the ability to detect for any effects against treatment groups. Longer exposure periods could potentially result in lower effective exposure concentrations, as we demonstrate for the EC_{50} in *V. fischeri* (for a 24 hour exposure compared with shorter test periods) and as has been shown for the ASRIT (Kümmerer *et al*. 2004). Extending exposure periods in growth tests however needs to ensure that this does not compromise the ability to distinguish for effects i.e. additional time does not result in the controls being limited in their growth dynamics by the available resources and thus affect the comparison with the treated groups. It needs to be recognized, however, that differences between test conditions optimized for different species (e.g. chemical constituents of the culture media, pH, temperature, light intensity and test length, to name just a few) could all impact the fate and behavior of the antibiotic and its bioavailability, distribution, metabolism and excretion in test organisms, which in turn may influence the perceived relative sensitivity. Distinction needs to be made on whether the exposure adopted is optimized for assessment of effects relative to controls (as is the case in the OECD 201 test guideline for green algae and cyanobacteria) or focused more on environmental relevance (for example in the ASRIT analyzing for impacts within hydraulic residence time in sewerage treatment works). Species sensitivity analyses and /or functional impacts are arguably better addressed under context specific conditions that consider the microbial community structure(s) and physicochemical conditions that occur in those natural systems.

Available study information was not sufficiently comprehensive to allow for consideration of these variables within our meta-analysis and we were thus restricted to endpoint data (EC_{50} and NOEC) that we derived from reliable studies. Further investigation is warranted into the physiological basis for the differences in sensitivity to antibiotics to help identify species, or groups of species, that best represent the phylum for their protection and the critical ecosystem services (e.g. primary productivity and food source) they provide.

V. fischeri and *Pseudomonads* were more sensitive than cyanobacteria to some antibiotics and may potentially provide valuable additional species for inclusion within the ERA. Furthermore, they already have internationally recognised test guidelines (ISO 1995; 2007). *V. fischeri*, is a marine bacterium that would not normally be considered in ERA for freshwaters, but is sometimes used in whole effluent assessments (ECETOC 2004). It is, nevertheless, a prokaryotic species and antibiotics and antibiotic resistant bacteria have been detected in estuaries and marine environments emanating from sewerage treatment plant discharges and manufacturing effluents (Schaefer *et al*. 2009; Webster *et al*. 2004; Zheng *et al*. 2011; Zou *et al*. 2011). The compiled data show that *V. fischeri* was more sensitive than cyanobacteria for six antibiotics, and for half of these by nearly an order of magnitude (flumequine, lomefloxacin and oxolinic acid). The inclusion of this test could therefore be of value to ERA if performed with an exposure time of 24 hours (results based on exposure lengths of less than 24 hours showed significantly less sensitivity). *Pseudomonads* have been shown to be less sensitive than the other soil bacteria to tetracycline, chlortetracycline, and oxytetracycline and in some instances by over an order of magnitude (Halling-Sørensen *et al*. 2002). The low sensitivity observed in *Pseudomonas* species has been attributed to their apparent high natural resistance to some antibiotics (Halling-Sørensen *et al*. 2002; Kittinger *et al*. 2016). Thus, our findings suggest that additional testing with *P. putida* could be of value to the ERA, but it may still not be protective of other soil bacteria. Any consideration to incorporate the test with *P. putida* in antibiotic ERA would need to first characterise the strain in terms of its chromosomal and plasmid resistance to help prevent biasing any function or growth based assessment (Brandt *et al*. 2015).

The ASRIT (OECD 2010) was several orders of magnitude less sensitive to antibiotics than cyanobacteria and other bacterial species, confirming reports that this test is largely insensitive to antibiotics (Kümmerer *et al*. 2004). As such, the ASRIT would not influence the outcome of the ERA. This lack of sensitivity may be due to several factors, including the short exposure time (3 hour) of the test (Kümmerer *et al*. 2004), the lack of antibiotic bioavailability due to adsorption to the sludge solids (Golet *et al*. 2002) or that the microbial community in the activated sludge has an innate resistance having been exposed previously to the antibiotic (Davies 2012). It was not possible to assess the effect of extending the ASRIT test duration due to a lack of available data

and because most ASRIT results are reported as censored data of >100 mg/L. Furthermore, the endpoint of respiration, may not be suitable for all mechanisms of actions (Brandt *et al*. 2015) and it does not equate with changes in bacterial diversity or community structure. We thus support the need to replace and/or complement the ASRIT with other assays (Brandt *et al*. 2015), which are relevant for all pharmaceuticals.

In order to build greater confidence in the ERA for antibiotics we sought to gain a better understanding on the differences observed in sensitivity between the species and to establish both how often and for which antibiotic classes these differences exceed the assessment factor of 10. Overall, across all the antibiotics assessed, cyanobacteria and CRB were equally sensitive to antibiotics (figure 1). Thus, neither CRB nor cyanobacteria were consistently more sensitive than the other. In this meta-analysis, the inclusion of CRB in ERA would drive the PNEC in 40% of cases further supporting a more holistic 'one health' approach that uses clinical and environmental data. There were, however, substantial differences in sensitivity to antifolates observed between the cyanobacterial species and CRB. The folate synthesis pathway that antifolates inhibit is present in cyanobacteria and so the reason for the apparent lack of sensitivity in some cyanobacteria is unknown. However, de Crécy-Lagard *et al*. (2007) reported that cyanobacteria possess a protein that may act as a folate transporter allowing the bypassing of some of the folate synthesis pathway. Our analysis suggests therefore that cyanobacteria may not always be a suitable representative for bacteria for full protection against antifolate antibiotics.

Macrophytes appear especially sensitive to antifolates and quinolones. The folate synthesis pathway in bacteria, algae and plants is fundamentally the same (Basset *et al*. 2005) and they are, therefore, all potentially susceptible to antifolates. Indeed, sulfamethoxazole has been reported to act as a competitive agonist to *p*-aminobenzoic acid in both *Lemna gibba* (Brain *et al*. 2008b) and *Arabidopsis thaliana* (Zhang *et al*. 2012). Macrophytes were also more sensitive than cyanobacteria to five quinolones. Quinolones cause toxicity by forming complexes with DNA gyrase or topoisomerase IV resulting in the inhibition of DNA replication and transcription (Aldred *et al*. 2014). Chloroplasts are descended from cyanobacteria (Falcon *et al*. 2010) and some plants and

red algae have been shown to contain DNA gyrases in their plastids (including chloroplasts) and mitochondria (Moriyama and Sato 2014; Wall *et al*. 2004). Quinolone antibiotics are reported to have anti-chloroplastic activity (Brain *et al*. 2008a; Brain *et al*. 2004; Ebert *et al*. 2011) which can affect photosynthesis in plants (Brain *et al*. 2008a). Indeed, organellar DNA gyrase has been shown to be the primary target of ciprofloxacin in *Arabidopsis thaliana* (Evans-Roberts *et al*. 2016). Thus, our findings indicate that for some antibiotics in these classes, macrophytes could potentially drive the protection goal. Consequently, these species should be considered for inclusion within risk assessment frameworks for antibiotics.

The metazoan taxa were never found to be the most sensitive compared with all bacterial taxa. This questions the necessity of resource intensive metazoan testing of antibiotics, as required by European Medicines Agency and Food and Drugs Administration guidance (EMA 2006). Inclusion of appropriate (and additional) bacterial testing in the ERA for antibiotics would potentially allow for the exclusion of some unnecessary testing on metazoan species, acknowledging the principles of the 3R's to replace, reduce and refine studies that use 'protected' animals, such as fish (Hutchinson *et al*. 2016; Scholz *et al*. 2013).

We performed this meta-analysis based on data that was deemed most reliable according to the CRED system (Moermond *et al*. 2016). The conclusions however, are still drawn upon data that were conducted in different labs, with different procedures and of varying quantity (in terms of test performance and meta-data) and quality of reporting. We strongly emphasise the need to collect and report suitable control data, chemical analysis and meta-data in order to assist in reliable comparisons of studies.

An analysis of appropriate additional bacterial species for inclusion in the ERA needs to consider potential differences in sensitivity due to pharmacokinetic considerations including bioavailability, charge, uptake, elimination, metabolism, degradation rates or binding affinities, or a combination of them. Differences in bacterial morphologies and innate resistance may also account for some of the differences in sensitivity between species. Some bacteria have several different growth forms depending on the environmental conditions. As an example, increased temperature and light intensity causes aggregation of S*ynechococcus*

elongates cells (Koblížek *et al*. 2000) and this aggregation may have an impact on the sensitivity of the cells to antibiotic exposure. Several studies have demonstrated that cells in biofilms are less sensitive/more protected from chemical exposure (Balcázar *et al*. 2015). A better understanding of how physiological and morphological differences in cells and community structure affect the toxicity of chemicals to bacteria is required to fully understand the risk posed by antibiotics in the environment.

Bacteria are fundamental to many vital ecosystem services, but little is understood regarding species loss and functional redundancy and thus, the resilience of ecosystem function. Some investigators, however, have begun to address this. For example, Lundström *et al*. (2016) found no change in the overall taxonomic diversity when biofilms were exposed to tetracycline, however, the community composition was altered and the functional diversity, as measured by utilization of carbon sources, decreased with increasing tetracycline concentrations. Ciprofloxacin exposure altered the bacterial community structure in marine sediments at 0.2 mg/L), resulting in a decrease in the community ability to degrade pyrene (Näslund *et al*. 2008). It was also found to increase overall biomass in salt marsh microbial communities, favouring gram negative and sulfate-reducing bacteria (Cordova-Kreylos and Scow 2007). Several studies have shown that bacterial diversity has a positive relationship with ecosystem function (Bell *et al*. 2005; Langenheder *et al*. 2010). (Delgado-Baquerizo *et al*. 2016) demonstrated that loss of diversity in aquatic bacterial communities caused a decrease in both broad (microbial respiration) and specialized (toxin degradation; of mycrocystin-LR and triclosan degradation) endpoints and the communities showed little or no functional redundancy. These studies indicate that a small drop in bacterial diversity may potentially impact negatively on the ecosystem services they provide.

From this, we conclude that the ERA framework for antibiotics needs to be based upon a suitable range of bacteria. This should include CRB and capture a wider range of ecologically important functional groups. Previous investigators have identified standard studies that may fulfill some of these data gaps e.g. nitrifying bacteria, methanogens and sulfate-reducing bacteria (Brandt *et al*. 2015) although more research is required to identify if these tests will be protective of all functional bacterial groups or if further standard tests will need

to be developed. The effect of antibiotics on these functional groups is currently outside risk assessment frameworks and environmental and non-therapeutic human impacts are considered in isolation. Furthermore, a measure of the change in community structure would add value, especially looking at diversity in terms of clinical and environmental relevance, and understanding to changes in functional endpoints in bacterial multispecies/community tests to determine whether ecological resilience is being compromised.

PNECs for AMR verses traditional ecotoxicological effects

AMR is a serious risk to human health globally and currently sits outside the ERA regulations. Both theoretical methodologies and empirical data available for assessing AMR selection and transfer in the environment are limited. Consequentially, evidence is lacking to assess the best approach for the risk of AMR development, how resistance in the environment may lead to enrichment of resistance in human pathogens and how the risk posed by antibiotics by AMR development compares to their effects upon ecosystem function and services. Previous investigators have explored resistance selection using a variety of approaches, for example, comparing predicted environmental concentrations with MICs (Kümmerer and Henninger 2003), using MICs to calculate potentially affected fractions of communities (Singer *et al*. 2011) and using growth and competition experiments to demonstrate resistance selection (Negri *et al*. 2000) and calculate MSCs (Gullberg *et al*. 2011). The theoretical approach proposed by (Bengtsson-Palme and Larsson 2016) is a recent contribution and provides a good basis for this discussion, using MIC data to assess reduction in antibiotic efficacy due to erosion by resistance. However, it is important to note that this approach assumes growth can be used to predict resistance and is not verified through direct testing of resistance markers and as such any conclusions drawn from this analysis must therefore be considered with this in mind.

Our findings suggest that the $PNEC_{RT)}$ defined by Bengtsson-Palme and Larsson (2016) is not always lower than the $PNEC_{SW}$; for 7 antibiotics $PNEC_{SW}$ was lower in four cases (figure 5). This may be due to either the $PNEC_{R(T)}$ underestimating the risk or cyanobacteria being more sensitive to some antibiotics compared with the CRB. Experimentally determined MSCs were derived largely from laboratory strain competition experiments (four of the five

cases; Figure 5B), where strains that differ in only the presence/absence of the resistance genes under investigation are compared (Gullberg *et al*. 2014; Gullberg *et al*. 2011). These strain competition experiments have limitations in scaling up to more complex microbial communities (Bengtsson-Palme *et al*. 2014). There are very few cases where analyses have been conducted for more complex communities but it is hypothesised that the combined effects of changes in community structure (due to loss of the most sensitive species), protective morphological forms (e.g. bacteria maybe less susceptible in biofilms compared to those within the water column (Balcázar *et al*. 2015)), difficulty in defining the 'true' antibiotic exposure concentration, and alternative selection pressures (e.g. nutrient limitation, predation and other chemical/physical stressors) may negate the fitness benefit of the resistance (Bengtsson-Palme and Larsson 2016; Brosche and Backhaus 2010; Day *et al*. 2015; Gullberg *et al*. 2014; Lundström *et al*. 2016; Quinlan *et al*. 2011). Most studies that have considered effects of antibiotics on complex communities have been taxon independent, assessing AMR gene copy number relative to 16SrRNA, rather than providing species specific information. Investigations into AMR following tetracycline exposure, however, have found that resistance was increased in periphyton at the lowest test concentration of 0.5 μg/L (Quinlan *et al*. 2011), horizontal gene transfer (HGT) was promoted at 10 μg/L (Jutkina *et al*. 2016) and resistant bacteria and resistance genes was increased in biofilms at concentrations below 1 μg/L (Lundström *et al* , 2016). Assuming an assessment factor of 10, from this data a $PNEC_{R(Exp)}$ would be 0.05 μg/L, which is 20 times lower than $PNEC_{R(T)}$ of 1 μg/L (Bengtsson-Palme and Larsson 2016). There is no NOEC data for tetracycline in cyanobacteria, but in *Microcystis aeruginosa* a EC_{50} is reported at 90 μg/L (Halling-Sørensen, 2000) and in *Anabaena* sp an EC_{10} of 2.5 mg/L (González-Pleiter *et al*. 2013), suggesting that resistance for tetracycline may occur at concentrations nearly 100-fold lower than effects on growth inhibition in cyanobacteria. This again emphasizes the need for a more holistic approach to the setting of protection goals for antibiotics and the development of validated assays to assess MSCs in complex and simple systems, as well as generating toxicity data for cyanobacteria and other environmental and/or clinical bacteria.

It should be recognized that although studies that are used to guide regulatory decision-making require standardized test methodologies to help ensure reliable and repeatable results, the link between these single species studies and those operating in the complex systems in the field is largely unknown and, as mentioned previously, the link to ecosystem services is not made. The application of mesocosm studies that enable community response and effects upon ecosystem functions to be assessed have good utility here to help provide insights into the development of AMR in environmentally realistic scenarios (Knapp *et al*., 2008; Knapp *et al*., 2010; Quinlan *et al*., 2011). In addition to living in complex communities in the environment, it is important to note that organisms are also likely to be exposed to antibiotic mixtures and the relationship between single exposure laboratory testing and mixtures toxicity is unknown and requires further research (Backhaus *et al*. 2000; Brosche and Backhaus 2010; González-Pleiter *et al*. 2013; Liu *et al*. 2014).

In the context of current regulatory guidance, MSCs derived from experimental data, albeit they are limited, in some cases supported the theoretically derived $PNEC_{R(T)}$. There were cases also where $PNEC_{R(T)}$ was not necessarily appropriate (optimal) for risk assessment for AMR. Nevertheless, until there is an internationally accepted method for the experimental determination of $PNEC_R$ - which may require further knowledge on resistance mechanisms, model variability and the application to mixed communities that vary over time and space - the theoretical approach advocated by Bengtsson-Palme and Larsson (2016), based on MIC data in the European Committee on Antimicrobial Susceptibility Testing database, provides a valuable alternative as part of a broader evidence-based approach to ERA. Moreover, it provides an efficient and cost effective method to address concerns and prioritise legacy antibiotics that have already been registered and are present in the environment. It should be noted, however, that there are clear limitations to this approach (as identified by the paper's authors). These include the test conditions for determining the MIC in CRB, that are largely environmentally irrelevant, the assumptions that growth inhibition can be used to predict selection for resistance. There is also an assumption that an assessment factor of 10 will provide a suitable safety margin to account for selection below the MIC and conversely that adjusting the MIC down to account for species numbers and then applying a further assessment factor of 10 isn't

overprotective. Finally, MIC-derived protection goals will change over time, as MICs are determined for more species with variable sensitivity and as a consequence periodic updates will be required.

Our analysis suggests that the susceptibility of species in European Committee on Antimicrobial Susceptibility Testing is not always protective of environmental bacteria, such as cyanobacteria and therefore a $PNEC_{R(T)}$ using CRB MIC data as a surrogate for resistance may not be protective of the risk of AMR development in environmental bacteria. Furthermore, we show that a $PNEC_{R(T)}$ may not be protective of ecosystem function traditionally determined using the growth inhibition test with cyanobacteria. From this we conclude that despite evidence that resistance will occur at lower concentrations than the effects on population density (Gullberg *et al*. 2011; Hughes and Andersson 2012), both a $PNEC_R$ and a $PNEC_{SW}$ are needed to establish safe concentrations for the protection of ecosystem function and against the development of resistance.

It is noteworthy that from an environmental health perspective (rather than human health), AMR can provide an ecosystem service or benefit. For example, bacteria expressing beta-lactamase enzyme activity degrade and reduce the environmental burden of beta-lactam antibiotics and this in turn could contribute positively in sewerage treatment plants where high antibiotic concentration might otherwise compromise functional efficiency.

Production discharge limits

In addressing the impact of antibiotic pollution on ecosystem function, AMR development and human health, safe discharge limits for antibiotic production facilities need to be established (Agerstrand *et al*. 2015; Larsson 2014; Pruden *et al*. 2013). However, there are few data available in the public domain to support the development of such limits and this is especially so for experimental data on AMR development. Most data that are available are based on growth inhibition tests and we have therefore identified the lowest NOEC values for 27 antibiotics representing sensitive phyla (cyanobacteria, *V. fischeri* and *P. putida*) and using these data we estimate the 5^{th} percentile to be 225 ± 71 ng/L. Thus, a conservative limit of 154 ng/L would account for uncertainty. Provided that these 27 antibiotics are representative of all antibiotics, the cyanobacterial NOECs are, with 95% confidence, likely to be higher than 154 ng/L.

The lowest MSC reported in the literature is 100 ng/L with many others between 10-1000 times higher (Brosche and Backhaus 2010; Gullberg *et al*. 2014; Gullberg *et al*. 2011; Lundström *et al*. 2016). Setting a threshold limit of 100 ng/L for antibiotic discharges would, therefore, appear to be protective of environmental bacterial populations (with 95% confidence) and match the lowest empirical evidence of AMR development. However, it would not be protective for 16% of the theoretical $PNEC_{R(T)}$s, described by Bengtsson-Palme and Larsson (2016) (Figure 6B) highlighting that safe discharge limits may need to be lower than this for some antibiotics in order to consider the potential to select for resistance in clinical and environmental isolates. It should be noted, however, that the $PNEC_{R(T)}$ incorporates a correction factor that adjusts the MIC according to the number of species it is based upon and a further assessment factor of 10 to account for AMR. In turn, the corrections could cause the $PNEC_{R(T)}$ to be over protective (as shown for some antibiotics in Figure 5B).

A single, protective threshold limit that could be applied as an interim measure in the absence of other reliable empirical clinical and or environmental data (and standardised methodologies for AMR), which is based on empirical data would be of great value. Based on the antibiotic compounds for which we were able to obtain NOECs from environmentally relevant bacteria and from the available MSCs in the literature, we suggest a production discharge limit of 100 ng/L for each antibiotic, applied in the mixing zone downstream of the point source discharge for protection of ecosystem function and the risk of AMR development. The use of a single protection goal rather than a range, for production facilities offers pragmatic benefits to industry and suppliers. Compliance with a single protection value provides simplicity and ease of implementation compared with the 111 values advocated for the different antibiotics suggested by Bengtsson-Palme and Larsson (2016), of which some would not be protective of the environment or the MSC. Consideration is required for how this limit would apply in the case of antibiotic mixtures, although this falls out of scope of this meta-analysis.

This approach could also help prevent the use of conflicting values for a single antibiotic. However, it is important to ensure that this value proves to be protective. So where other data are available (e.g. empirical or $PNEC_{R(T)}$) that suggest a lower limit is required to be protective, the 100 ng/L should be

adjusted accordingly to provide the required protection. Equally, a higher limit may be applicable where there are substantive data to support its increase. We advocate this as an interim measure only until more data are obtained to support the risk analysis for antibiotics. Furthermore, as methodologies for the assessment of AMR are developed these values should also be incorporated and protection goals updated.

Concluding remarks and considerations for ERA

Our analysis shows that frameworks for ERA and human health protection (through protection for the risk of AMR) for antibiotics need to consider the impact of antibiotics on relevant vulnerable species and the essential ecosystem services they provide. The current framework for ERA based on just one cyanobacterial species is, in many cases, inadequate and it does not address risk to critical ecosystem services. There is also an urgent need to better establish the effects of antibiotics on bacterial diversity, community structure, ecosystem function and resilience in order to better understand the effects of antibiotics in the environment.

We emphasise that the presence of antibiotics in the environment does not necessarily lead to the development of AMR in bacterial communities and studies are required that better establish the toxic effects of antibiotics, AMR and the relationship between them in environmentally relevant contexts. In the environment other selection pressures (e.g. nutrient availability and predation) may be more significant than that posed by exposure to low levels of antibiotics. As a consequence AMR may not be observed at the same concentrations as in the laboratory studies. However, it is also the case that the fitness cost of carrying some resistance genes may be very low or even neutral and therefore the genes coding for resistance could remain in the bacterial communities after only a short exposure. Understanding these complexities in AMR development in the environment is crucial for establishing interrelationships with human pathogens and in turn managing and mitigating the risk of antibiotics in the environment for the protection of human health.

From our analyses on relative species sensitivity we highlight the following as key considerations for the use, and development of human and ERA frameworks for antibiotics.

1. The need for inclusion of a larger selection of bacterial species for testing to account for the variability in sensitivity between species and for greater confidence in the protection of bacterial communities and the ecosystem services they provide.

    a. Brandt *et al*. (2015) have identified a number of suitable established standard tests for other bacteria (including *P. putida*) and for ecosystem services (e.g. nitrification and carbon transformation) and these should be considered as additional tests in the ERA of antibiotics.

    b. We show that pre-clinical MIC data of CRB could be used to increase the diversity of bacterial species represented in ERA at little cost. The use of pre-clinical and clinical data is often advocated to identify environmental risk (Boxall *et al*. 2012) but the realisation of this is limited with 'bridging' studies and methods still being developed.

    c. We reaffirm that the only required community test, the ASRIT, is not sensitive to antibiotics and thus its suitability for determining the effect of antibiotics to environmental bacteria and sewerage treatment plant microorganism communities is questionable. Consideration for its replacement by tests to assess the effects on bacterial community function or impacts on population growth are warranted.

2. Testing of antibiotics on metazoans may not be required.

    a. Metazoans were generally 2 to 4 orders of magnitude less sensitive to antibiotics than cyanobacteria. Further investigation is required to assess and confirm these results on a wider series of empirical *in vivo* exposures, however this meta-analysis provides a starting point for this discussion and the possible reduction in the use of metazoans in antibiotic testing.

3. Our meta-analysis highlights that the relative high sensitivity of microalgae and macrophytes to some antifolate and quinolone antibiotics (compared with cyanobacteria) supporting their inclusion in risk

assessment frameworks for these compound classes. Further research into the relative sensitivity of macrophytes and microalgae to these classes of antibiotics is warranted.

4. Test systems to determine PNEC or MSC for AMR development are urgently required for clinical and environmental species. Our analysis, suggests that the CRB in the European Committee on Antimicrobial Susceptibility Testing database are not always representative of the diversity of sensitive bacteria in nature. This illustrates that ERA needs to incorporate both $PNEC_{SW}$ and $PNEC_{R}$. There is a need to develop a standardised method to experimentally determine an MSC in environmental and clinical bacteria, exemplified by three out of five experimental values being lower than the theoretical value.

5. A discharge limit of 100 ng/L maybe a protective and pragmatic approach to address environmental concerns around antibiotic production in the absence of sufficient reliable clinical and environmental data, whilst urgently needed methodologies and empirical data are obtained to draw firmer conclusions. Where data exists that suggest a higher or lower concentration is required to be protective that value should be used instead.

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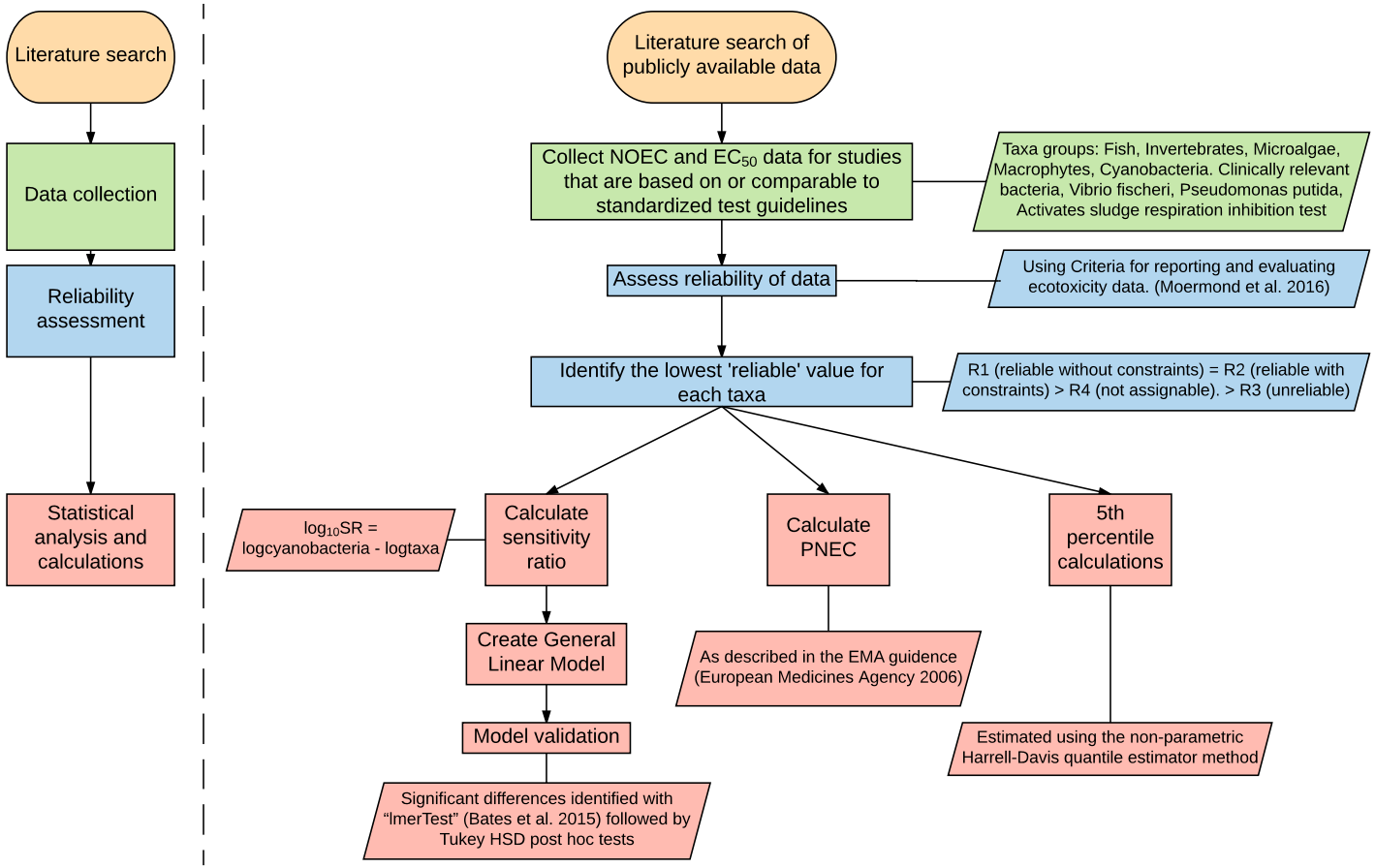
Chapter 2 - Supplemental Material

Supplementary Material

Integrating human and environmental health in antibiotic risk assessment: a critical analysis of protection goals, species sensitivity and antimicrobial resistance

Figure S1 - Flowchart of data colection and statistical processes



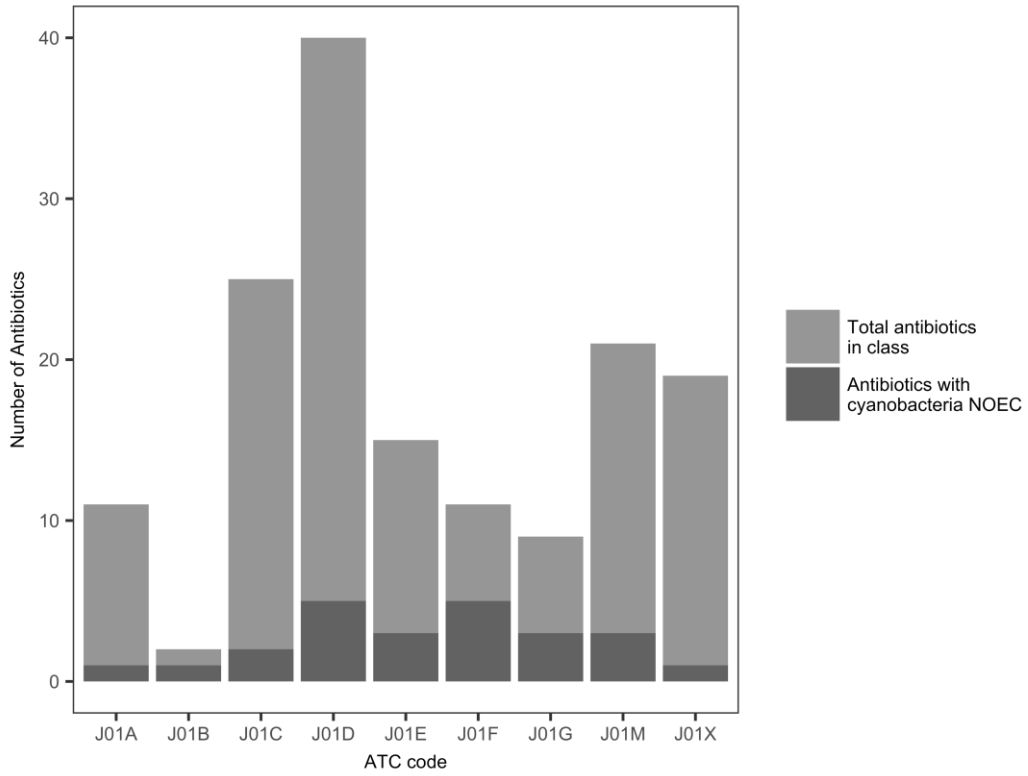


Figure S2. Number of regularly approved antibiotics by anatomical therapeutic chemical (ATC) classification system codes, identified in www.fass.se and (Santos *et al*. 2017), and number of antibiotics that have cyanobacteria no observed effect concentration data in this meta analysis. This figure also includes data for tedizolid phosphate, enrofloxacin and tiamulin that were not included in www.fass.se or (Santos *et al*. 2017).

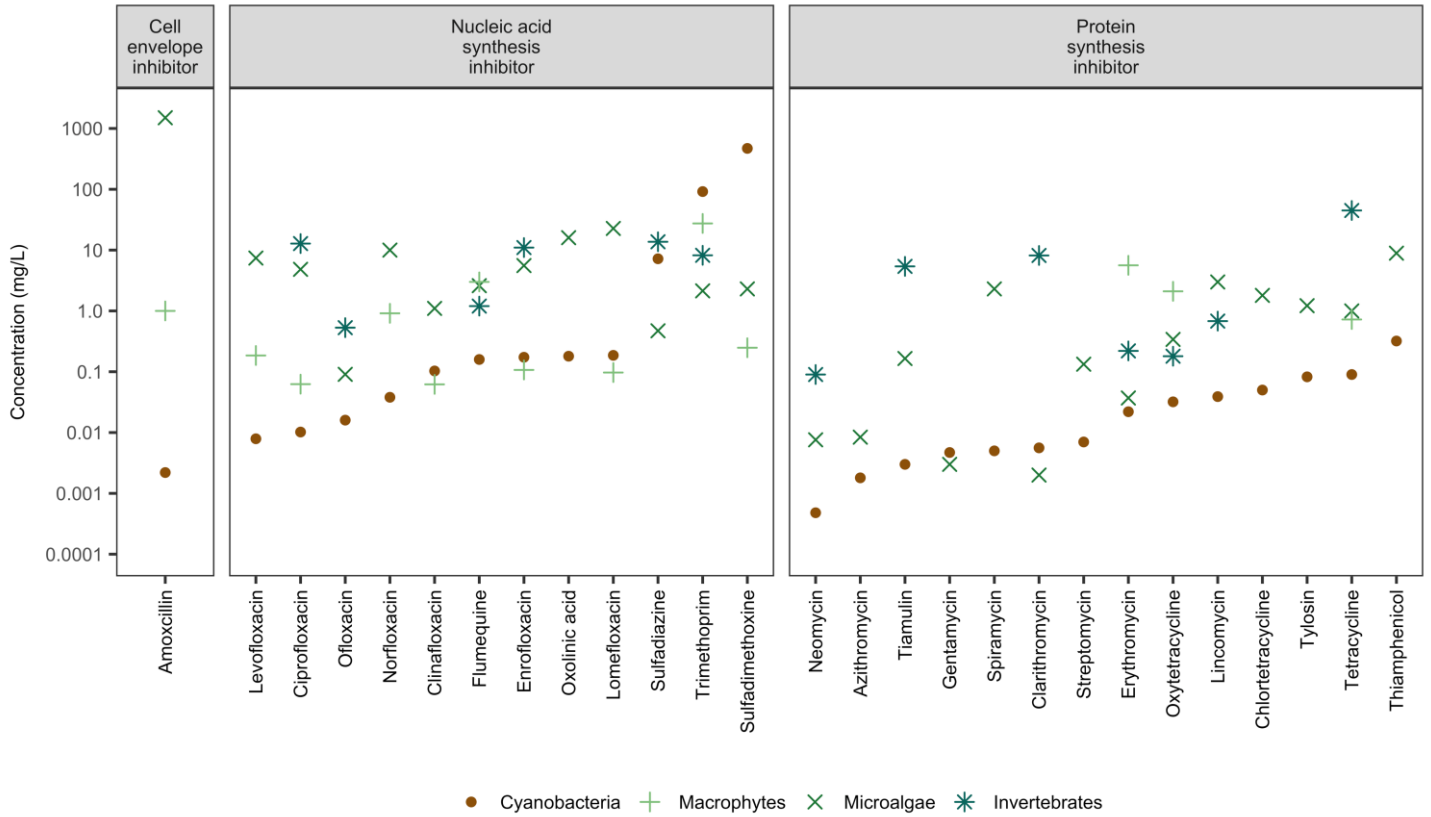


Figure S3. Chronic exposure effects of antibiotics on cyanobacteria compared with microalgae, macrophytes and invertebrate 50% effective concentrations

API	EC50 (mg/L)	Species
Amoxcillin	0.0022	*Synechococcus leopoldensis*
	0.0037	*Microcystis aeruginosa*
	56.3	*Anabaena CPB4337*
Ampicillin	0.14	*Anabaena cylindrica*
	3.3	*Anabaena flos-aquae*
	2.2	*Anabaena variabilis*
	0.002	*Microcystis aeruginosa*
	0.013	*Microcystis wesenbergii*
	>200	*Nostoc sp.*
	0.083	*Synechococcus leopoldensis*
	0.0069	*Synechococcus sp.*
Ciprofloxacin	0.005	*Microcystis aeruginosa*
	0.0102	*Anabaena flos-aquae*
Enrofloxacin	0.049	*Microcystis aeruginosa*
	0.173	*Anabaena flos-aquae*
Erythromycin	0.022	*Anabaena CPB4337*
	0.0035	*Anabaena cylindrica*
	0.27	*Anabaena flos-aquae*
	0.43	*Anabaena variabilis*
	0.023	*Microcystis aeruginosa*
	0.023	*Microcystis wesenbergii*
	0.2	*Nostoc sp.*
	0.16	*Synechococcus leopoldensis*
	0.23	*Synechococcus sp.*
Gentamycin	0.069	*Synechococcus leopoldensis*
	0.0047	*Anabaena flos-aquae*
Levofloxacin	4.8	*Anabaena CPB4337*
	0.0079	*Microcystis aeruginosa*
Lincomycin	0.053	*Anabaena flos-aquae*
	0.039	*Synechococcus leopoldensis*
Norfloxacin	5.6	*Anabaena CPB4337*
	0.053	*Anabaena cylindrica*
	0.29	*Anabaena flos-aquae*
	0.19	*Anabaena variabilis*
	0.062	*Microcystis aeruginosa*
	0.038	*Microcystis wesenbergii*
	1.7	*Nostoc sp.*
	0.63	*Synechococcus leopoldensis*
	0.63	*Synechococcus sp.*
Oxytetracycline	0.21	*Microcystis aeruginosa*
	0.032	*Anabaena cylindrica*
	0.39	*Anabaena flos-aquae*
	0.36	*Anabaena variabilis*
	0.23	*Microcystis aeruginosa*
	0.35	*Microcystis wesenbergii*

API	EC50 (mg/L)	Species
	7	*Nostoc sp.*
	1.1	*Synechococcus leopoldensis*
	2	*Synechococcus sp.*
Sulfadiazine	0.14	*Microcystis aeruginosa*
	7.2	*Anabaena flos-aquae*
Sulfadimethoxine	480	*Anabaena cylindrica*
	>2000	*Anabaena flos-aquae*
	1500	*Anabaena variabilis*
	500	*Microcystis aeruginosa*
	470	*Microcystis wesenbergii*
	>2000	*Nostoc sp.*
	1100	*Synechococcus leopoldensis*
	760	*Synechococcus sp.*
Tetracycline	6.2	*Anabaena CPB4337*
	0.09	*Microcystis aeruginosa*
Thiamphenicol	1.3	*Anabaena cylindrica*
	13	*Anabaena flos-aquae*
	14	*Anabaena variabilis*
	0.32	*Microcystis aeruginosa*
	0.43	*Microcystis wesenbergii*
	3.5	*Nostoc sp.*
	0.36	*Synechococcus leopoldensis*
	0.67	*Synechococcus sp.*
Trimethoprim	112	*Microcystis aeruginosa*
	253	*Anabaena flos-aquae*
	>200	*Anabaena cylindrica*
	>200	*Anabaena flos-aquae*
	11	*Anabaena variabilis*
	150	*Microcystis aeruginosa*
	>200	*Microcystis wesenbergii*
	53	*Nostoc sp.*
	>200	*Synechococcus leopoldensis*
	>200	*Synechococcus sp.*
	91.677	*Anabaena flos-aquae*
	>100	*Synechococcus leopoldensis*
Tylosin	0.034	*Microcystis aeruginosa*
	0.0842812	*Anabaena flos-aquae*
	0.082449	*Synechococcus leopoldensis*
Ofloxacin	0.016	*Synechococcus leopoldensis*
	0.021	*Microcystis aeruginosa*

Table S3. Cyanobacteria EC_{50} for antibiotics where more than one EC_{50} is available.

Data	Number of antibiotics	5th percentile (µg/L)	Standard error (µg/L)
Lowest CRB MIC (LOEC)	103	2.028	0.087
Lowest CRB MIC rounded down to next concentration in concentration range (NOEC)	103	1.014	0.043
Adjusted MIC as calculated in Bengtsson-Palme and Larsson (2016) (NOEC)	103	0.457	0.059
All environmental bacteria and CRB (NOEC and lowest MICs rounded down)	107	0.703	0.253
Environmental bacteria (NOECs)	27	0.225	0.071
Cyanobacteria only (NOECs)	27	0.225	0.072

Table S4. 5^{th} percentiles and standard error of different bacteria no observed effect concentration (NOEC) and/or minimum inhibitory concentration (MIC) datasets. Clinically relevant bacteria (CRB) MICs were obtained from Bengtsson-Palme and Larsson (2016). The lowest MIC is broadly equivalent to a traditional lowest observed concentration (LOEC) the MIC rounded down to the next test concentration is broadly equivalent to a traditional NOEC. The adjusted MIC has been statistically adjusted down to account for antibiotics with fewer CRB MIC data. 5^{th} percentiles and standard error were calculated using Harrell-Davis quantile estimator method that accounts for the non-normal distribution of the data. Analysis was conducted in R (version 3.3.0; R Project for Statistical Computing, Vienna, Austria) using the hdquantile function in the 'Hmisc' package (Harrell Jr 2016).

Extended method and results for the analysis of sensitivity ratios

Sensitivity ratios (SRs) were analysed using a general linear model (GLM). GLMs were constructed using the 'lmer' package with the restricted maximum likelihood method (Bates *et al*. 2015) in R (version 3.3.0; R Project for Statistical Computing, Vienna, Austria). Model residuals were checked to assess the validity of the GLM and significant differences from 0 (value of cyanobacteria SR) were identified using the "lmerTest" package in R (Kuznetsova *et al*. 2013). Further statistical differences between taxa were identified using TukeyHSD procedure. A more detailed method is provided below:

1. A GLM was constructed for all antibiotics, followed by further GLMs based upon mode of action groups and individual antibiotic classes. GLM were only constructed where there were SRs for 3 or more antibiotics. Where a taxa group had only one SR in any antibiotic class these were removed from the GLM analysis. Cyanobacteria SRs (which were 0) were removed from the data before constructing the GLMs and the intercept of the model was forced through 0. Identifying significant differences from the intercept (0) and removing the cyanobacteria SRs prevented 1) the over-representation of cyanobacteria SRs in the model that caused the standardised residuals to be non-normal and 2) the cyanobacteria being accounted for twice in the analysis (once in the ratio calculation and secondly in the statistical analysis of the GLM).

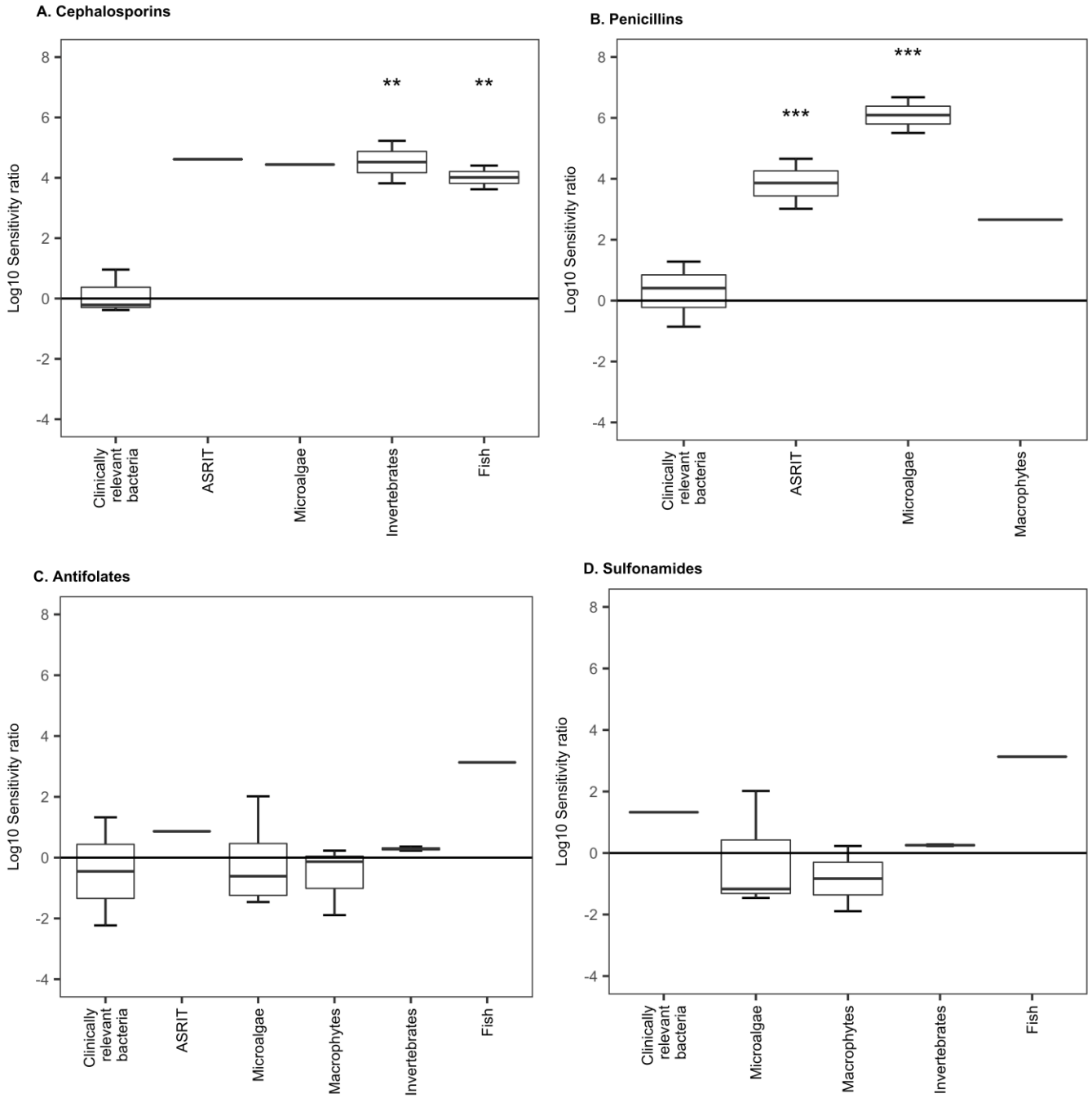
2. The SRs used to construct each model were checked for normality using a histogram, QQ-plot, and the Shapiro-Wilk normality test and equal variances were checked using Levene test in the Lawstat package in R (Gastwirth J L *et al*. 2015).

3. A GLM was constructed in the 'lmer' package using the following basic model outline:

```
Model <- glm(SR ~ Taxa -1,
family = gaussian (link ="inverse" or "identity"),
na.action = na.exclude,
data = data)
```

4. The error family was Gaussian and link function (identity or inverse) was chosen using the GLM with the lowest akaile information criterion.
5. Model validation was conducted to assess
    a. Normal distribution of standardised residuals
    b. Homoscedasticity of residuals
    c. Collinearity
    d. Auto-correlation
    e. Leverage of data

Boxplots and statistical results from the GLM analysis are provided for all antibiotic classes in supplemental material, part 1 Figures 1A-K below.


A. Cephalosporins
B. Penicillins
C. Antifolates
D. Sulfonamides
Log10 Sensitivity ratio
Clinically relevant bacteria
ASRIT
Microalgae
Invertebrates
Fish
Macrophytes
**
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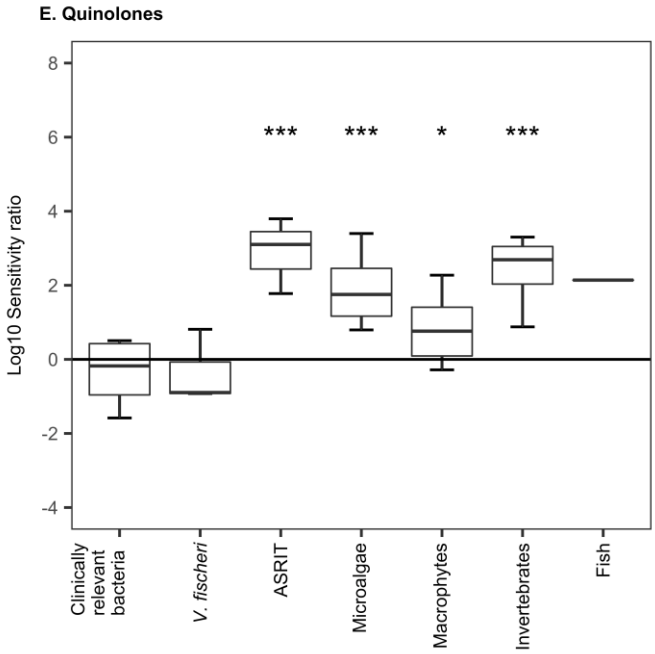
E. Quinolones
Log10 Sensitivity ratio
8
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4
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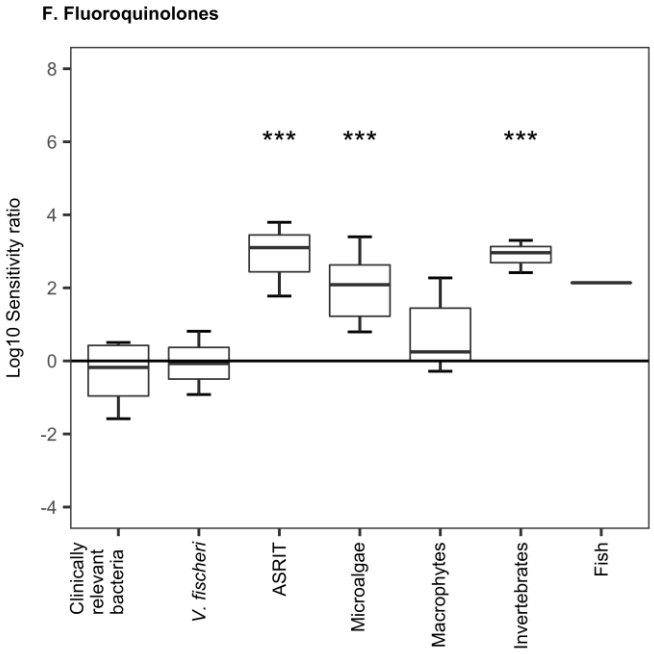
Clinically relevant bacteria
V. fischeri
ASRIT
Microalgae
Macrophytes
Invertebrates
Fish


F. Fluoroquinolones
Log10 Sensitivity ratio
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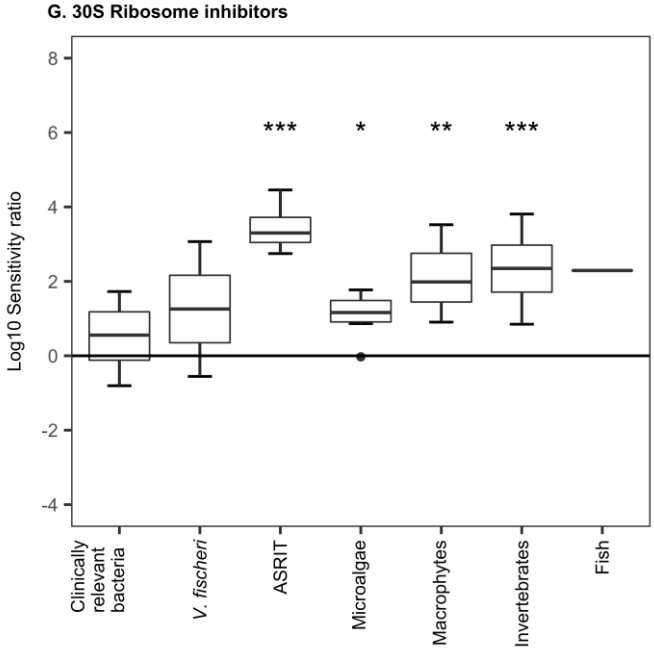
Clinically relevant bacteria
V. fischeri
ASRIT
Microalgae
Macrophytes
Invertebrates
Fish


G. 30S Ribosome inhibitors
Log10 Sensitivity ratio
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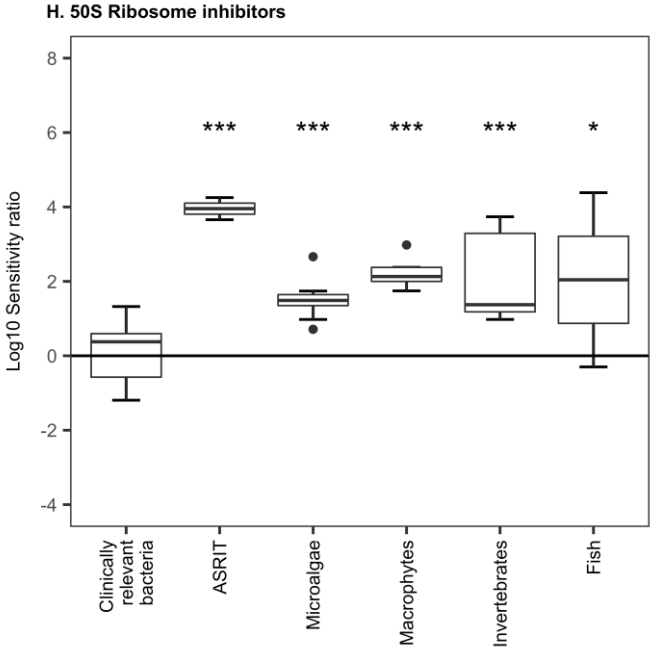
Clinically relevant bacteria
V. fischeri
ASRIT
Microalgae
Macrophytes
Invertebrates
Fish


H. 50S Ribosome inhibitors
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Clinically relevant bacteria
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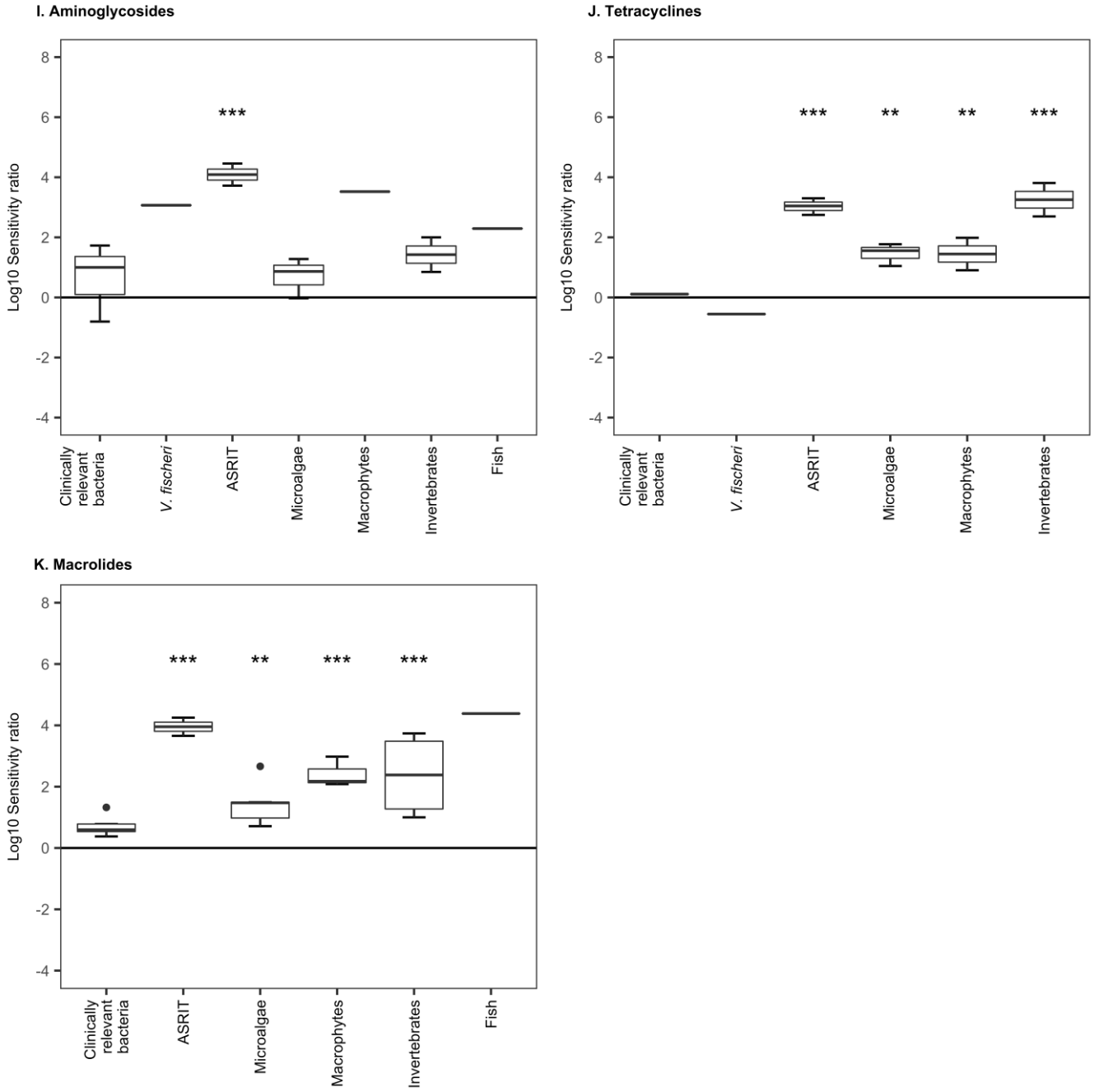

Supplemental material, part 1 Figures 1A-K. Boxplots of Log_{10} sensitivity ratio (SR) between cyanobacteria and other species/phyla for A) cephalosporins (n=3), B) penicillins (n=4), C) antifolates (n=4), D) sulfonamides (n=3), E) quinolones (n=9), F) fluoroquinolones (n=4), G) 30S ribosome inhibitors (n=7), H) 50S ribosome inhibitors (n=10), I) aminoglycosides (n=4), J) tetracyclines (n=3) and K) macrolides (n=5). SR calculated by log_{10}cyanobacteria NOEC or EC_{50} – log_{10}taxa NOEC or EC_{50}. Where SR = 0 the sensitivity of the taxa is equal to cyanobacteria, represented by horizontal line, where SR >0 a lower sensitivity and <0 indicates higher comparative sensitivity. Significant differences of SR from 0 (cyanobacteria SR = 0) in the generalised linear mixed models are indicated by: * p<0.05; ** p<0.01; *** *p*<0.001. Statistical tests were not performed for taxa with only one SR.

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Chapter 3

Antibiotic risk assessment needs to protect both environmental and human health

Gareth Le Page, Lina Gunnarsson, Jason Snape, Charles R. Tyler;
published in *Environmental International* (2018) 115: 397-399.

This article is a published response to a commentary article by Bengtsson-Palme and Larsson (2018) published in *Environment International* 111: 352-353 that has a series of criticisms on the previous published chapter. We discuss the consequences of comparing a no observed effect concentration with a minimum inhibitory concentration and the limited impact that it likely has upon our conclusions in chapter 2. We also address questions raised by Bengtsson-Palme and Larsson regarding the setting of protection limits for manufacturing discharges.

I carried out the first drafting of the paper. All authors were actively involved in later discussions on the work and in the preparation of the manuscript for submission. This paper is included in the thesis appendix in its published format.

Antibiotic risk assessment needs to protect both environmental and human health

In our recent meta-analysis on antibiotic ecotoxicity data published in *Environment International* (Le Page *et al*. 2017) we suggest that because of the great diversity in species sensitivity, environmental risk assessment (ERA) would be improved by testing a more diverse range of bacteria (including both environmental bacteria and clinically relevant bacteria (CRB)). We also conclude that tests on antibiotics should consider endpoints of relevance to ecosystem function. Comparing the protection goals for environmental heath with those for human health (protection against antimicrobial resistance (AMR) development) we, furthermore, identify that neither protection goal is always protective of the other whilst using current methodologies (with surrogate endpoints for each goal and very limited bacterial biodiversity tested); supporting the need for both in any comprehensive health protection system for antibiotics.

In a correspondence to our paper Bengtsson-Palme and Larsson (2018) point out a bias in our sensitivity analysis favouring environmental bacteria (including cyanobacteria). We acknowledge this, but equally in this correspondence we challenge some of their points made on how this impacts on the significance of our data. We also address points relating to the lack of clarity on protection goals for antibiotics in the discussion of our paper and discuss what data are most suitable for establishing those protection goals. We emphasise that the main conclusion drawn from our original paper has not changed and we maintain that a holistic approach including both environmental health and resistance selection is required to drive an effective overall protection limit for antibiotics.

Sensitivity analyses skews

Bengtsson-Palme and Larsson (2018) rightfully point out that our analysis skews the apparent sensitivity in favour of the environmental bacteria because

the endpoints compared for CRB (minimum inhibitory concentrations, MIC) and environmental bacteria (no observed effect concentrations, NOEC) for growth inhibition are derived from different ends of the dose response curve; MICs are derived from the top of the dose-response curve (full inhibitory effect on growth) and the NOECs for environmental bacteria from the bottom of the response curve (concentration with no inhibition). In some cases therefore CRB may be more sensitive than environmental bacteria than our analysis suggests. However, it should be highlighted that this doesn't necessarily mean that environmental bacteria will not represent the most sensitive taxa for individual antibiotics. This is because, in the first instance, in the cases where environmental bacteria were more sensitive by an order of magnitude or more compared with CRB in our analysis, environmental bacteria are likely to be comparable, if not more sensitive to those antibiotics. In our meta analysis this would be the case for 6 out of 24 antibiotics (including azithromycin and ampicillin). Secondly, very large differences in sensitivity can occur between different species of bacteria (our meta analysis showed sensitivity spanned five orders of magnitude in 8 species cyanobacteria exposed to ampicillin) and because of the far greater species number and diversity tested in CRB compared with environmental bacteria there is likely to be a sensitivity bias in favour of CRB. The size-adjusted MIC value used as our comparative endpoint for CRB was calculated from the MICs of up to 70 species in up to 5 families (Bengtsson-Palme and Larsson 2016). In stark contrast to CRB, cyanobacteria antibiotic test data were generally derived from only one or two species giving far greater uncertainty in the sensitivity calculation for this group.

Uncertainty in protection targets.

ERA for antibiotics in the European Union is legislated by the Medicinal Products for Human Use directive (EC 2001) where the protection goal is to prevent "any risk of undesirable effects on the environment". Current practice is to calculate a PNEC using chronic growth and/or reproduction data on single species, which for antibiotics is normally based on the $PNEC_{SW}$ driven by a cyanobacterium. The relationship however, between individual species sensitivity, ecosystem function and functional redundancy is not well understood (Antwis *et al*. 2017) and what constitutes an "undesirable effect" is unclear. As Bengtsson-Palme and Larsson (2018) point out, clarity is,

therefore, required in the definition and objectives of these protection goals. The issue of functional redundancy, and to what extent it is possible to eradicate or lose a microbial species without compromising that ecosystem function is a hugely important consideration for environmental protection. There is some evidence that microbial communities may be less functionally redundant than macroorganism communities (Delgado-Baquerizo *et al*. 2016). Thus, although we re-iterate our support of the inclusion of ecosystem function based tests, given the uncertainties relating to functional redundancy, at this time ecosystem level protection may be best served by a conservative protection goal based upon bacterial biodiversity (and therefore inherently ecosystem function).

Bengtsson-Palme and Larsson (2018), highlight that the risk of AMR and human health concerns are generally the main driving force for antibiotic protection goals but they also agree with our conclusions that a holistic approach that considers both environmental health and AMR should be taken. The meta analysis shows that for some antibiotics the environmental protection limits may be lower than the protection limits predicted for AMR (using current methodologies and surrogate endpoints for biodiversity and AMR). To illustrate this, here (Fig 1) we compare the $PNEC_r$ determined using the size-adjusted MIC data (Bengtsson-Palme and Larsson 2016) and $PNEC_{sw}$ calculated from the lowest NOEC in our meta analysis with the $PNEC_{fw}$ (PNEC in freshwater) determined for the 5 antibiotics in the European commission environmental quality standards watch list (Carvalho *et al*. 2015). In each case the $PNEC_r$ represents the highest PNEC for each antibiotic (i.e. is least protective as a whole).

As Bengtsson-Palme and Larsson (2018) point out, protection against antibiotic pollution for environmental health is more of a localised impact, whereas AMR has a wider and more pervasive global significance, directing stakeholders towards the need for two different protection targets determined from appropriate data and methodologies. We still maintain however, that an overall protection limit should protect both environmental and human health. Environmental protection and associated legislation differs across countries, but equally there is a social responsibility to ensure that product provenance is conducted to the highest possible levels.

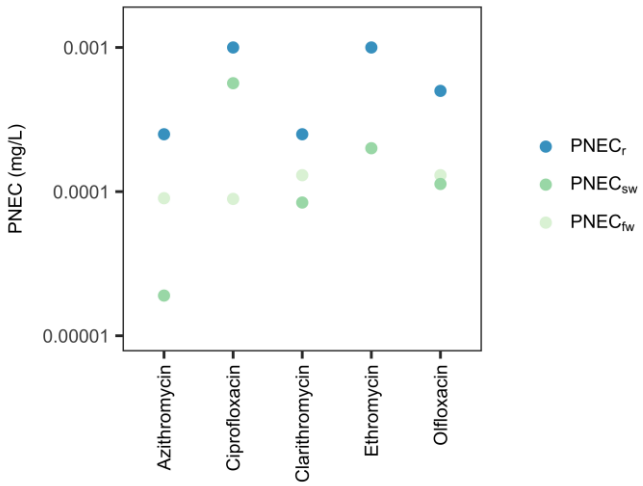

Fig 1. Predicted no effect concentrations (PNEC) for the antibiotics in the European commission watch list under the environmental quality standards directive (Carvalho *et al*. 2015). $PNEC_{fw}$ is the PNEC that is determined for freshwater in the European commission directive (Note that the assessment factor for $PNEC_{fw}$ may be up to 50 rather than 10 in these examples due to the lack of a full phase II base set of data – algae/cyanobacteria, invertebrates and fish (EMA 2006). The $PNEC_{fw}$ for ciprofloxacin is thus most likely overprotective); $PNEC_r$ is the PNEC calculated from minimum inhibitory concentrations (Bengtsson-Palme and Larsson 2016); $PNEC_{sw}$ is the PNEC determined from the lowest, publically available, environmental bacteria no observed effect concentration (Le Page *et al*. 2017). $PNEC_{sw}$ uses an assessment factor of 10 for each antibiotic.

Discharge limit

In response to stakeholder calls to address the risk of antibiotics released from manufacturing operations, which currently sits outside of the regulatory ERA framework, in our original paper we proposed an interim production discharge limit of 100 ng/L for each antibiotic, to be applied in the mixing zone to both protect environmental bacteria populations and reduce the risk of AMR development. This interim limit recognised that (i) because most antibiotics were authorised before the current guidelines came into force, many either lack or have very limited ecotoxicology data, and (ii) the need to establish science-based limits in the absence of such data. We were explicit in our paper to point out, however, that as sufficient data become available for mode of action relevant species we support the use of higher or lower protection limits based on these empirical data. Bengtsson-Palme and Larsson (2018) questioned this conservative limit for antibiotics because it may incur higher manufacturing costs through the need for infrastructure investment to reduce discharges and

based on the fact that some antibiotics have relatively low toxicity and do not exert a strong selection pressure for antibiotic resistance. These are important points to debate. A single interim value helps the pharmaceutical industry, many of whom are currently reviewing their antibiotic manufacturing operations, to prioritise interventions and actions. These interventions may include generating relevant environmental toxicology data where empirical data does not exist or when a possible risk is identified at a site. A single value will also enable the pharmaceutical industry to benchmark existing suppliers more effectively to identify best practice in waste management. The requirement for infrastructure investments, as highlighted by Bengtsson-Palme and Larsson (2018), represents a last resort and these would only be required where risks could not be refined and managed through other interventions. Where infrastructure upgrades are required to meet scientifically robust limits, then the costs of these upgrades will need to be evaluated and justified as part of a wider socio-economic assessment into the stewardship of antimicrobial chemotherapy. In most cases, however, these interventions are not likely to incur excessive costs; the manual wipe down of equipment prior to cleaning washes, separation and incineration of the wastewater from the first wash of equipment, or the installation of inline filters to remove undissolved material can all significantly reduce environmental concentrations of APIs, in most cases by >90% (Hargreaves *et al*. 2017). The logistics for antibiotic supply can be extremely complex with many suppliers manufacturing a whole range of antibiotics for numerous contractors and there can be language barriers and many suppliers lack the expertise to determine safe concentrations for themselves. In this case the use of a single interim limit has practical as well as scientific value. It may help remove conflicting limits (e.g. where two contractors provide different safe values or no level of protection), and minimise confusion amongst the pharmaceutical industry and their suppliers in the absence of data.

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Chapter 4

Development and application of a microplate assay for toxicity testing on aquatic cyanobacteria

Gareth Le Page, Lina Gunnarsson, Jason Snape, Charles R. Tyler;

This article is in preparation for submission for publication at the time of submitting this thesis. It is a paper that details the development and validation of a microplate assay for testing the growth inhibition of eight species of cyanobacteria.

I carried out all experimental planning and work, performed the analysis and first drafting of the paper. All authors were actively involved in later discussions on the work and in the preparation of the manuscript.

Development and application of a microplate assay for toxicity testing on aquatic cyanobacteria

Abstract

Environmental risk assessment (ERA) relies on a growth inhibition test performed upon a single species of cyanobacteria and conducted in a cumbersome shake flask assay, to represent all bacterial diversity when establishing a protection limit. Recently, the ability of this approach to account for the wide range of interspecies differences in sensitivity has been questioned and there is therefore a requirement for empirical data for additional species to further investigate the effectiveness of ERA. To this end, we present the development and validation of a microplate assay that is able to obtain growth rate inhibition data for eight cyanobacteria and which is comparable to the traditional shake flask test, whilst also being cost effective and time efficient. We use phycocyanin fluorescence as a surrogate for cell density and optimise the assay to ensure a comparison of cyanobacteria sensitivity under exponential growth in the same experimental conditions. Finally the test is validated using the reference compound potassium dichromate to show reproducible results across assays over time and comparable results with the OECD 201 shake flask test design. This assay is also suitable for the screening of ecotoxicology data-poor antibiotics to prioritise further investigation and for new compounds in drug development.

Introduction

Bacteria, including cyanobacteria, play key functional roles in our aquatic ecosystem including primary production, biogeochemical cycles and processing pollutants (Díaz 2004; Falkowski *et al*. 2008; Finlay *et al*. 1997). In particular, cyanobacteria are autotrophs (although many are additionally able to produce energy through other mechanisms) that are ubiquitous across all habitats having evolved to perform many ecological functions essential to supporting life, such as, among others, nitrogen fixation, oxygen generation and photosynthesis-mediated calcification (Falkowski *et al*. 2008; Zhu and Dittrich 2016).

There is concern however, that in the environmental risk assessment (ERA) of antibiotics, microorganisms are underrepresented and that the protection limits determined may not be fully protective of bacteria populations nor of the ecological functions that they provide (Agerstrand *et al*. 2015; Brandt *et al*. 2015; Le Page *et al*. 2017). Additionally, the vast majority of antibiotics were registered before new EU regulations were introduced in 2006 when effects upon the bacterial compartment were not considered in ERA. Thus there is only limited data in the published literature regarding the environmental effects of antibiotics from which to establish their potential risk.

Regulatory studies supporting ERA that are responsible for representing all global bacterial diversity, currently rely upon the activated sludge respiration inhibition test (which is not considered to be sensitive to antibiotics (Kümmerer *et al*. 2004; Le Page *et al*. 2017)) and a cyanobacteria growth inhibition test based upon just one species, normally *Anabaena flos-aquae*. Since antibiotics are selected to inhibit growth or kill pathogenic bacteria without affecting mammals, cyanobacteria are likely to have the antibiotic drug targets which are unlikely to be evolutionally well conserved in the other eukaryotic species normally required for regulatory testing (green algae, daphnid and fish).

Ideally, testing on bacterial should consider different bacteria phyla and determine effects upon communities and the ecological functions that they perform (Brandt *et al*. 2015; Le Page *et al*. 2017). However, the requirement for standardised test methodologies and robust results that are consistent across laboratories in both space and time has favoured more simplistic single species

testing on growth rate and/or reproduction endpoints. As with green algae in the regulatory testing of other chemicals, the endpoint determined to establish the effects of antibiotics on cyanobacteria is population growth inhibition based upon cell density. In a recent meta-analysis we demonstrate that there can be considerable differences in sensitivity to the effects of antibiotics between cyanobacteria species and in that work it was concluded that testing on a more diverse species selection would provide greater confidence in the protection goal derived (Le Page *et al*. 2017). In support of this, a low-cost and medium throughput screening assay suitable for testing a range of cyanobacteria species would be of great value for use in both i) assessing the toxicity of new antibiotics to inform regulatory testing and ii) in considering the toxicity of legacy antibiotics that were registered previous to 2006 so that the risk to the environment from antibiotics with limited environmental data can be evaluated. Furthermore, the screening of such antibiotics would allow for the prioritisation of antibiotics that pose the greatest potential risk for further testing.

The internationally accepted shake flask test is traditionally used for determining the growth inhibition of chemicals to green algae and cyanobacteria, especially for studies that support regulatory dossiers (ISO 2004; OECD 2011). However, considerable work has gone into the development, validation and adoption of miniaturised microplate versions of the shake flask test in 24, 48 and 96 well microplates (Eisentraeger *et al*. 2003; Paixão *et al*. 2008; Pavlic *et al*. 2006; Rojíčková *et al*. 1998; Schrader *et al*. 1997; Wells *et al*. 1997). A microplate assay has considerable benefits over the shake flask method in regards to the operator time (further benefited by the increased potential for automation), space required and the quantity and cost of consumables, including the test substance of which there may be very small quantities available in early drug development. These microplate tests generally show good correlation in regards to sensitivity with the shake flask method and some national guidelines now advocate the use of a microplate method over the traditional shake flask method (e.g. Environment Canada (2007)).

Although successfully utilised by some investigators (Churro *et al*. 2009; Churro *et al*. 2010; Nagai *et al*. 2013), the optimisation of microplate methods has primarily focused upon green algae, with less consideration given to cyanobacteria. In order to address the requirement for a cost efficient and

medium throughput screen to assess the effects of antibiotics on cyanobacteria, here we set out to modify and optimise a microplate approach using eight different cyanobacteria species. The selected species were *Anabaena flos-aquae*, *Synechococcus leopoliensis*, *Anabaena cylindrica*, *Synechococcus elongates*, *Synechococcus sp*, *Synechocystis sp*, *Cyanobium gracile* and *Geminocystis herdmanii*. Species of the genus *Anabaena* are filamentous, nitrogen fixers that occur globally. Considerable focus has been given to this genus as a model organism for nitrogen fixation and its role in harmful algal blooms. *Synechococcus* species, of which three are represented here, are diverse unicellular picoplankton, that have a wide global distribution and are important primary producers, especially in oligotrophic environments (Callieri *et al*. 2007; Palenik *et al*. 2003). Species belonging to the Synechocystis genus are widely distributed in the environment and the strain selected here, Synechocystis sp (PCC 6803), was the first fully sequenced photosynthetic autotroph (Kaneko *et al*. 1996) and having biochemical similarities with plant chloroplasts it has become an important photosynthesis model organism. *C. gracile* is also a picoplanktonic species (cell length up to approximately 1µm) and *G. herdmanii* has a relatively large cell size (with diameter reaching 5µm;(Jana *et al*. 2009).

The test was designed to provide data that fulfil two objectives: 1) allow the direct comparison of the sensitivity to antibiotics across eight species of cyanobacteria and 2) ensure results obtained were consistent with that obtained for the traditional shake flask test design so that data derived were acceptable for informing regulatory testing, although we stress we do not intend for this to replace the shake flask test for regulatory purposes. The work included performing reference toxicity testing with potassium dichromate to establish the sensitivity of each species, ensure valid and consistent performance over time and allow for a comparison of results with the shake flask test. Our data derived from the microplates show a strong correlation with that from the traditional shake flask method for seven of the eight species and is the first protocol to allow for the direct comparison of sensitivity of this number of cyanobacteria species at once.

Methods

Cyanobacteria strains and maintenance

Eight cyanobacteria species were selected for testing. These were: *Anabaena flos-aquae* (CCAP 1403/13A), *Synechococcus leopoliensis* (CCAP 1405/1), *Anabaena cylindrica* (PCC 7122), *Synechococcus elongates* (PCC 6301), *Synechococcus sp* (PCC 6312), *Synechocystis sp* (PCC 6803), *Cyanobium gracile* (PCC 6307) and *Geminocystis herdmanii* (PCC 6308). *A. flos-aquae* (CCAP 1403/13A) and *S. leopoliensis* (CCAP 1405/1) were both selected based on the fact that they are species recommended in the OECD 201 test guideline (OECD 2011). The remaining six test species were selected based upon the fact that they fulfilled the following criteria: 1) their growth rate was sufficient enough to reach exponential growth and achieve measurable differences in biomass to enable toxicity testing within a 72 hour period under similar laboratory conditions as the other species (media, temperature, shaking and light intensity); 2) they are environmentally relevant; and 3) The test species also provided a wide range of phylogenetic diversity within those species of cyanobacteria with sequenced genomes in Shih *et al*. (2013). *Microcystis aeruginosa* (PCC 7806) was also considered in some of the early analyses due to its high sensitivity to antibiotics (Le Page *et al*. 2017) and importance in harmful algal blooms, but subsequently excluded due to its slow and variable growth in this test system.

Cyanobacteria were maintained as continuous aqueous cultures in 100mL conical flasks with approximately 50mL BG-11 medium ((Rippka *et al*. 1979); laboratory grade constituents of >97% purity). In order to provide a continuous source of exponentially growing cells, cultures were regularly transferred (twice a week) to new medium and incubated in Multitron II incubators (Infors) under test conditions. Before their use, cultures were visually examined using an inverted light microscope to ensure cells appeared healthy and that there was an absence of biological contamination.

Growth measurements and optimisation

Cyanobacteria have multiple fluorophores for light harvesting that include chlorophyll and the phycobiliproteins: phycoerythrin, phycocyanin and allophycocyanin (Glazer 1994). These fluorophores are commonly used as a

surrogate for cell biomass along with optical density (absorbance). In developing our microplate-based assay we identified the wavelengths for phycocyanin fluorescence in the cyanobacteria and compared its fluorescence with chlorophyll a and optical density (OD) to determine the most suitable surrogate for cell density. After a suitable surrogate was selected we assessed the growth of each species under varying temperature, light intensities and starting inoculums in order to develop a test design that allowed a direct comparison of species sensitivity within 72 hours.

Spectral scans

Spectral scans were performed to identify the optimum wavelengths for measurement of *in vivo* phycocyanin fluorescence. Supplementary material A gives details on the selection of optimal emission wavelengths for phycocyanin in all species. Fluorescence and OD were determined using a Spectromax M5 with Softmax® Pro software (Molecular Devices).

Cell density determination

Optical density and *in vivo* fluorescence of phycocyanin and chlorophyll a were assessed to establish the most suitable surrogate for cell density in order to calculate population growth inhibition. For each species a dense culture of cells in the stationary phase of the growth curve was serial diluted (50% dilutions) and three 200μL replicates of each dilution were pipetted into a microplate. OD (at 750nm), phycocyanin and chlorophyll A fluorescence (excitation and emission wavelengths of 590 & 650 nm and 420 & 681 nm respectively) were determined. Direct cell counts were conducted in a haemocytometer for *A. flos-aquae, A. cylindrical, Synechocystis sp and G. herdmanii*. Species with very a small cell size (*S. leopoliensis, S. elongates*, *Synechococcus sp,* and *C. gracile*) could not be accurately counted in the haemocytometer.

Calculation of limits of detection for phycocyanin fluorescence

The limit of detection for phycocyanin fluorescence in this system was determined to be 1.1 arbitrary fluorescence units (AFU) and the limit of quantification (LOQ) 3.6 AFU.

Optimising test conditions

In order to establish optimal test conditions for obtaining exponential growth rates the effects of temperature, starting inoculum and light intensity on the growth rate of each species were investigated over 72 hours (with exception of light intensity on *C. gracile*). Temperature ranges tested were between 20 and 30°C; starting inoculum levels between 2 – 10 AFU (phycocyanin fluorescence); and light intensity between 4000 – 12000 lux. Testing was carried out in microplates as described below.

Growth curves

Growth curves, based upon phycocyanin fluorescence, for cultures in the exponential growth phase were determined in microplates under the test conditions selected for the exposure (28°C, 2AFU starting inoculum (phycocyanin fluorescence) and 4000 lux) over 96 hours in BG-11.

Reference toxicity testing

Reference toxicity testing was performed using potassium dichromate (Sigma-Aldrich, product no P5271, CAS 7778-50-9, purity ≥99.5%). Potassium dichromate was selected due to i) having been previously and regularly used reference toxicant, ii) being stable in solution and unlikely to absorb to the plastic microplates or glass flasks, thus not requiring chemical analysis and, iii) have a large body of literature regarding toxic effects to a variety of organisms.

Seven microplate assays, performed in three groups (a, b and c) were used to assess the performance and repeatability of the assay with each species of cyanobacteria. Assays were performed as described below.

Microplate assays in each group (a, b or c) shared the same batch of medium and parent inoculum, i.e. the same parent culture was used to prepare the starting inoculum stock in all plates within a group (Table 1). To compare how the microplate performed with the shake flask test design, *A. flos-aquae, S. leopoliensis, Synechocystis sp., C. gracile* and *G. herdmanii* were tested using a shake flask test (as described below) and these ran concurrently with the microplate groups and shared the same batch of medium and parent inoculum as the microplate assays run in that group (Table 1).

Assay	Assay group
MP1	A
MP2	A
MP3	B
MP4	B
MP5	C
MP6	C
MP7	C
SF *A. flos-aquae*	B
SF *S. leopoliensis*	C
SF *Synechocystis sp*	B
SF *C. gracile*	A
SF *G. herdmanii*	A

Table 1. Table of assay groupings indicating which assays were performed at the same time using the same medium and parent inoculation culture. MP = Microplates (all species were tested in each MP assay); SF = Shake flask. Control pH for each group was as follows: A = 8.1, B = 8.0, C= 8.4.

Microplate toxicity assay

To ensure cells entering our tests were growing exponentially a pre-culture (50mL) for each species was prepared 3-4 days prior to the start of the test in 100mL Erlenmeyer flasks. These were grown under exposure conditions and growth rate was determined, using phycocyanin fluorescence as a surrogate for cell desnity, to verify cells inoculated into the test had been growing exponentially.

A geometric series of test concentrations were prepared in BG-11 medium at twice the nominal test concentrations. For each replicate, 100μL of the relevant test solution was then added to 100μL of cyanobacteria inoculum in BG-11 medium at 4AFU (twice the nominal starting inoculum) to achieve a final cyanobacteria concentration at 2AFU at the nominal test concentration in each well. Inoculums were based upon phycocyanin fluorescence measurement rather than cell density.

When fluorescence is used as an end measure black plates are generally adopted to prevent cross-over of fluorescence (the detection of fluorescence from neighbouring wells). However in this work, non-transparent, rather than black, 96 well plates were used (Greiner Bio-one item no. 650201) to allow sufficient light penetration to enable cyanobacteria growth. As found by Nagai *et al*. (2013) in a similar experimental design, cross-over was not observed in our assays. Plates were sealed with AMPLIseal™ sealer (Greiner Bio-one item no. 676040) to prevent water loss due to evaporation over the test period and to decrease the risk of possible contamination between wells. The seal prevented

gaseous exchange, including for CO_2 and O_2, between the wells and exterior atmosphere. The sealant had no effect on growth of all test species within the exposure period (between 24 and 72 hours depending on the species). pH fluctuations of the controls ranged between acceptable limits of ±0.2 as defined by most standardised test guidelines ((OECD 2011).

The plate layout for the incubations described is provided in supplementary material B (Figure S.B1). All exterior wells on the outer edge of the plate consist of cyanobacteria blanks (test solution only with an absence of cyanobacteria) in order to i) identify any fluorescent signal from a test compound and provide, if required, test concentration specific blanks to subtract from the exposure wells with cyanobacteria to obtain cyanobacteria fluorescence only and ii) the analytical determination of the test compound concentration in order to compare with the exposure well and thus allow the assessment of loss due to the presence of cells.

Cell density in each well was measured by phycocyanin fluorescence (excitation = 590nm, emission = 650n, cut-off = 635nm) daily from the start to end of the test. pH was determined in the test solution stocks at the beginning of the test and in one replicate of each test concentration for each species at the end of the exposure.

In some replicates, the starting inoculum was determined to be <LOQ of 3.6 AFU. This is because in our test system we prepared a cyanobacteria inoculum stock at a nominal 4 AFU which was then 50% diluted by the addition of the test solution, thus starting the test at a nominal 2 AFU. As such, some replicates had a starting fluorescence of below the LOQ (3.6 AFU).

Shake flask test procedure

In order to assess the performance of the microplate test in relation to more traditional growth inhibition assays used in regulatory testing, reference toxicity testing was performed upon *A. flos-aquae, S. leopoliensis, Synechocystis sp., C. gracile* and *G. herdmanii* using a shake flask test procedure according to the OECD 201 test guideline (OECD 2011). All test conditions were as conducted for the microplate test. Cell density was measured by sampling 200µl from each replicate flask in triplicate and placing these into a microplate to measure phycocyanin fluorescence as described previously. pH was determined in the

test solution stocks at the beginning of the test and in one replicate of each test concentration for each species at the end of the exposure.

Statistical analysis

Growth rate calculations

Growth rate of cyanobacteria was calculated according to equation 1.

Equation 1:

$$Growth\ Rate = \frac{\ln X_j - ln X_i}{t_j - t_i}$$

where

X = cell density at time i and j

t = time at time i and j

Yield or biomass integral were not calculated as growth rate is more appropriate for comparing the sensitivity across organisms. This is because the effects of different test durations and absolute growth are taken into account within the growth rate calculation and differences in species growth rate do not affect 'apparent' sensitivity (Eberius *et al*. 2002; Ratte *et al*. 1998). Additionally, growth rate tends to be the endpoint utilised within ERA (EM(E)A 2006).

In this assay, growth rate reflects the increase in phycocyanin fluorescence over time, which is used as a surrogate for cell density. This allows for the rapid measurement required of a medium-high throughput assay. There are some limitations and considerations associated with this approach, such as the loss of proportionality of phycocyanin fluorescence with cell density at the lowest cell numbers or the potential for some chemicals to inhibit phycocyanin fluorescence but not affect cell density depending on the mode of action. These concerns are described further in the discussion.

Test condition selection

To identify the effect of temperature, light intensity and starting inoculum a generalised least squares model was constructed using the 'nlme' package (J Pinheiro *et al*. 2017) with the restricted maximum likelihood method (Bates *et al*. 2015) in R (version 3.3.0; R Project for Statistical Computing, Vienna, Austria). Where the data was not homoscedastic the model was weighted by the

variance structure in the fixed variable (temperature/light intensity or starting inoculum) to account for the variance structure in the data. Models were compared using the Akaike information criterion and the best fitting model used. Pairwise comparisons were performed using the lsmeans package (Lenth 2016).

Dose-response modelling

Dose-response curves were fitted in r (version 3.3.0; R Project for Statistical Computing, Vienna, Austria) using the drc package (Ritz *et al*. 2015). The data handing and standard code used for each species are presented in supplementary material C. Several known distributions (3, 4 and 5 parameter log-logistic distributions and 4 and 5 parameter Weibull 1 and 2 distributions) were fitted to the growth rate data and the optimal distribution selected based on log-likelihood score. From this fitted distribution estimates of the effective concentrations 10 and 50 (EC_x) and associated confidence limits were determined and compared to assess the repeatability of the microplate assay and to compare results with the traditional shake flask test design. Coefficient of variations were also calculated to assess variability in assay performance.

Results

Cell density determination

With the exception of *A. flos-aquae* phycocyanin and chlorophyll a fluorescence were highly positively correlated with cell density at fluorescent values of more that 2 AFU for all species (Fig 1 - r^2 for phycocyanin fluorescence = 0.97 - 0.99; Supplementary material D). OD correlated less well at the lowest cell densities tested (Fig S.D1). Direct cell counting by haemocytometer was not possible for the smallest sized cells (*S. elongates*, *Synechococcus sp., S. leopoliensis* and *C. gracile*), but where they were obtained, for Synechocystis sp., *A. flos-aque, A. cylindrical* and *G. herdmanii*, they showed a similar linear relationship to the fluorescence measurements. It should be noted that in the lowest cell densities the reliability of direct counting was lower and this is demonstrated by the decrease in confidence around the modelled relationship (e.g. *A. cylindrical* Figure S.D1).

The reason for the non-linear relationship between phycocyanin fluorescence and cell density in *A. flos-aque* is unknown, however the fluorescence over the highest eight cell densities tested was consistent with the other species before taking a second linear relationship with a shallower slope over the lower cell densities (Figure 1). This change in relationship between phycocyanin fluorescence and cell density was also observed in the chlorophyll a fluorescence in *A. flos-aque* and in the OD of all species.

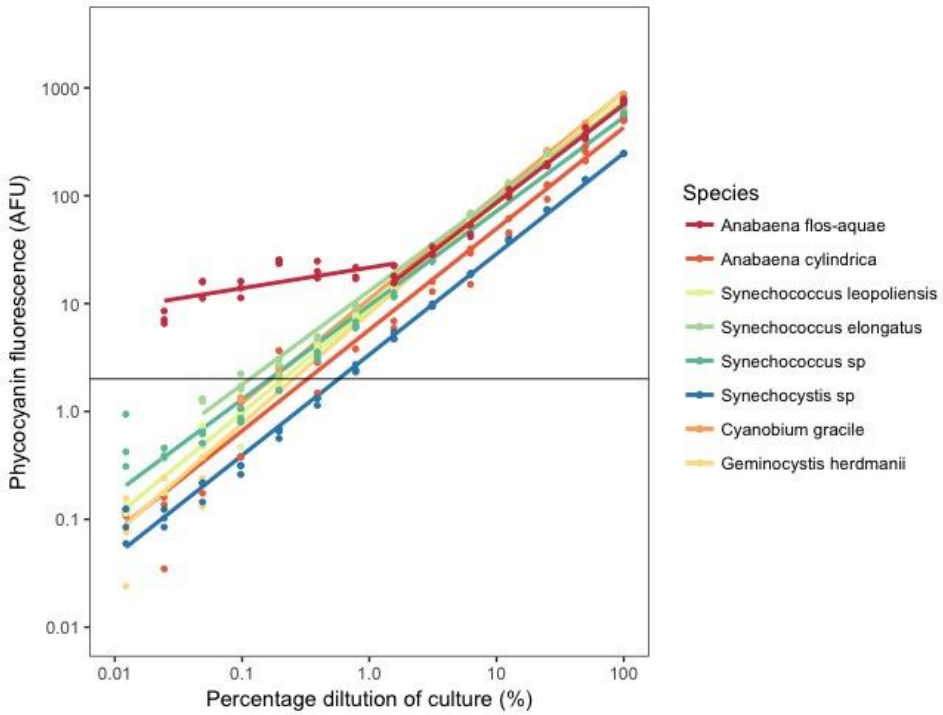

Figure 1. Relationship between phycocyanin fluorescence and cell density. Dilution of the culture is considered to be a surrogate for cell density (see supplementary material X). *A. flos-aquae* does not show a linear relationship over all cell densities and has been split into 2 (one each for the lower and higher cell densities). Regression equations and r^2 values for each species are as follows: *A. flos-aquae* (lower cell densities) - *Y = 1.33 + 0.191x; r2 = 0.047; A. flos-aquae* (higher cell densities) - *Y = 1.03 + 0.908x; r2 = 0.989; A. cylindrical - Y = 0.757 + 0.937x; r^2 = 0.968; S. leopoliensis - Y = 0.956 + 0.968x; r^2 = 0.994; S. elongates* Y = 1.12 + 0.869x; r^2 = 0.995; *Synechococcus sp*, Y = 0.983 + 0.872x; r^2 = 0.981; *Synechocystis sp* Y = 0.526 + 0.934x; r^2 = 0.995; *C. gracile* - Y = 1.04 + 0.961x; r^2 = 0.999; and *G. herdmanii*.- Y = 0.914 + 1.03x; r^2 = 0.987.

Test condition optimisation

Temperature

Figure 2 shows the relationship between temperature and growth rate (further analysis including a table of all pairwise comparisons can be found in supplementary material E). Temperature generally had a significant positive relationship with growth rate over 72 hours, with maximum growth rates occurring at 28 or 30°C (Figure 2; Table S.E1; Supplementary material E). In the case of *A. cylindrica* there was no significant difference in growth rate for temperatures at and above 25°C. The growth rate of *G. herdmanii* did not significantly differ for incubation temperatures between 20 and 30°C. Data for *C. gracile* was not obtained at 28 and 30°C due to problems in the growth of the pre-culture. In all species except *Synechocystis sp.* higher temperatures resulted in increased variability in growth rates. This could negatively affect the ability of the growth inhibition assay to identify statistical differences between test concentrations (e.g. no observed effect concentrations) and increase uncertainty around estimates derived from the dose-response curve (EC_xs). The growth rate of some species (including *C. gracile* and *Synechocystis sp.*) at temperatures of 20 and 22°C was too slow to establish a reliable dose-response over 72 hours. Since the primary aim of the microplate assay is to provide a medium to high throughput test, priority was given to a high growth rate that enabled a full test in 72 hours or under, as in shake flask assays used for regulatory studies. Based upon these results, therefore, a test temperature for the growth inhibition assay of 28°C was selected for all species as it consistently promoted high growth rates.

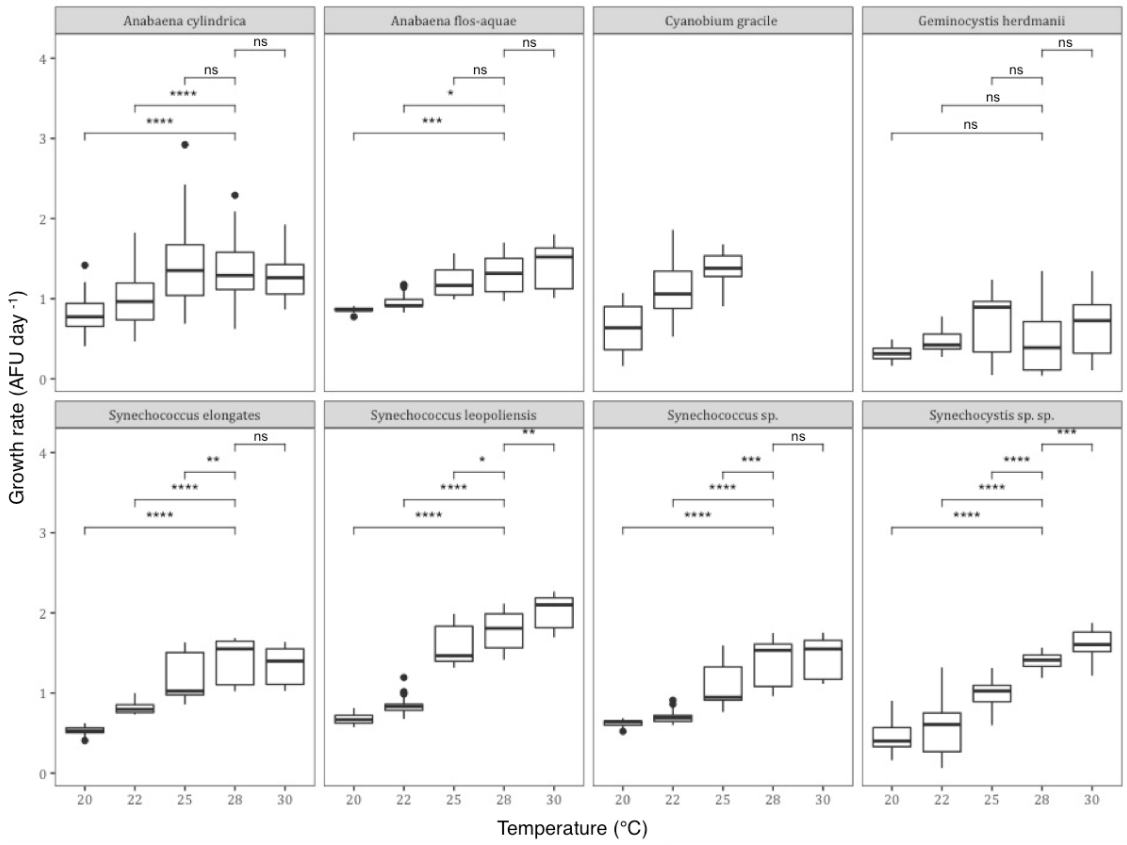

Figure 2. Effect of temperature on daily growth rate of cyanobacteria over 72 hours with a starting inoculum of phycocyanin fluorescence at 2AFU and light intensity of 4000 lux. NS – not significant; * - p = <0.05; ** - p = <0.01; *** - p = <0.001; **** - p = <0.0001.

Light intensity

Light intensity did not show any major effect on growth rate over 72 hours for any of the cyanobacteria (Figure 3; Table S.E2). For the slower growing species in these tests, however, including *A. flos-aque*, *A. cylindrical* and *G. herdmanii*, growth rate tended to be higher at a light intensity of around 4000 lux. Data for *C. gracile* was not obtained and were therefore not available for analysis due to problems in the growth of the pre-culture. As such, 4000 lux was selected as the light intensity for the assay across all species. Additionally, the use of a lower light intensity is advantageous to reduce any potential photo degradation of the chemical test substances during the test period.

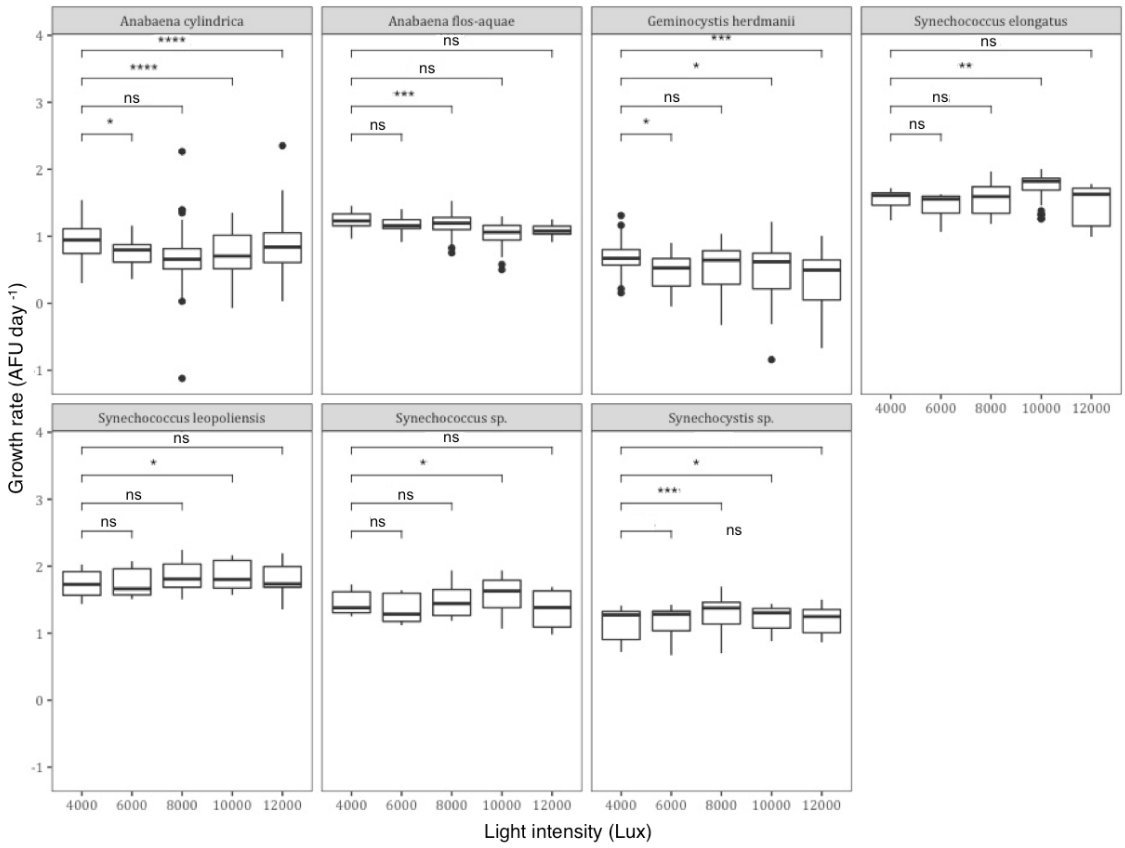

Figure 3. Effect of light intensity on daily growth rate of cyanobacteria over 72 hours with a starting inoculum of phycocyanin fluorescence at 2AFU and at a temperature of 28°C. NS – not significant; * - p = <0.05; ** - p = <0.01; *** - p = <0.001; **** - p = <0.0001.

Inoculum cell density:

With exception of *G. herdmanii,* increasing the cell density, based upon phycocyanin fluorescence, of the starting inoculum had a significant negative effect on daily growth rate over the 72 hour period (Figure 4; Table S.E1). Data for *C. gracile* was not obtained and were therefore not available for analysis due to problems in the growth of the pre-culture. From these results a starting cell density equivalent to a phycocyanin fluorescence measurement of 2 AFU was selected for use in the microplate assay for all species.

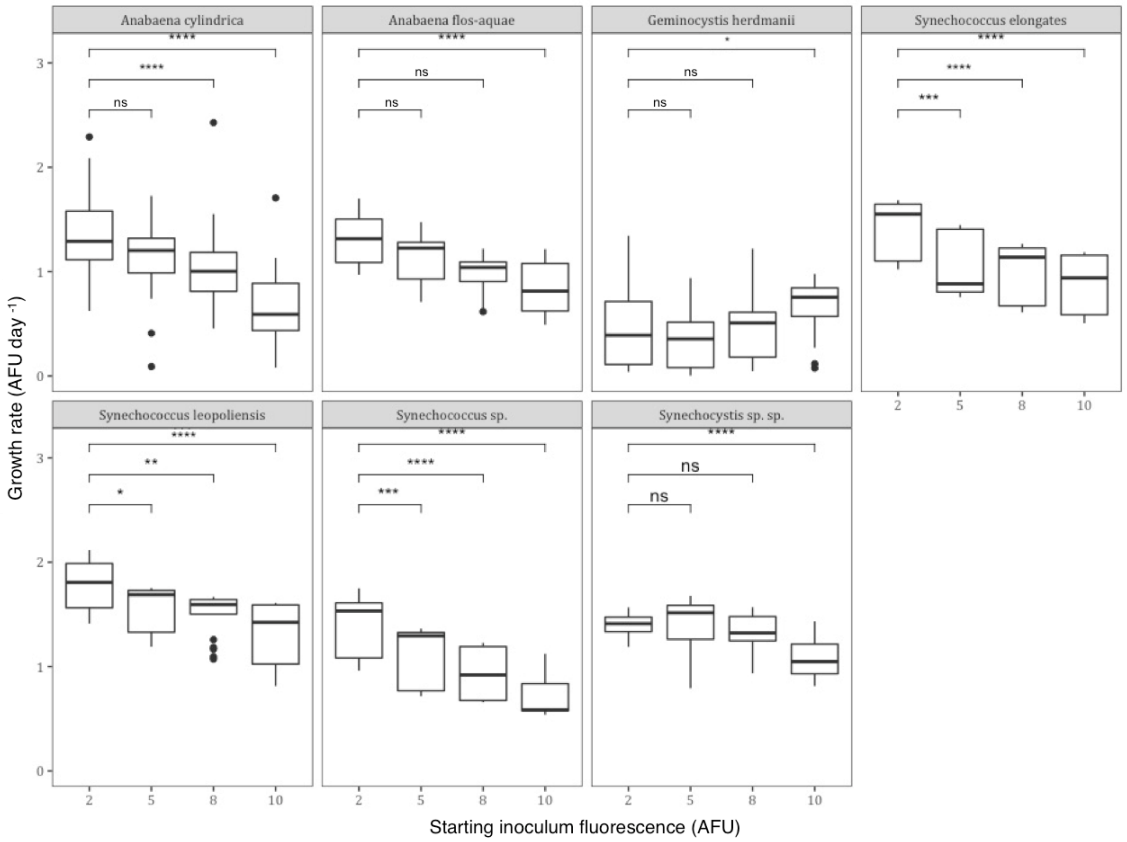

Figure 4. Effect of starting inoculum, measured by phycocyanin fluorescence, on daily growth rate of cyanobacteria over 72 hours at a temperature of 28°C and light intensity of 4000 lux. NS – not significant; * - *p* = <0.05; ** - *p* = <0.01; *** - *p* = <0.001; **** - *p* = <0.0001.

Growth curves

Figure 5 presents the growth curves for each species, under the selected test conditions of 28°C, 4000 lux, 140 rpm (shaking speed was not investigated here) and a starting cell density of 2 AFU based on phycocyanin fluorescence. To obtain a dose-response curve based upon the exponential growth of each species the following exposure periods were selected for toxicity testing: i) for the fastest growing cyanobacteria, *S. leopoliensis, S. elongates* and *Synechococcus sp*, that reached stationary growth phase rapidly (within approximately 24 hours) an exposure period of 24 hours was adopted; ii) for *A. flos-aque* and *Synechocystis sp.* an exposure period of 48 hours was selected where they reach the stationary phase of the growth curve; iii) for the slower growing species (*A. cylindrical, C. gracile* and *G. herdmanii*) that had a lag phase over the first 24 hours that was followed by an increase in growth rate, reaching exponential growth between 48 and 96 hours, a 72 hour exposure period was selected.

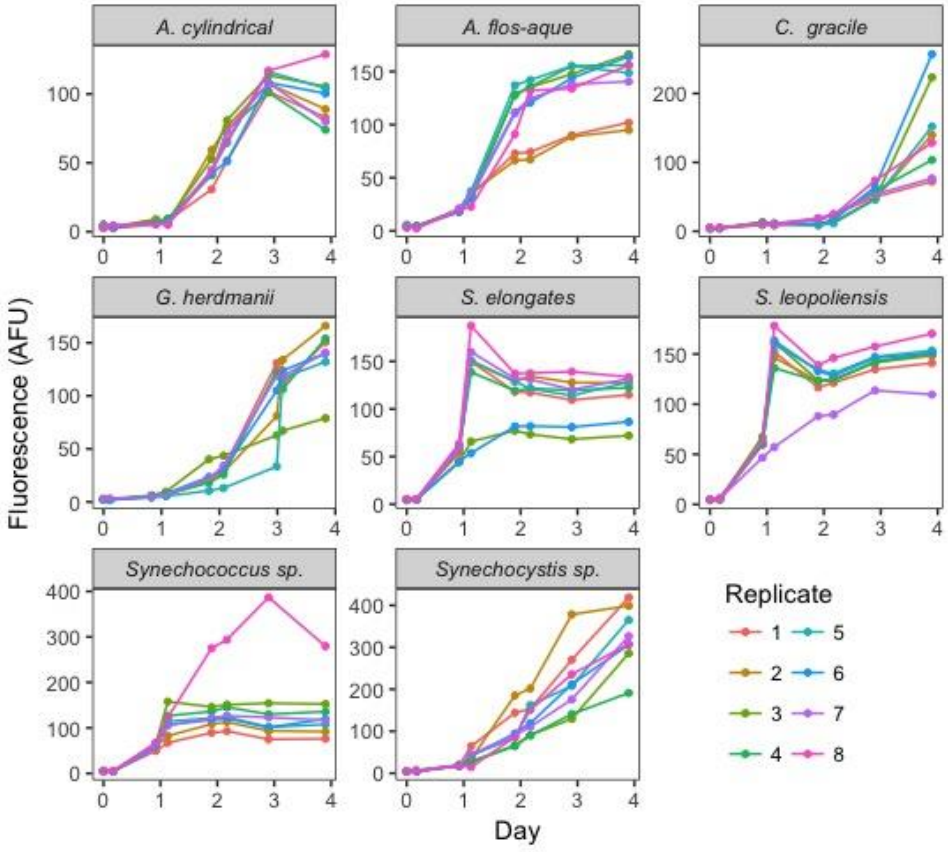

Figure 5. Growth curves of cyanobacteria species in microplates over 4 days with a starting inoculum of 2 AFU, temperature of 28°C, light intensity of 4000 lux and shaking the plates at 140rpm. Each replicate represents growth in one well of a microplate.

Reference toxicity testing

The EC_{10} and EC_{50}s after exposure to the reference toxicant, potassium dichromate, for each microplate and shake flask test are presented in Figure 6. The dose-response curves for each species obtained from both test designs are presented in supplementary material F along with the percentage growth inhibition across all assays for each species. *Synechocystis sp*. and *C. gracile* were the most sensitive species to potassium dichromate, followed by *G. herdmanii*. *A. flos-aque* was an order of magnitude less sensitive than these whilst the three species in the *Synechococcus* genera (*S. leopoliensis, S. elongates* and *Synechococcus sp*.) were the least sensitive overall. Variability in *A. cylindrical* growth rate was high in all concentrations and thus dose-response curves were not very reliable and estimates of effective concentrations spanned a wide range (e.g. EC_{50}s of 0.96 to 154 mg/L). *Synechococcus sp*. also had high variability in growth rate in control conditions

that was reflected in the relatively wide confidence limits around the EC_x estimates, but this did not appear to result in high variability between EC_x estimates as seen in *A. cylindrical.*

The results in Figure 6 show that, in most cases, the cyanobacteria show a consistent response to potassium dichromate in the microtitre well format and the results between assays were repeatable with EC_x estimates within the 95% confidence limits of the other assays. Table 2 provides the coefficient of variation (CoV) in EC_{10} and EC_{50} estimates across all microplate assays and for assay groups that shared medium and parent inoculum cultures (Table 1). Most variability in responses occurred between assay groups whilst those that shared the same medium and parent inoculum had considerably less variability between assays.

The pH of the controls of the different batches of media ranged between 8.2 ± 0.2 and the pH of the highest test concentrations of potassium dichromate (100 and 200 mg/L) decreased the pH to approximately 7.8 ± 0.2.

Microplate assays were consistent with the traditional shake flask test design for *A. flos-aque, S. leopoliensis, Synechocystis sp*. and *G. herdmanii* with EC_x estimates determined to be within the confidence limits calculated from the shake flask data (Figure 6). This was not the case in only *C. gracile* where the microplate assays estimated EC_xs that were an order of magnitude higher than for the shake flask test. For *G. herdmanii and S. leopoliensis* wide confidence limits were observed in the shake flask tests.

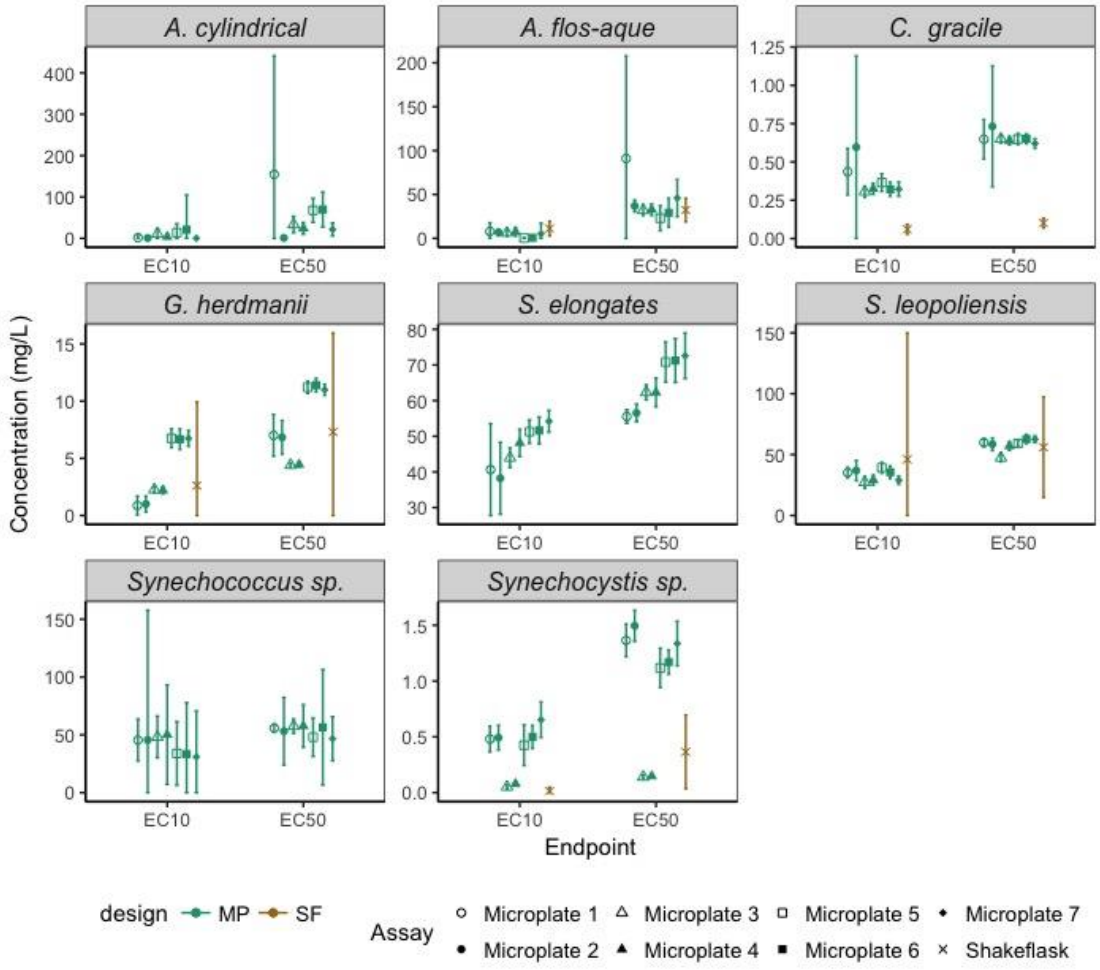

Figure 6 – Effective concentrations for growth inhibition of 50 and 10% of the population (EC_{50} and EC_{10}) and the associated 95% confidence intervals for eight cyanobacteria species in microplate and shake flask test designs exposed to potassium dichromate. Seven microplate assays and one shake flask test were run for each species (excluding a shake flask test for *S. elongates* and *Synechococcus sp*.). Assays run using the same media batch and parent starting inoculum: microplates 1 and 2 (circular symbols) with shake flask tests for *C. gracile* and *G. herdmanii*; microplates 3 and 4 (triangular symbols) with shake flask tests for *A. flos-aque* and *Synechocystis sp.* and; microplate 5,6 and 7 (square symbols) with shake flask for *S. leopoliensis.*

		Coefficients of variation			
Species		*MP 1 & 2*	*MP 3 & 4*	*MP 5, 6 & 7*	All MPs
A. cylindrical	EC_{10}	50.2	69.8	89.2	106.4
	EC_{50}	139.7	22.9	51.4	96.5
A. flos-aque	EC_{10}	10.3	11.3	134.9	64.6
	EC_{50}	59.9	1.3	36.3	55.3
C. gracile	EC_{10}	22.0	4.4	7.5	27.4
	EC_{50}	8.6	1.3	2.7	5.4
G. herdmanii	EC_{10}	9.7	2.0	0.7	73.9
	EC_{50}	1.8	0.8	1.8	38.9
S. elongates	EC_{10}	4.4	6.4	3.0	12.9
	EC_{50}	1.3	0.0	1.3	11.0
S. leopoliensis	EC_{10}	3.3	4.6	15.0	14.0
	EC_{50}	1.6	13.3	3.2	9.0
Synechococcus sp.	EC_{10}	0.03	2.9	4.8	19.5
	EC_{50}	3.4	0.2	10.6	8.5
Synechocystis sp.	EC_{10}	1.9	30.7	22.1	59.6
	EC_{50}	6.5	3.4	9.4	59.5

Table 2 – Coefficients of variation of the effective concentrations for growth inhibition 50 and 10% of the population (EC_{50} and EC_{10}) obtained from the microplate assays. MP=microplate. Plates grouped by those sharing media batch and parent starting inoculum.

Discussion

Bacteria, including cyanobacteria, play key roles in a variety of ecological functions on which ecosystems rely upon (Falkowski *et al*. 2008), but there is concern that protection limits determined in ERA are not protective of environmental bacteria populations and therefore neither the functional roles that they play. We developed a microplate assay for eight species of cyanobacteria, using the same environmental conditions to allow for the comparison of species sensitivity. After examining the performance with the reference toxicant potassium dichromate, the assay proved to be repeatable and consistent with a shake flask test design for seven out of the eight species.

Cell density determination

Both phycocyanin and chlorophyll a fluorescence were identified to be a suitable surrogate for quantifying biomass in the experimental design developed. Cyanobacteria have higher levels of phycocyanin than chlorophyll a (Nagai *et al*. 2013; Watras and Baker 1988) and phycocyanin fluorescence showed higher sensitivity and less variability in measured fluorescence at the lowest cell densities (Fig S.D1). The direct counting of *Synechocystis sp*., *A. flos-aque, A. cylindrical* and *G. herdmanii*, showed a similar linear relationship to the fluorescence measurements with the percentage dilution of the cultures, further supporting fluorescence as a suitable surrogate for cell density. These results are consistent with that reported by other authors showing that fluorescence of a phytochrome (chlorophyll or phycobiliproteins) has higher detection sensitivity than OD, allowing quantification at lower cell densities (Eisentraeger *et al*. 2003; Van Wagenen *et al*. 2014). Cyanobacteria cell density however, has not always been well correlated with phycocyanin fluorescence in environmental samples (Beutler *et al*. 2002; Kasinak *et al*. 2014). It is possible that the poor correlation observed in these environmental samples is due to mixed phytoplankton communities being measured where cell size and morphology varies within and between species thus affecting estimation accuracy in cell density determinants. This is in contrast with our assay that uses single species cultures and controlled laboratory conditions encouraging more uniform growth. It is noteworthy that, in some instances, such as when assessing compounds that target or indirectly affect the light harvesting pigments, phycocyanin fluorescence may not always be a suitable

surrogate for cell density and care should therefore be taken when interpreting results from such assays (Debelius *et al*. 2009; Hadjoudja *et al*. 2009).

In the case of *A. flos-aque*, there appeared to be two separate linear relationships between cell density and each surrogate measure (fluorescence of phycocyanin and chlorophyll A and OD). At higher cell densities the linear relationship had a similar gradient of slope, and thus appeared consistent with the other cyanobacteria. Whilst at lower cell densities (equivalent to 8AFU and below for phycocyanin fluorescence) a second linear relationship took a shallower slope (Fig 1). The reason for this is unknown and requires further investigation. If phycocyanin fluorescence in lower cell densities does indeed have a different linear relationship than at high cell densities, this measure may overestimate cell numbers below 8 AFU for *A. flos-aque* and effect the growth rate determined. These levels of fluorescence (≤8 AFU) are considerably lower than the fluorescence readings in control growth that range from 30 to over 100 AFU over a 48 hour test period. The starting inoculum fluorescence is proportionately higher to the final fluorescence in wells that have had less growth and it might therefore be expected that this will have a disproportionate effect on the growth rate calculations in the highest test concentrations where growth is slowest. In this case, the uncertainty and potential overestimation caused by this is likely to affect the lower end of the dose-response curve whilst the higher end is less likely to be less affected. Any EC_x calculations that are based on concentrations at which there is a fluorescence of 8 AFU or less (e.g. EC_{80}) will therefore be less reliable whilst those based on concentrations with higher fluorescence (e.g. EC_{10} and EC_{20}) more likely to be reliable. This should be carefully considered when interpreting the results.

Test condition selection

Growth rates and toxicity are influenced by many factors, including the metabolic state of the cells in the starting inoculum, the cell density of the starting inoculum, temperature and light intensity (Franklin *et al*. 2002; Yu *et al*. 2007). In the development of this assay each of these were investigated in order to optimise the experimental conditions such that each species grew exponentially over a 24 to 72 hour period. The results showing the effects of temperature, light intensity and starting inoculum on growth rate all supported the ability to adopt the same test conditions across the eight species. This is of

importance because the primary objective of this microplate assay was to compare cyanobacteria sensitivity to test chemicals and consistent test conditions allow for a more robust species comparison which include eliminating the potential for differences in chemical behaviour in the test system (e.g. photo degradation rates can be assumed consistent if light intensity is the same across all species).

A low starting inoculum was selected primarily in order to maximise the growth rate over the exposure. However there are additional benefits to using a low starting cell density; i) higher cell densities could lower toxicity estimation due to the dilution of compound relative to the total cell surface area (Franklin *et al*. 2002); ii) higher cell densities can result in cell aggregation and/or higher quantities of extracellular polymeric substance that, in turn, is likely to affect the bioavailability and integrity of the test substance (Khunjar and Love 2011); iii) rapid pH changes that may affect bioavailability or test substance speciation are more likely to occur in closed systems, such as in this test design, when you have higher cell densities (Franklin *et al*. 2002); and iv) test assays with lower cell densities than those used in traditional ecotoxicity testing are more environmentally realistic. These examples are likely to be even more pronounced for chemicals such as antibiotics where the toxicity is observed at very low concentrations.

For studies assessing growth inhibition, it is important that the population is in balanced growth (exponential growth phase) and indeed this is a pre-requisite of many standardised test regimes (Environment Canada 2007; ISO 2004; OECD 2011). Balanced growth is where the cells are replicating at the maximum growth rate possible given the environmental conditions that they are in with the assumption that reproducing cells are equally distributed across of all stages of the cell cycle and thus, at any given time interval the cell density increases at the same rate including the cellular components (ribosomes, proteins etc) (Campbell 1957; Schaechter 2015). This is of particular importance for growth inhibition studies as only a population in balanced growth can ensure reproducible results over time (Schaechter 2015) because cells are replicating at a constant rate defined by the environmental conditions, removing variation between assays. Populations in other phases of the growth cycle (lag and stationary phases) on the other hand, have cells that vary in metabolic

states and replication rates, leading to variability between one lag or stationary phase and the next. For example, in the lag phase cells are preparing themselves for exponential growth by synthesising all the cellular components required (RNA, proteins, enzymes) (Rolfe *et al*. 2012) and the lag phase will differ depending on the environmental conditions the cells were in previously and their metabolic state on inoculation. This is of even more importance in growth assays when using a surrogate such as phytochrome fluorescence for cell density because the energy invested in light harvesting pigments may vary depending on the metabolic state of the cells and their recent lighting environment (MacIntyre and Cullen 2005; Van Wagenen *et al*. 2014).

If the toxicity assay is performed on cultures that are not in exponential balanced growth there may thus be some variation observed in assays performed over time. It isn't however always possible to avoid initiating a lag phase. Indeed, in the case of our microplate assay, in order to obtain a single culture of cyanobacteria with a high enough cell density to inoculate the entire test the parent culture needs to be grown in a volume larger than what a microplate well will allow. As such, the transfer from (in this case) 50mL volume in 100mL conical flask to a 200uL microplate well, will initiate a lag phase as the cells readjust to the new environment and enter balanced growth. Some species will require more readjustment to the new conditions (longer lag phase) than others and of the species tested in our assay (and under our sampling regime) cyanobacteria in the *Synechococcus* genera to showed no evidence of a lag phase after inoculation whilst C. *gracile* had a lag phase that lasted between 24 and 48 hours. This is unavoidable and as such consideration must be given to how this may affect the dose-response curve and any calculations derived from it. For example, a lag phase at the beginning of the test may increase the apparent affect if exposed replicates take proportionately longer than the controls to exit the lag phase and enter balanced growth. Whereas if no lag phase was initiated and cells were continuously in balanced growth for the whole test the affect observed can be entirely attributed to the test chemical. Conversely, if the exposure period is long enough that the growth rate of the controls reaches stationary phase before the end of the test, the toxically affected (and slower growing) replicates may 'catch up', skewing the data and driving toxicity endpoints calculated to higher concentrations (smaller apparent affect). It is therefore important to both report the maximal growth rate and run

regular reference toxicity tests over time to be able to quantify the variability that may be expected and identify if a population is in balanced growth at a rate within expected norms.

To best ensure toxicity testing was carried out during balanced growth for each species whilst avoiding the lag or stationary phase, two approaches could have been adopted. The first was to adopt exactly the same exposure conditions for all species and adjust the exposure time. The alternative was to adjust the exposure conditions (temperature, light intensity etc) to slow/increase the growth rate for each species and have the same length of exposure. This compromise reflects the biology of the species and is thus unavoidable. In these studies it was decided to keep test conditions the same and adjust the exposure period for reasons outlined previously given the objectives of the test to examine interspecies sensitivity. Since the exposure period changes between species, consideration must therefore be given to the properties of the test chemical, especially in regards to degradation. Where degradation occurs rapidly in <24-48 hours, it is crucial to base the dose-response on measured, rather than nominal, chemical concentrations.

Reference toxicity testing

The reference toxicity results showed that there was a very wide range of cyanobacteria sensitivity to potassium dichromate (based on EC_{50}s there was over 2 orders of magnitude difference in sensitivity between the most sensitive species (*C. gracile* and *Synechocystis sp*.) and the least sensitive (*S. leopoliensis* and *S. elongates*)). These results are in accordance with published data for cyanobacteria. For example, Gupta *et al*. (2013) obtained 9 day EC_{50}s for *S. elongates* and *Synechocystis sp*. of 44.1 and 3.53 mg/L respectively which are comparable with our EC_{50}s of 56 - 76 mg/L for *S. elongates* and 0.14 – 1.5 mg/L for *Synechocystis sp*. Microcystis sp., which was found to have generally high sensitivities to antibiotics compared to other cyanobacteria (Le Page *et al*. 2017), was found to have an EC_{50} of 0.211 mg/L (Halling-Sørensen 2000) which is comparable with the two most sensitive species tested here, *Synechocystis sp*. and *C. gracile*. Yamagishi *et al*. (2016) determined an EC_{50} for a marine *Cyanobium sp*. of 4.61 mg/L that was around and order of magnitude higher than our EC_{50}s that ranged from 0.31 to 0.60 mg/L, although this may be expected since marine algae have been suggested to be more

tolerant to metals than freshwater species (Ebenezer and Ki 2013; Yamagishi *et al*. 2016).

Chromium (VI) (the oxidation state of dichromate) is stable, highly soluble and enters bacterial cells via the sulphate uptake pathway (Mishra and Bharagava 2016). Once in the cell chromium (VI) has several toxic effects due to its reduction into other, less stable, forms. For example, chromium (V) is genotoxic and chromium (III) binds to phosphates in the DNA (Labra *et al*. 2007; Mishra and Bharagava 2016; Plaper *et al*. 2002). Changes in morphology such as cell elongation and enlargement have been recorded in bacteria following exposure to chromium (VI) that may be due to effects upon membrane ligands (Mishra and Bharagava 2016). Combined, these various modes of action inhibit normal cell functioning, cell division and thus growth (Mishra and Bharagava 2016; Yamini Shrivastava *et al*. 2004). Although the mode of action once in the cell has been well investigated across many organisms, none have focused upon cyanobacteria and what drives the differences in interspecies sensitivity observed in in these results is therefore unknown.

Some EC_x estimates had wide confidence intervals due to increased uncertainty due to either i) not having enough test concentrations placed between the EC_5 and EC_{90} of the dose-response curve; and/or ii) due to one or two replicates that have a disproportionate influence on the dose-response model (i.e. some replicates having a much higher or lower growth rate compared to the others in that test solution). In many instances these data with a 'disproportionate influence' could be considered a statistical outlier and removed from analysis based upon a statistical test, however due to relatively high replication (10 for controls and five for exposure wells) it was considered more informative to include this variability in the calculations.

The microplate assay results may be cause for concern in ERA because both species recommended for use in regulatory testing (*A. flos-aquae* and *S. leopoliensis*) were more than an order of magnitude less sensitive to potassium dichromate than the most sensitive species. Even with an assessment factor of 10 applied to determine the predicted no effect concentration (PNEC) in ERA, employed to account for interspecies sensitivity and differences between laboratory and field environments, PNEC would not be protective of the two

most sensitive species here. Indeed, *C. gracile* was on average 50 times more sensitive than *A. flos-aquae* and 104 times more sensitive than *S. leopoliensis*.

The microplate assays proved to be consistent across time with interassay variation, as determined by CoV, for five of the eight species was generally low, giving comparable EC_x estimates between plate runs (CoV = 5 to 39% based on EC_{50}s). For *A. flos-aque, Synechocystis sp*. and *A. cylindrical* however, variation was higher with CoVs based on interassay EC_{50}s of 55, 60 and 97% respectively. When considering the CoVs, much variability was accounted for by the group in which the assay was run (groups a, b or c). Thus, either the different batches of media, test solution preparation and/or the different parent starting inoculum cultures are likely to be the main cause of the variability observed. One possible explanation could be due to differing pH between media batches since the pH of the different batches of media did differ slightly with a range of 8.2 ± 0.2. Furthermore, the pH of the highest test concentrations of 100 and 200 mg/L were approximately 7.8 ± 0.2 and there is therefore the potential for the pH to *i*) affect the toxicity of potassium dichromate and *ii*) affect the growth rate of the cells across treatments. This however, is not considered to explain the observed variation between assays here because where the EC_{50}s differed between microplate runs (Figure 6), the difference was not consistent across species within the same assay grouping that shared the same media and starting pH. For example, the group in which EC_xs were lower/higher than the remaining assays for *G. herdmanii*, *C. gracile* and *Synechocystis sp* were C, A and B respectively and if media pH was the cause of this we would expect to see the same effect in all species. Neither is there evidence from the published literature that suggests that differences in media pH would affect the toxicity. On the contrary, pH did not affect the toxicity of potassium dichromate on the green algae *Raphidocelis subcapitata* (Mayer *et al*. 1998), although the effects of pH on the cyanobacteria growth rate cannot be ruled out.

It is much more likely therefore that the EC_{50} variability will be due to differences in the parent inoculum culture and the subsequent affects on the lag phase and balanced growth as discussed earlier. It is possible that a difference in cellular metabolic state in the parent culture when inoculating the assay and thus affecting the lag phase and time to balanced growth is responsible for this variability. Indeed, other authors have also identified the importance of cellular

metabolic state on growth assays (MacIntyre and Cullen 2005; Van Wagenen *et al*. 2014).

Even with this potential source of variability, we consider that these results are adequate for the objectives of this assay: to provide a rapid screen to allow the comparison of cyanobacteria sensitivity. But if the data was to be used for regulatory purposes, further investigation is required to examine the affects the lag phase of these species, how to further optimise the tests to reduce any impact and if these species can even be considered suitable for regulatory testing within this test system.

The reference toxicity testing showed that the microplate test produced comparable results to the shake flask test design for all species except *C. gracile.* The reason for the difference in performance in test designs observed for *C. gracile* here is unknown, but it may be due to differences in the lag phase and maximal growth rate (once in balanced growth) caused by the different environments in the shake flask and microplate test designs. Similar microplate assays using the green algae *Desmodesmus subspicatus* (Eisentraeger *et al*. 2003) and five periphytic riverine species (the green alga (*Desmodesmus sp*.), three diatoms (*Achnanthidium sp., Nitzschia sp., Navicula sp.*) and the cyanobacteria (*Pseudanabaena sp.*) (Nagai *et al*. 2013) also found that there was no significant difference in sensitivity between the 96 well microplate assay and their shake flask test designs.

There are some instances where a microplate assay is either not applicable or is perhaps less appropriate than the shake flask test as an approach to determining toxicity to growth rate. These are mainly circumstances when plastic microplates are not suitable for the physical-chemical properties of the test chemical, where bioavailability is compromised due to the compound sticking to the plastic plate or alternatively due to loss by volatility. Riedl and Altenburger (2007) concluded that microplate test systems shouldn't be used for chemicals with a log K_{OW} higher than three or a Henry coefficient log K_{AW} higher than −4. There are ways, however, to mitigate some of the problems caused by test chemical properties including glass coated plates, airtight seals and obtaining measured exposure concentrations by analytical assessment of the test compound at the start and end of the test.

Overall, these results demonstrate that the microplate assay we present would be suitable for the screening of toxic effects of chemicals on the growth rate of cyanobacteria. Compared to the shake flask test, the approach developed offers numerous advantages that include the use of less resources (time, substance, space and media etc.) and the ability to automate the majority of the test protocol. These advantages are particularly important in regards to the need for environmental data early in antibiotic development and in the screening of legacy chemicals registered previous to 2006 with little environmental data in order to prioritise compounds that may pose a risk to bacterial populations.

We stress however, that we don't advocate this assay as a replacement to the shake flask method for regulatory testing, although the results from these screens would be able to inform and guide such testing. We also highlight that although we consider the data from all species examined here to be useful in the context of examining cyanobacteria sensitivity, some species, including *A. cylindrical* and potentially *Synechocystis sp*. (due to their higher variability) and *A. flos-aque* (due to the non-linear relationship between cell density and fluorescence) may not be as suitable as the others.

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Supplementary material A

Phycocyanin spectral analysis

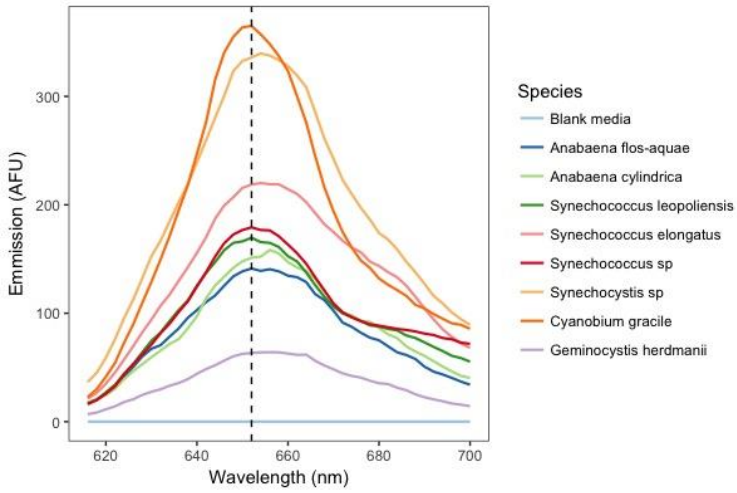

Figure S.A1. Emission spectra for eight species of cyanobacteria and blank BG-11 media following excitation at 590nm. The dotted line indicates 652nm.

A spectral scan of each species was undertaken on a Spectromax M5 microplate reader (Molecular Device Inc., USA) using the SoftmaxPro 3.0 software (Molecular Devices). Briefly, to identify the optimal emission wavelength for phycocyanin, using an excitation wavelength of 590nm (Sobiechowska-Sasim *et al*. 2014) an emission scan was obtained for each species between the wavelengths of 620 and 700nm with an interval of 2nm. All species had emission spectra that peaked between 650 – 655nm with an average of approximate 652nm (Figure S1). As such they confirm the suitability of using excitation and emission wavelengths for phycocyanin at 590nm and 650nm respectively. However, it is likely that measuring the fluorescence at these wavelengths will capture both phycocyanin and allophycocyanin to different degrees due to the spectra overlap for these pigments (Beutler *et al*. 2002; Sobiechowska-Sasim *et al*. 2014). Since our primary aim is to develop a simple, medium throughput assay, attempts to correct for this overlap and relate cell density to just one pigment was not deemed necessary in this instance as; *i*) fluorescence at these wavelengths was found to be proportional to cell density and; *ii*) distinguishing between the two pigments will not effect growth rate calculations.

It should be noted however that wavelengths can change between species and may differ between measurements on extracted vs in vivo methods due the effects of membranes and proteins in the cell (Sobiechowska-Sasim *et al* 2014). Thus, further optimisation would be required if introducing extra species for use in this microtitre assay.

Supplementary material B

Microplate layout

Control BL	T1 BL	T2 BL	T3 BL	T4 BL	T5 BL	T6 BL	T7 BL	T8 BL	T9 BL	T10 BL	Control BL
Control BL	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	Control BL
Control BL	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	Control BL
Control BL	Control	Control	Control	Control	Control	Control	Control	Control	Control	Control	Control BL
Control BL	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	Control BL
Control BL	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	Control BL
Control BL	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	Control BL
Control BL	T1 BL	T2 BL	T3 BL	T4 BL	T5 BL	T6 BL	T7 BL	T8 BL	T9 BL	T10 BL	Control BL

Figure S.B1. A schematic plate map of the layout of replicates in the mirotitre plate.

The ten control replicates run along the centre of the plate in parallel to the concentration gradient where a significant directional effect, caused by uneven environmental conditions across the plate (e.g. light intensity) or by the contamination from surrounding wells (e.g. volatile compounds) can be identified. Five replicates of each test concentration run from column 2 to 11. The outer cells of the 96 well microplate will have no addition of algal inoculum, creating 16 control blanks and two blanks per concentration.

If a solvent control is required it is run in the spaces of the blanks in row H.

Supplementary material C

Dose-Response model code

Dose-response models were established using the using the drc package (Ritz *et al*. 2015) in r (version 3.3.0; R Project for Statistical Computing, Vienna, Austria).

Before establishing the model, the data was examined to identify replicates where growth rates that could not be calculated. The three reasons for this and our rules for the handling of them are given below:

1. The fluorescent signal at the start of the test is a negative number because the background/blank value is higher than the exposure well. The log of the negative value cannot be calculated and therefore neither could the growth rate.

    a. In these cases the replicate was excluded from further analysis as a reliable starting value could not be established in which to calculate growth rate

2. The growth rate is negative as the fluorescence at the end of the test is less than that at the beginning. Although the population size decreased (grew negatively) it is unknown from this fluorescence data if cells were alive or dead (with phycocyanin still fluorescing).

    a. In these cases the growth rate was fixed at 0.

3. The fluorescent signal at the end of the test is a negative number because the background/blank value is higher than the exposure well. The log of the negative value could not be calculated and therefore neither could the growth rate.

    a. In these cases the replicate considered to have zero growth since there was effectively no fluorescence from the cells at the end of the test.

The data were then examined and the optimal distribution was selected for the data based on log-likelihood score (distributions examined were 3, 4 or 5 parameter log-logistic distributions and 4, or 5 parameter weibull 1 or 2 distributions). Model residuals were examined for normality and heteroscedasticity and residuals with a disproportionate effect on the model were identified using cooks distance (>0.5 is considered to have a disproportionate effect). Any outliers were individually examined and excluded if biologically justified and the dose-response model re-run. Replicates that did have a disproportionate effect on the model but without any reason to suggest an experimental error (e.g. not inoculated correctly) were included in the analysis rather than excluded as an outlier, even if statistically justifiable, in order to include the inherent variability of the species and test designs. This may result in wider confidence intervals and/or less statistical power.

On the following page is an example of the code used for each dose-response curve:

```
library(drc)
library(dplyr)

# Make starter model
SpeciesX.m1<-drm(GR~Conc,
 data=Data,
 fct=LL.3(),
 na.action = na.omit)

# Identify best distribution of data for dose response curve:
mselect(CYAN.m1, list(LL.3(), LL.4(), LL.5(), W1.3(), W1.4(), W2.4()))

# Make best fitting model
SpeciesX.m1<-drm(GR~Conc,
 data= Data,
 fct=LL.4(),
 na.action = na.omit)

# Plot model
plot(SpeciesX.m1, xlab = bquote('Concentration ('*mu~'g/L)'), ylab = "Growth rate (per day)",
 main = "SpeciesX",
 broken = TRUE, type = "all",
 col = TRUE, legendPos = c(10,2), cex.legend = 0.5)

# Estimate EC50, EC20 and EC10

ED(SpeciesX.m1, c(10, 20, 50), interval = "delta")

# Summarise the model parameters:

summary(CYAN.m1)

# Graphical analysis of residuals – check for heteroscedasticity

plot(residuals(CYAN.m1) ~ fitted(CYAN.m1), main="Residuals vs Fitted") +
 abline(h=0)

# Graphical analysis of residuals – check for heteroscedasticity

qqnorm(residuals(CYAN.m1))
qqline(residuals(CYAN.m1))

# Identification of outliers – cooks distance of > 0.5 indicates data had a disproportionate
 effect on model

plot(cooks.distance(CYAN.m1))
```

Supplementary material D

Cell density validation

For each species a dense culture of cells was serial diluted 15 times by 50% with the assumption that with each 50% dilution the cell density was also halved. The percentage dilution, therefore, was considered to be proportional to cell density.

In three replicates for each dilution optical density (at 750nm), phycocyanin and chlorophyll A fluorescence (determined at excitation and emissions wavelengths of 590 & 650nm and 420 & 681nm respectively) were measured. Where the cell size and density allowed reliable results, cell density was also counted in a haemocytometer. The correlation between the different cell density measurements with the percentage dilution was determined to assess which was the most suitable surrogate for cell density.

The species that had cell sizes that enabled reliable direct counts were *A. flos-aque*, *A. cylindrical*, *Synechocystis sp*. and *G. herdmanii*. The optical density and chlorophyll A were not measured for *S. elongates*.

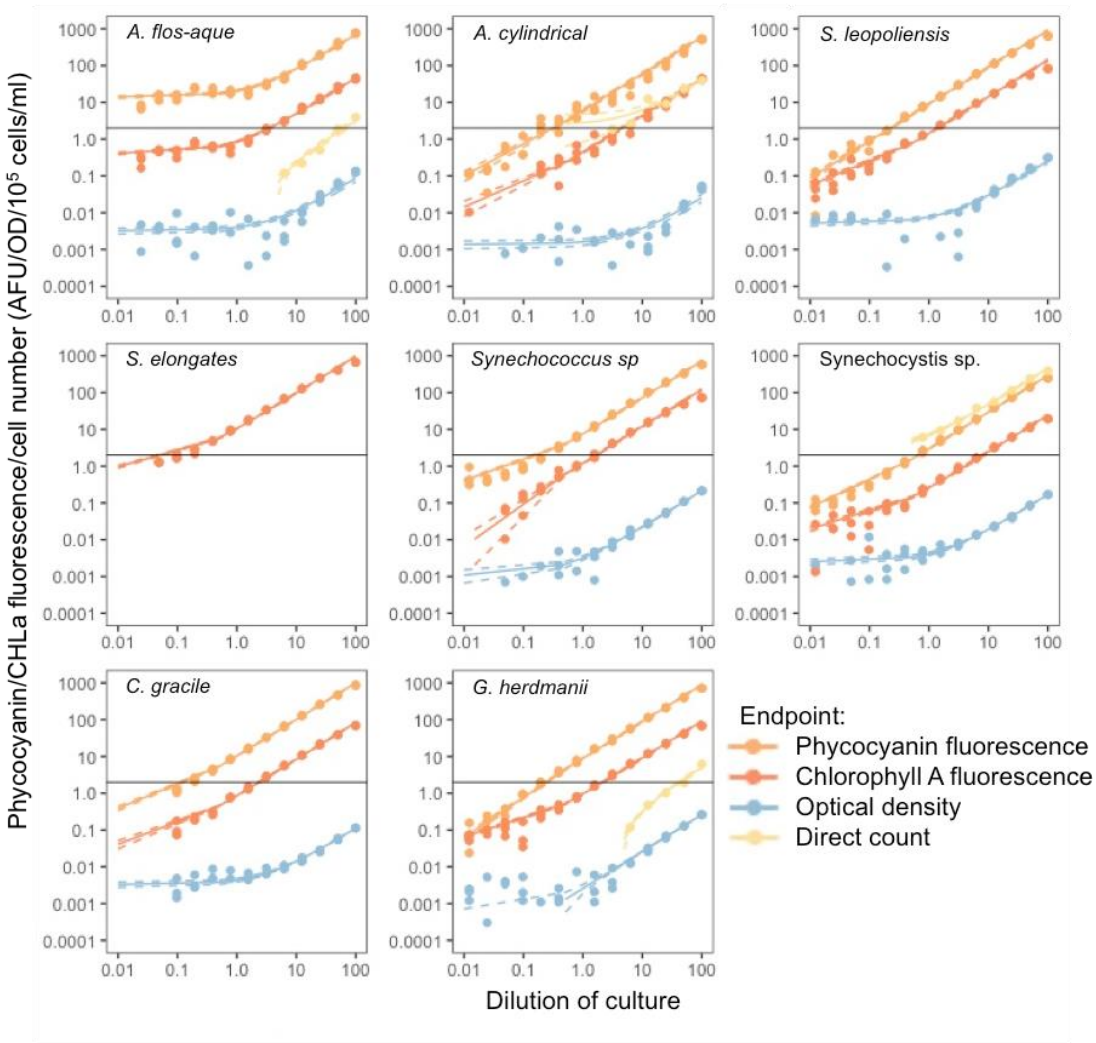

Figure S.D1. The relationship between cell density and phycocyanin florescence, chlorophyll A fluorescence, optical density and direct counting in a haemocytometer (where cell size and density allowed) in cyanobacteria. Solid lines show the general linear model and dashed lines showing the 95% confident limits

Supplementary material E

Effects of temperature, starting inoculum and light intensity on growth rate

The effects of temperature, starting inoculum and light intensity on the growth rate of the cyanobacteria species was investigated to provide data that allowed the selection of suitable growth conditions for each species in the microplate assay.

Temperature (ranging from 20 to 30°C) and starting inoculum (ranging from 2 to 10 AFU of phycocyanin fluorescence at excitation and emission wavelengths of 590nm and 650nm respectively) was assessed at the same time under a constant light intensity of 6000 Lux and with shaking at 140rpm. The growth rate of 15 replicates (quarter of a 96 well microplate with dilution water control (DWC) blanks in rows A and G) of each species at each combination of temperature and starting inoculum was determined over 72 hours.

The effect of light intensity (4000 – 12000 lux) on growth rate was assessed at 28°C and with a nominal starting inoculum of 2 AFU and shaking at 140 rpm. The growth rate 42 replicates (half a plate with DWC blanks in all outside wells) were determined at each light intensity over 72 hours.

The effects of temperature and starting inoculum were investigated by establishing a generalised least squares model using the 'nlme' package (J Pinheiro *et al*. 2017) with the restricted maximum likelihood method (Bates *et al*. 2015) in R (version 3.3.0; R Project for Statistical Computing, Vienna, Austria). Where the data was not homoscedastic the model was weighted by the variance structure in the fixed variable. The two models were compared using the Akaike information criterion and the best fitting model used. Pairwise comparisons were performed to establish significant differences on growth rate caused by temperature (using data from a starting inoculum of 2 AFU only) and significant differences in growth rate caused by starting inoculum (using data from 28°C only) using the 'lsmeans' package (Lenth 2016). The effects of light intensity were investigated using a wilcoxon test performed in 'ggpubr' package (Kassambara 2018).

The results showing the effect on growth rate of temperature and starting inoculum are both presented in figure S.E1 and the results of the pairwise comparisons are presented in table S.E1.

The results showing the effect on growth rate of light intensity are presented in figure 2 and the results of the pairwise comparisons are presented in table S.E

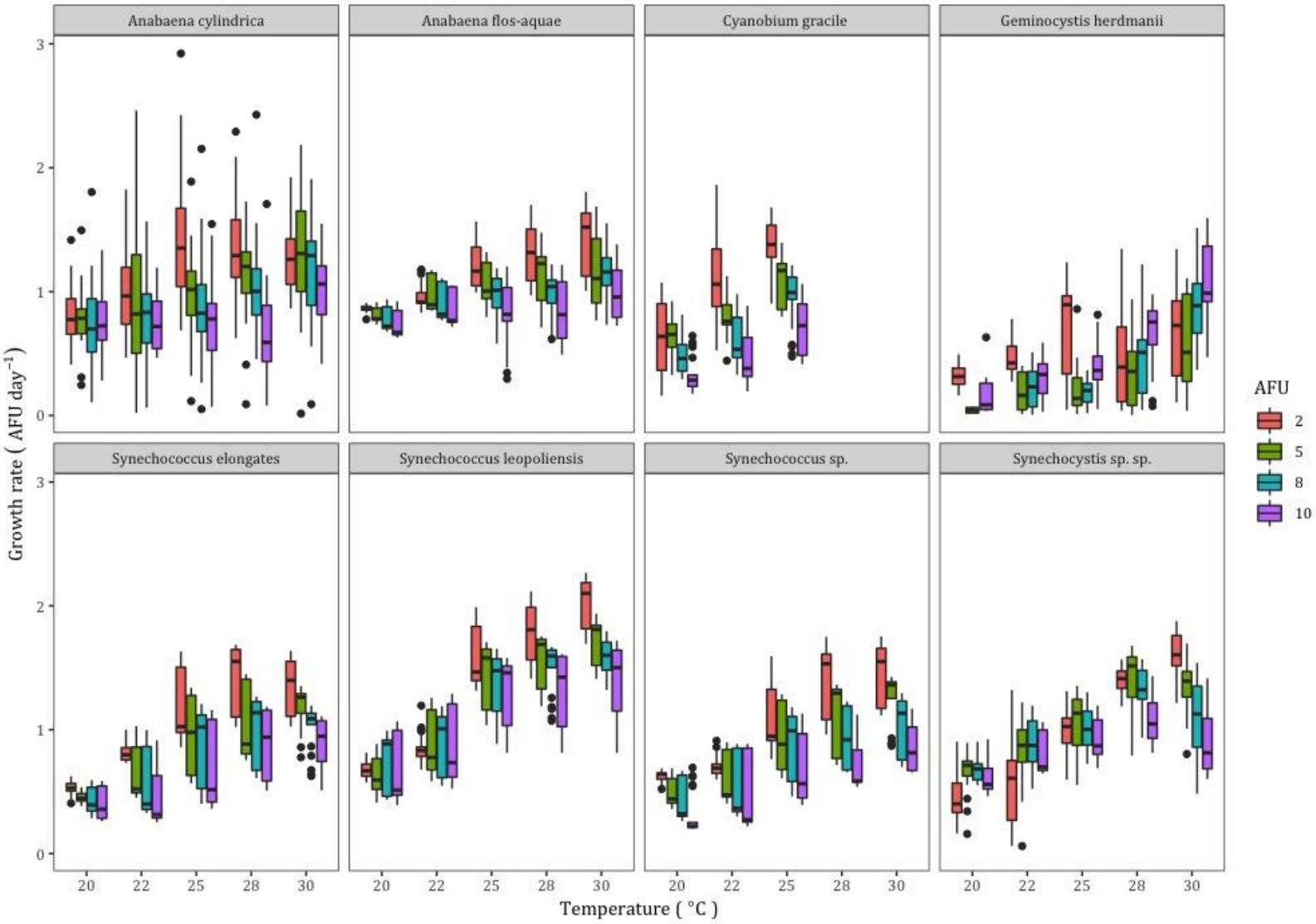

Figure S.E1 The effects of temperature and starting inoculum on the growth rate of cyanobacteria.

	Significance level (*p*)							
Temperature comparison (°C)	**ANA1**	**ANA2**	**SYNch 1**	**SYNch 2**	**SYNch 3**	**SYNcy**	**CYAN**	**GEM**
20 – 22	< 0.001	NS	< 0.001	< 0.001	< 0.01	NS	< 0.001	NS
20 – 25	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	NS
20 – 28	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	N/A	NS
20 – 30	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	N/A	NS
22 – 25	< 0.001	< 0.01	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	NS
22 – 28	< 0.001	< 0.05	< 0.001	< 0.001	< 0.001	< 0.001	N/A	NS
22 – 30	< 0.001	NS	< 0.001	< 0.001	< 0.001	< 0.001	N/A	NS
25 – 28	NS	NS	< 0.05	< 0.001	< 0.001	< 0.001	N/A	NS
25 – 30	< 0.05	NS	< 0.001	NS	< 0.001	< 0.001	N/A	NS
28 – 30	NS	NS	< 0.01	NS	NS	< 0.001	N/A	NS
Starting inoculum comparison (AFU)								
2 – 5	< 0.05	NS	< 0.05	< 0.001	< 0.01	NS	N/A	NS
2 – 8	< 0.001	NS	< 0.01	< 0.001	< 0.001	NS	N/A	NS
2 – 10	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	N/A	< 0.05
5 – 8	NS	NS	NS	NS	NS	NS	N/A	NS
5 – 10	< 0.01	< 0.01	< 0.01	NS	< 0.001	< 0.001	N/A	< 0.001
8 – 10	NS	< 0.05	NS	NS	< 0.05	< 0.001	N/A	NS

Table S.E1 Table showing results of the pairwise comparisons (*p* values) of the effect of temperature and starting inoculum on the growth rate of eight cyanobacteria species. NS – Not Significant (*P* >0.05); N/A – Not Applicable; ANA1 - *A. flos-aque*; ANA2 - *A. cylindrical*; SYNch 1 - *S. leopoliensis*; SYNch 2 - *S. elongates*; SYNch 3 - *Synechococcus sp.*; SYNcy - Synechocystis sp.; CYAN - *C. gracile*; GEM - *G. herdmanii*

	Significance level (*p*)							
Light intensity comparison (Lux)	**ANA1**	**ANA2**	**SYNch 1**	**SYNch 2**	**SYNch 3**	**SYNcy**	**CYAN**	**GEM**
4000 – 6000	< 0.01	< 0.01	NS	< 0.001	< 0.01	NS	N/A	< 0.001
4000 – 8000	< 0.0001	< 0.05	< 0.05	NS	NS	< 0.0001	N/A	NS
4000 – 10000	< 0.05	< 0.0001	< 0.01	< 0.0001	< 0.01	< 0.05	N/A	< 0.05
4000 – 12000	NS	< 0.0001	< 0.05	NS	NS	NS	N/A	< 0.001
6000 – 8000	< 0.05	NS	< 0.05	< 0.01	< 0.001	< 0.001	N/A	NS
6000 – 10000	NS	< 0.001	< 0.01	< 0.0001	< 0.0001	NS	N/A	NS
6000 – 12000	NS	< 0.01	< 0.05	NS	NS	NS	N/A	NS
8000 – 10000	NS	< 0.001	NS	< 0.0001	< 0.05	< 0.05	N/A	NS
8000 – 12000	< 0.01	< 0.001	NS	NS	< 0.05	< 0.01	N/A	< 0.05
10000 – 12000	NS	NS	NS	< 0.0001	< 0.001	NS	N/A	NS

Table S.E2 Table showing results of the pairwise comparisons (*p* values) of the effect of light intensity on the growth rate of eight cyanobacteria species. NS – Not Significant (*P* >0.05); N/A – Not Applicable; ANA1 - *A. flos-aque*; ANA2 - *A. cylindrical*; SYNch 1 - *S. leopoliensis*; SYNch 2 - *S. elongates*; SYNch 3 - *Synechococcus sp.*; SYNcy - Synechocystis sp.; CYAN - *C. gracile*; GEM - *G. herdmanii*

Supplementary material F

Reference toxicity with potassium dichromate

Dose-response curves

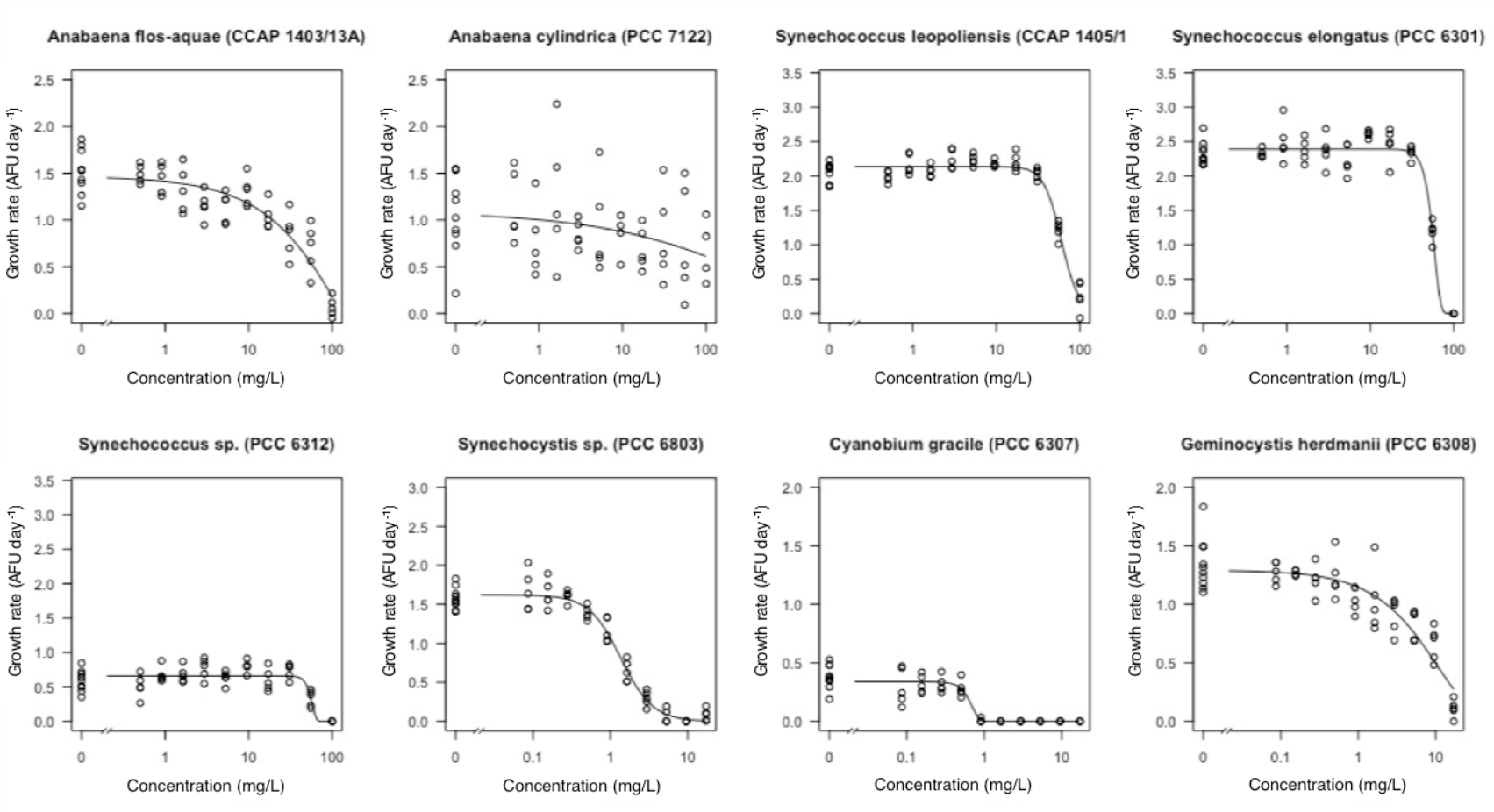

Figure S.F1 – Dose-response curves of effects of potassium dichromate on the growth rate of cyanobacteria. Assay number - Microplate 1.

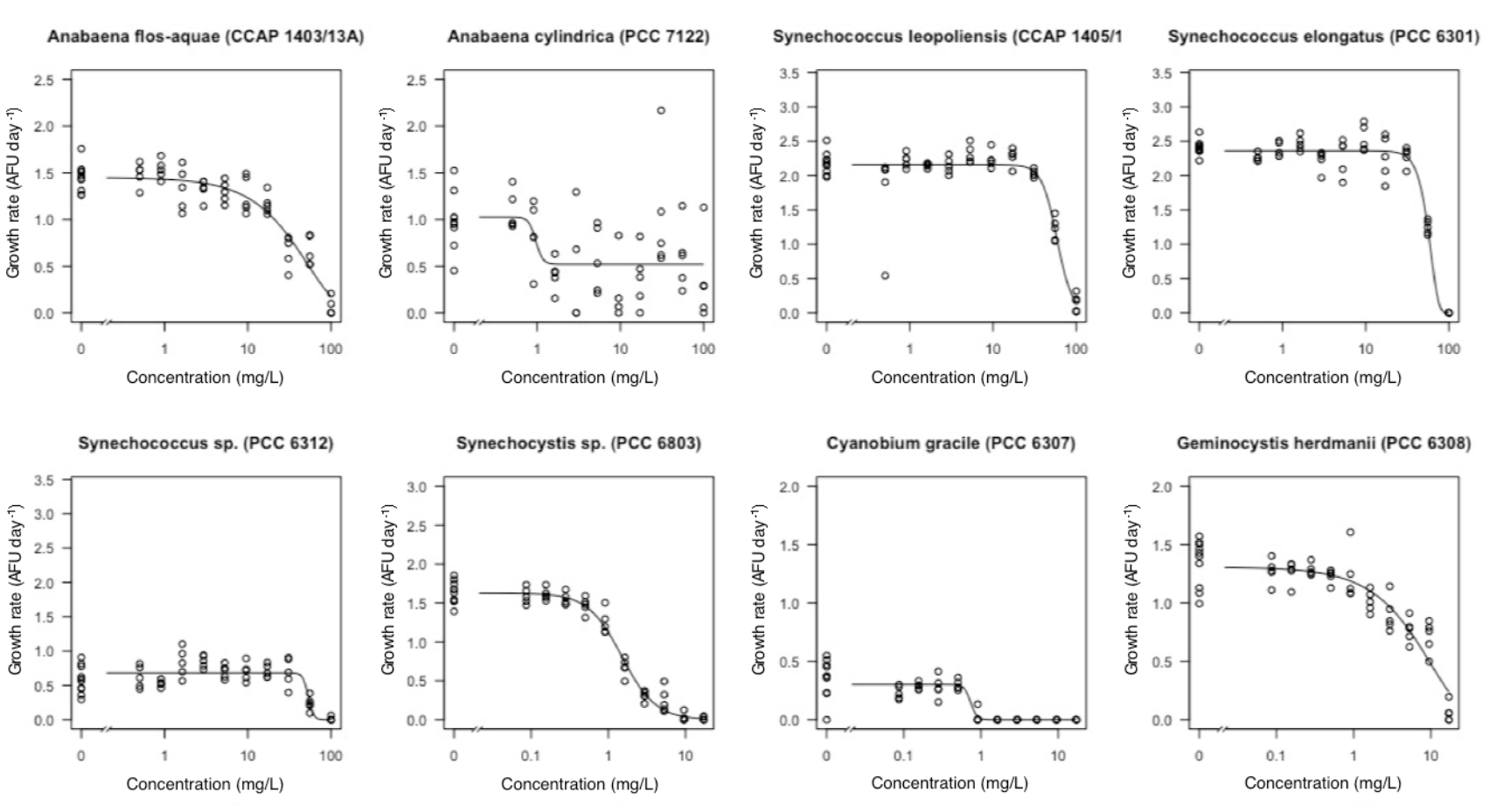

Figure S.F2 – Dose-response curves of effects of potassium dichromate on the growth rate of cyanobacteria. Assay number - Microplate 2.

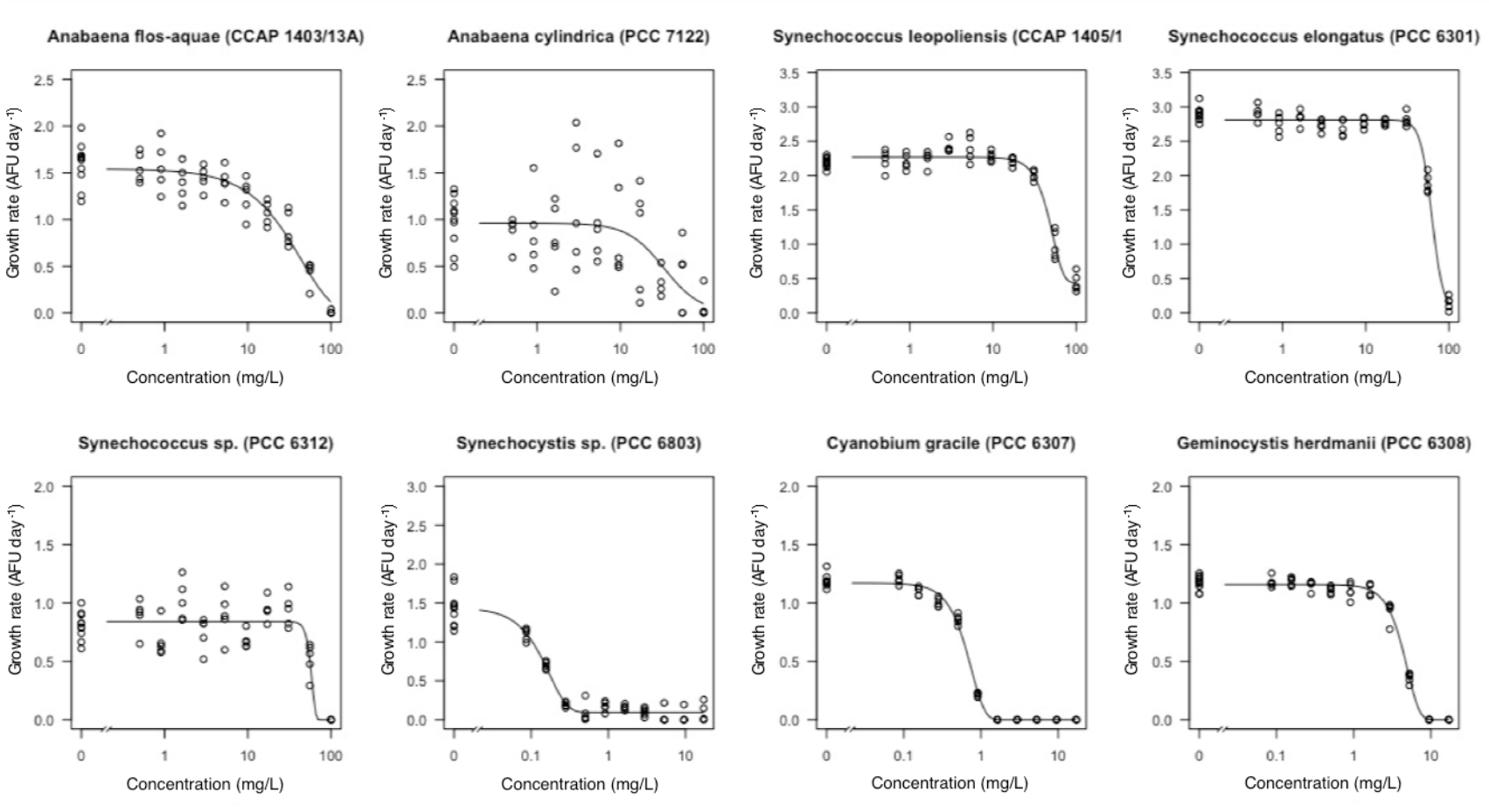

Figure S.F3 – Dose-response curves of effects of potassium dichromate on the growth rate of cyanobacteria. Assay number - Microplate 3.

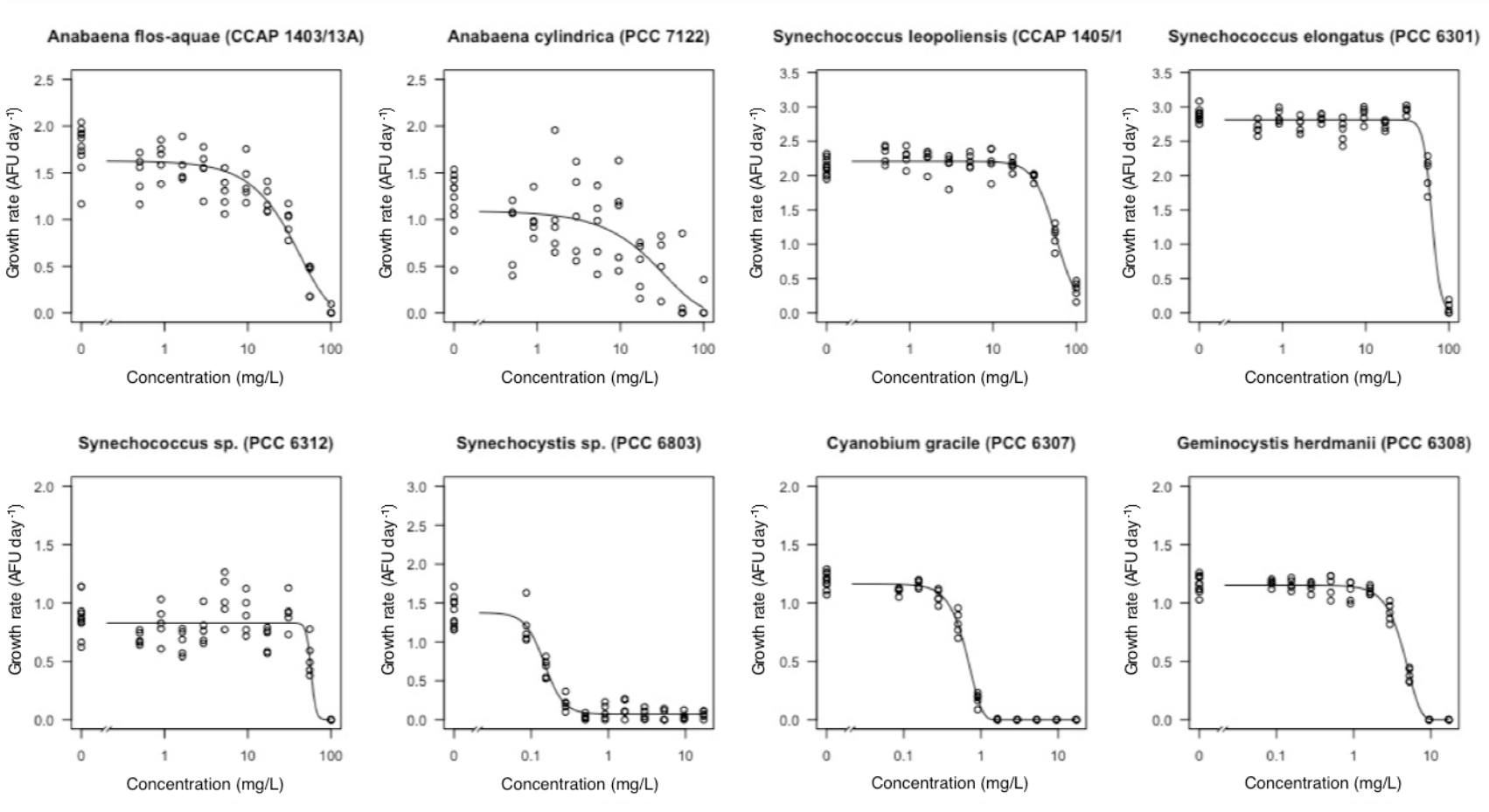

Figure S.F4 – Dose-response curves of effects of potassium dichromate on the growth rate of cyanobacteria. Assay number - Microplate 4.

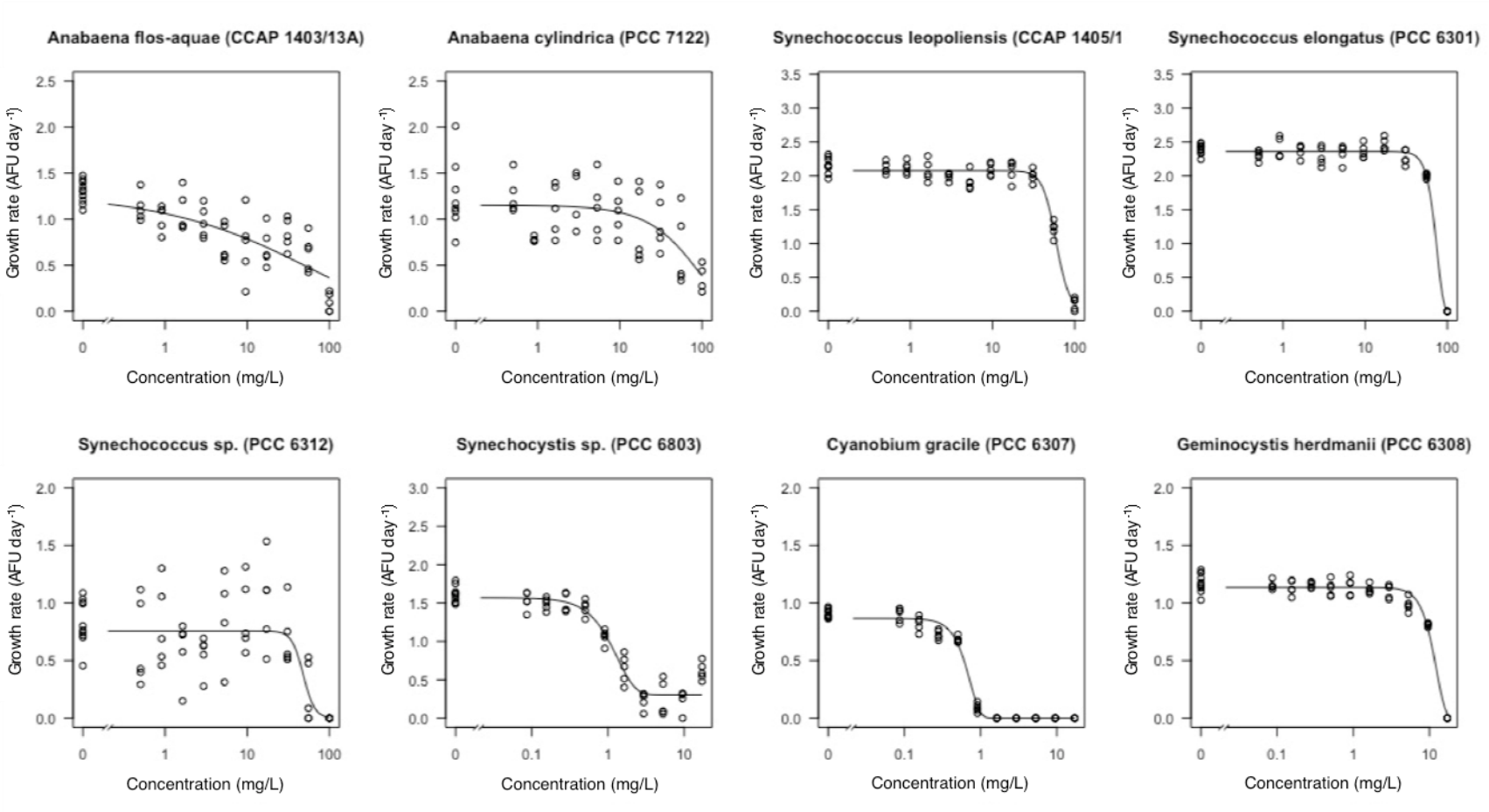

Figure S.F5 – Dose-response curves of effects of potassium dichromate on the growth rate of cyanobacteria. Assay number - Microplate 5.

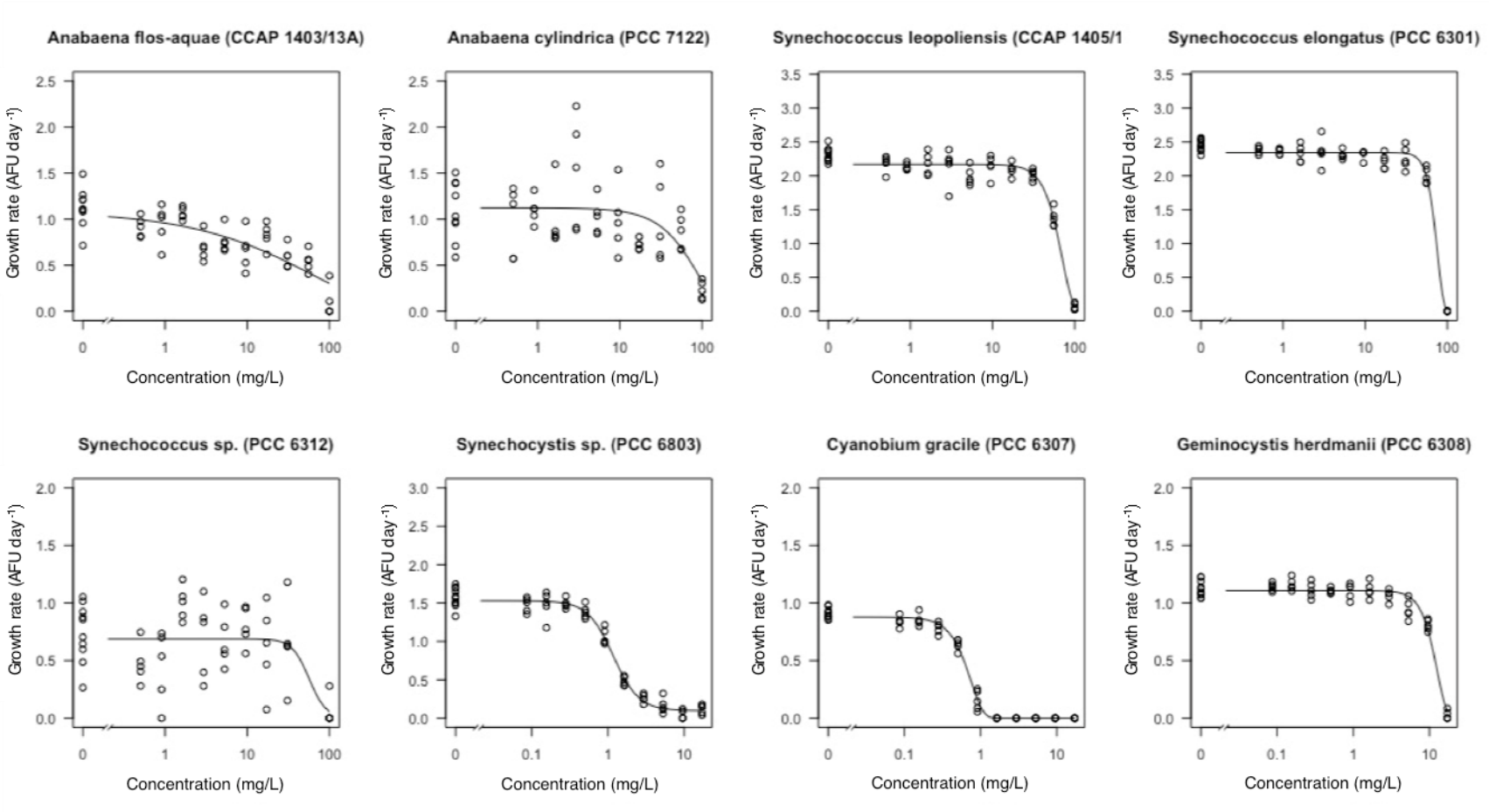

Figure S.F6 – Dose-response curves of effects of potassium dichromate on the growth rate of cyanobacteria. Assay number - Microplate 6.

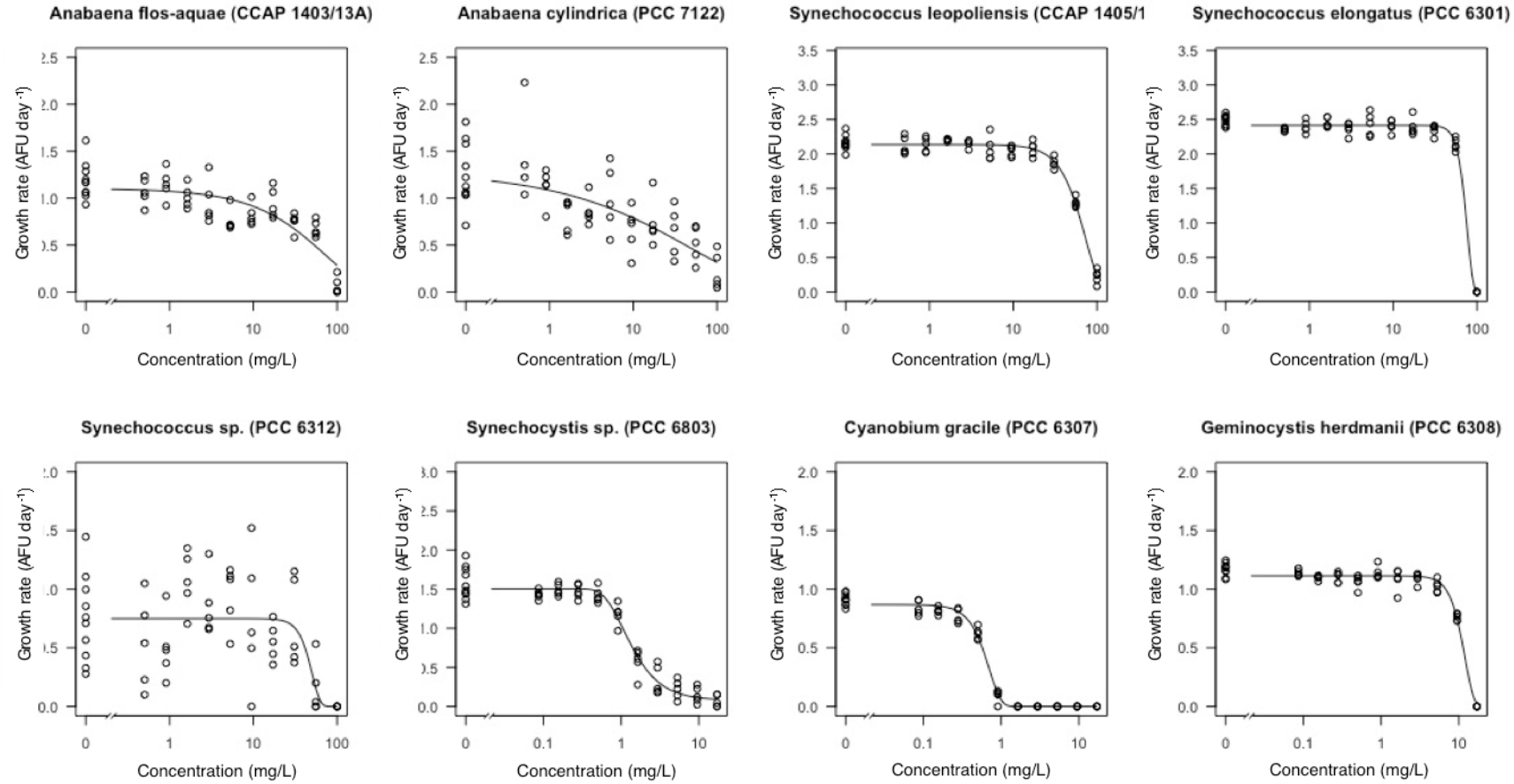

Figure S.F7 – Dose-response curves of effects of potassium dichromate on the growth rate of cyanobacteria. Assay number - Microplate 7.

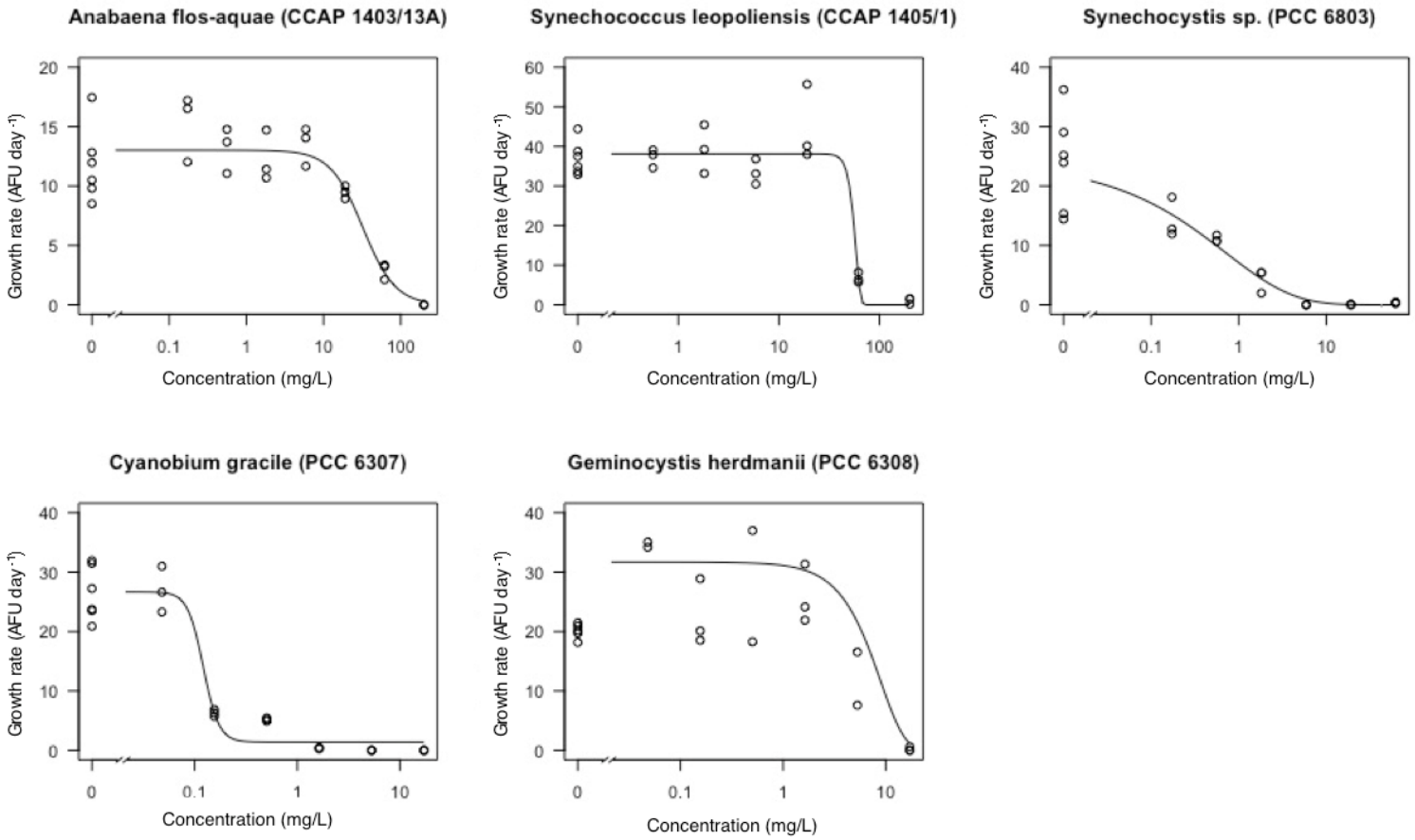

Figure S.F8 – Dose-response curves of effects of potassium dichromate on the growth rate of cyanobacteria. Shake flask assays.

Percentage growth inhibition of microplate assays

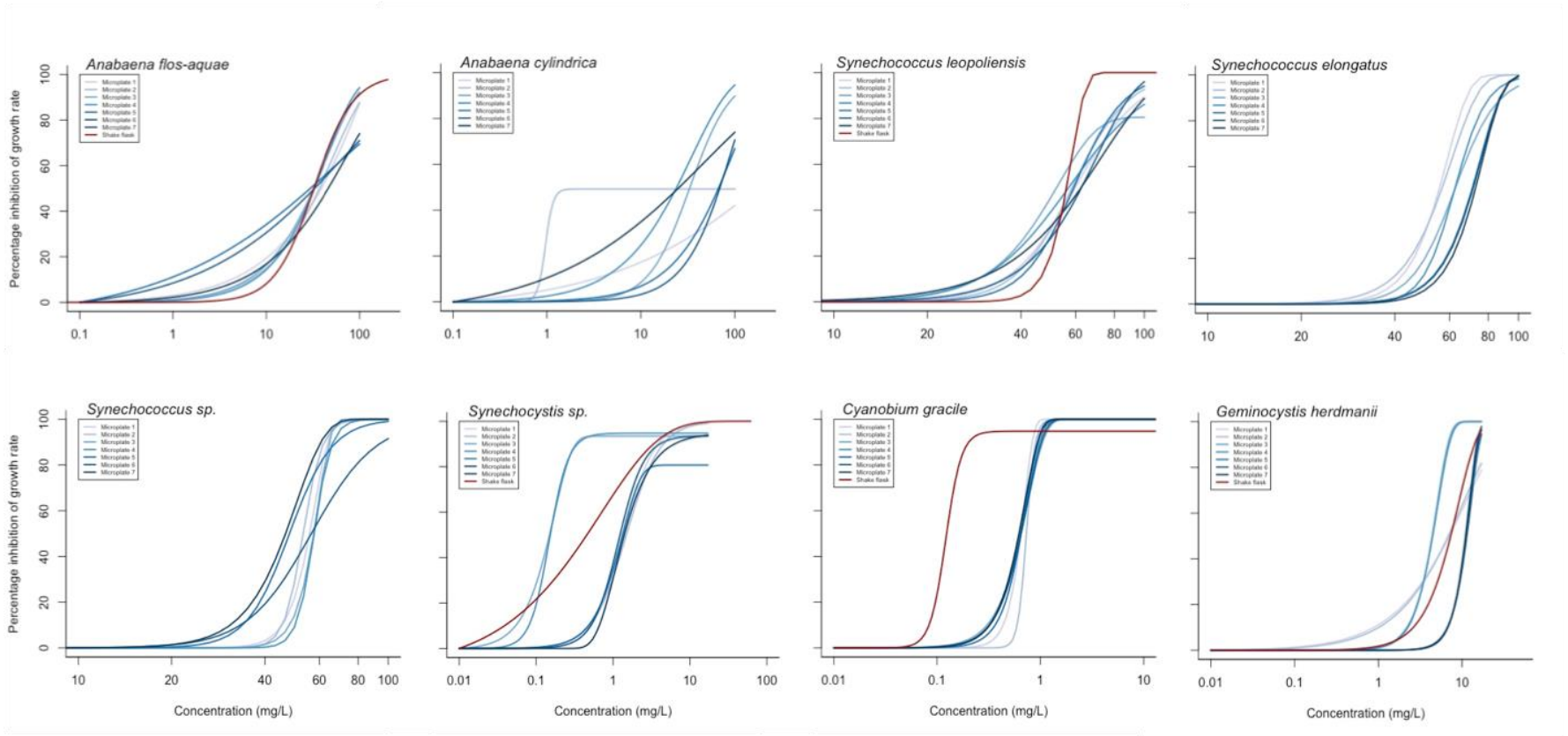

Figure S.F9 – Percentage inhibition of growth rate for eight species of cyanobacteria exposed to potassium dichromate. Blue lines represent the microplate assay test results (7 assays) and the red line represents the shake flask test (one assay). Assays run using the same media batch and parent starting inoculum: microplates 1 and 2 with shake flask tests for *C. gracile* and *G. herdmanii*; microplates 3 and 4 with shake flask tests for *A. flos-aque* and *Synechocystis sp.* and; microplate 5,6 and 7 with shake flask for *S. leopoliensis.* Growth rate units are in AFU day^{-1}

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Chapter 5

Variability in cyanobacteria sensitivity to antibiotics and implications for Environmental Risk Assessment.

Gareth Le Page, Lina Gunnarsson, *Maciej Trznadel, Malcolm Hetheridge*, *Jason Snape, Charles R. Tyler*

This article is in preparation for submission for publication at the time of submitting this thesis. It is a paper that examines the interspecies variability of cyanobacteria using the microplate test developed in chapter 4.

I carried out all experimental planning and work with exception of the chemistry analysis that was conducted my Maciej Trznadel and Malcolm Hetheridge. I performed all subsequent analysis and first drafting of the paper. All authors were actively involved in later discussions on the work and in the preparation of the manuscript.

Variability in cyanobacteria sensitivity to antibiotics and implications for Environmental Risk Assessment.

Abstract

Once released into the environment antibiotics can kill or inhibit the growth of bacteria, leading to affects upon community structure and ecosystem function. It is the role of environmental risk assessment (ERA) to establish protection limits at which adverse effects are of an acceptable level, but recent evidence suggests that the current approach may not be adequate to do so. In this study we assess the differences in interspecies sensitivity of eight species of cyanobacteria to seven antibiotics across three different modes of action (cefazolin, cefotaxime, ampicillin, sufamethazine, sulfadiazine, azithromycin and erythromycin). We used a microplate assay to measure growth rate inhibition that was specifically designed to allow for direct assessment of interspecies sensitivity across equivalent culturing conditions. For these species and antibiotics, we found that interspecies variability is dependent on the mode of action and can vary by up to 70 fold for β-lactams. As a consequence, a protection limit based on one of the regulatory approved cyanobacteria species was not protective for cefazolin. We also found that cyanobacteria may be inappropriate organisms for the setting of protection limits for sulfonamides due to their relative insensitivity. Our findings support calls for additional and more diverse bacteria testing of antibiotics within ERA.

Introduction

Antibiotics are designed to kill or inhibit the growth of bacteria and are fundamental in the treatment of pathogens in human and veterinary healthcare. Following the release of antibiotics into the environment however, non-target bacteria may be affected and the vital ecosystem services they facilitate may be at risk from being disrupted (Grenni *et al*. 2018; Kümmerer 2009). Aquatic ecosystems may be especially at risk due to antibiotic inputs received from manufacturing plant and hospital effluents, wastewater treatment plants (WWTP), and run-off from agriculture. Indeed, effluents from manufacturing plants and hospitals can be sources of very high environmental inputs of antibiotics, where they have been measured in milligrams per litre concentrations (Batt *et al*. 2007; Brown *et al*. 2006; Jaimes-Correa *et al*. 2015; Larsson 2014; Larsson *et al*. 2007; Li *et al*. 2008; Watkinson *et al*. 2009).

The purpose of environmental risk assessment (ERA) is to assess the risk chemicals pose to organisms and populations; and from this to establish suitable protection limits for environmental communities and the ecological functions they perform. For bacterial communities, ecosystem functions potentially at risk include primary productivity, nutrient cycling and the immobilisation and transformation of contaminants (Dopheide *et al*. 2015). The ability of ERA to establish adequate protection limits for antibiotics has recently been questioned because of the limited focus on relevant bacteria species that provide these ecosystem services (Agerstrand *et al*. 2015; Brandt *et al*. 2015; Le Page *et al*. 2017). This is because ERA relies upon just two prokaryotic toxicity tests; the activated sludge respiration inhibition test (ASRIT) and a cyanobacteria growth inhibition test. The ASRIT however, is not sensitive to antibiotics (Kümmerer 2009; Le Page *et al*. 2017), and consequently just a single species of cyanobacteria is used to represent all bacterial diversity in an assay that only concerns a single functional endpoint conveyed by bacteria; primary productivity.

The predicted no effect concentration (PNEC) in ERA is derived to protect wildlife populations, ecosystem structure and function. This is calculated by taking the no observed effect concentration (NOEC) from the most sensitive species in the ERA test battery (likely to be cyanobacteria in the case of most antibiotics) and applying an assessment factor of 10 to account for differences

in species sensitivity and extrapolation from the laboratory to field environments. Tests on other species, including an invertebrate and fish, are also included within the antibiotic ERA, but these tend not to be sensitive to antibiotics (antibiotics are designed not to be toxic to vertebrates at therapeutic levels; (Le Page *et al*. 2017)).

In this study we focus on cyanobacteria due to their current key role within ERA and because they are a very diverse bacterial clade of photoautotrophs that are ubiquitous in both aquatic and terrestrial environments, play key roles in many bacterial communities, and they have a range of important ecological functions such as primary production and nitrogen fixation (Falkowski 1997).

In a recent meta-analysis of all publicly available literature we identified that the sensitivity of different species of cyanobacteria may vary by up to five orders of magnitude, far exceeding the assessment factor of 10 used when establishing the PNEC used in risk assessment frameworks (Le Page *et al*. 2017). The afore mentioned meta-analysis was based on an assessment of published data and although it adopted best practice in the prioritisation of these data in accordance with the 'Criteria for reporting and evaluating ecotoxicity data' system (Moermond *et al*. 2016), ultimately it was reliant upon studies performed using different methodologies and test conditions in different laboratories by different study personnel. Accurate numeration and confidence in relative sensitivities to antibiotic exposure in cyanobacteria species are best derived through comparative experiments conducted under the same test design without inter-laboratory variation.

To this end we optimised a microplate growth inhibition assay to assess the effects of antibiotic on population growth for eight species of phylogeneticaly diverse cyanobacteria (as assessed by their genome sequences (Shih *et al*. 2013)) culturable under laboratory conditions that are of environmental relevance, namely, *Anabaena flos-aquae*, *Synechococcus leopoliensis*, *Anabaena cylindrica*, *Synechococcus elongates*, *Synechococcus sp.*, *Synechocystis sp.*, *Cyanobium gracile* and *Geminocystis herdmanii*.

Seven antibiotics were selected that spanned both a range of antibiotic classes across the main antibiotic modes of action (MoA). These included three cell envelope synthesis inhibiting antibiotics, namely cefazolin and cefotaxime (1st

and 3rd generation cephalosporins respectively) and ampicillin (penicillin); the DNA synthesis inhibitors, sulfadiazine and sulfamethazine (sulfonamides); and the protein synthesis inhibitors erythromycin and azithromycin (macrolides). The macrolides, azithromycin and erythromycin are priority compounds in both the EU watch list (Carvalho *et al*. 2015) and US EPA contaminate list 3 (US EPA 2009). Cefazolin and sulfamethazine also have no ecotoxicological data available for cyanobacteria in the open literature. Sulfadiazine, cefotaxime and azithromycin have very limited ERA relevant ecotoxicological data (Le Page *et al*. 2017).

Cephalosporins and penicillins, that are β-lactams, target penicillin binding proteins that catalyse the building of the peptidoglycan cell membrane of bacteria. They normally enter the environment in their parent form after excretion from humans (80% and 40-60% of cefazolin and cefotaxime respectively, are unchanged after excretion in urine (El-Shaboury *et al*. 2007)). They are therefore most commonly found in WWTP effluents (Mutiyar and Mittal 2014; Ribeiro *et al*. 2018). Ampicilin, on the other hand is largely removed in WWTPs through adsorption to the sewerage sludge (Li and Zhang 2010). Once released in the environment cefazolin, cefotaxime and ampicilin are considered to be relatively stable and to undergo relatively slow rates of hydrolysis under natural conditions, but they can be rapidly degraded via photolysis or β-lactamase enzymes released from environmental bacteria (Arsand *et al*. 2018; Fabbri *et al*. 2015; Jiang *et al*. 2010; Li and Zhang 2010; Wang and Lin 2012).

Sulphonamides inhibit DNA synthesis by preventing the production of folic acid, a key precursor in the DNA synthesis pathway. They do this by acting as a structural analogue of para-aminobenzoic acid and competitively inhibit the enzyme dihydropteroate synthase that catalyses the reaction between para-aminobenzoic acid and dihydropteroate diphosphate to create dihydropteroic acid (Bermingham and Derrick 2002). As with the β-lactams, sulphonamides also tend to be resistant to hydrolysis and environmentally persistent, although they too are reported to be light sensitive (Kümmerer 2009). Sorption to sewerage sludge is an important removal mechanism in WWTPs for sulphonamides (Kümmerer 2009; Tolls 2001).

The drug target for the macrolide antibiotics is the 50S subunit of the ribosome, which is reported to be evolutionarily conserved across bacterial species

(Lecompte *et al*. 2002; Yutin *et al*. 2012). Macrolides work by binding to a section of the nascent peptide exit tunnel of the ribosome from which the protein being synthesised emerges. When a specific amino acid sequence, termed a macrolide arrest motif, reaches the peptidyl transferase centre of the ribosome (where amino acids are bound together to build the protein), the presence of the macrolide prevents the ribosome from catalysing the reaction and thus stalls the ribosome and stops the protein from being formed (Vázquez-Laslop and Mankin 2018). Both azithromycin and erythromycin are considered to be fairly persistent in nature and enter the environment largely via WWTP effluents (Leung *et al*. 2012; Schafhauser *et al*. 2018; Xu *et al*. 2007).

We have previously established a microtitre plate assay to assess toxicity for population growth effects in cyanobacteria with equivalence to the traditional shake flask approach used in regulatory studies for ERA (Chapter 4). Using this assay we compared the effects of seven antibiotics (with a range of MoAs) on the growth rate of eight species of cyanobacteria and consider the implications of our findings for ERA.

Materials and methods

Test organisms and maintenance

We selected eight cyanobacteria species: *Anabaena flos-aquae* (CCAP 1403/13A), *Synechococcus leopoliensis* (CCAP 1405/1), *Anabaena cylindrica* (PCC 7122), *Synechococcus elongatus* (PCC 6301), *Synechococcus sp* (PCC 6312), *Synechocystis sp* (PCC 6803), *Cyanobium gracile* (PCC 6307) and *Geminocystis herdmanii* (PCC 6308). *A. flos-aquae* and *S. leopoliensis* were both obtained from the Culture Collection of Algae and Protozoa (CCAP) and are recommended species in the OECD 201 test guideline and as such represent species that may drive an ERA. The remaining species were obtained from the Pasteur Culture collection of Cyanobacteria (PCC) and were selected based on the following: i) there was an exponential growth rate of a magnitude to achieve measurable differences in biomass within 72 hours using the same laboratory conditions as the other selected species (media, temperature, shaking and light intensity); 2) environmental relevance; and 3) a wide range of phylogenetic diversity within the genome sequenced cyanobacteria in (Shih *et al*. 2013). *Anabaena,* more generally, are filamentous, nitrogen fixers that are

found globally and are important species in harmful algal blooms. Cyanobacteria from the genus *Synechococcus* are diverse unicellular picoplankton, with a large global distribution and represent important primary producers, especially in oligotrophic environments (Callieri *et al*. 2007; Palenik *et al*. 2003). Species belonging to the *Synechocystis* genus are also widely distributed and *Synechocystis sp* (PCC 6803), the strain utilised in this study, was the first fully sequenced photosynthetic autotroph (Kaneko *et al*. 1996) and is an important model organism for photosynthesis. As with the Synechococcus, *C. gracile*, is also a picoplanktonic species and *G. herdmanii* has a much larger cell size with a diameter reaching up to 5µm (Jana *et al*. 2009).

Continuous cultures of exponentially growing cyanobacteria were maintained in 50mL BG-11 medium (Rippka *et al*. 1979); laboratory grade constituents of >97% purity). Cultures were incubated in Multitron II incubators (Infors) under test conditions. Cultures were visually examined using an inverted light microscope to ensure cells appeared healthy before testing.

Antibiotics

Seven antibiotics were selected: cefozolin sodium salt (CAS: 27164-46-1; purity ≥98%; Tokyo Chemical Industry UK Ltd (TCI)), cefotaxime sodium salt (CAS: 64485-93-4; purity ≥ 91.6%; Sigma-Aldrich), ampicillin trihydrate (CAS: 7177-48-2; purity ≥98%; TCI), sulfadiazine (CAS: 68-35-9; purity ≥99%; Sigma-Aldrich), sulfamethazine (CAS: 57-68-1; purity ≥98%; TCI), azithromycin dihydrate (CAS: 117772-70-0; purity ≥98%; TCI) and erythromycin (CAS: 114-07-8; purity ≥98%; TCI). A summary of the chemical properties is given in table 1.

Growth inhibition assays

Growth rate inhibition assays were performed in 96 well microplates that followed a procedure adapted from the (Environment Canada 2007) and (OECD 2011) test guidelines. Biomass was measured using phycocyanin fluorescence as a surrogate (excitation = 590nm, emission = 650nm, cut-off = 635nm; bottom read mode; Spectromax M5 with Softmax® Pro software (Molecular Devices)). This has been previously demonstrated to have a linear relationship with cell density for all species except *A. flos-aquae* that had a

shallower gradient at cell densities below eight artificial fluorescence units (AFU; chapter 4).

A pre-culture for each species was prepared three to four days prior to the start of the test in 50 mL of BG-11 and under exposure conditions (and in the absence of the antibiotic) in order to obtain exponentially growing cells. A cyanobacteria inoculum was prepared in BG-11 medium at a phycocyanin fluorescence of 4 AFU (twice the nominal starting inoculum). Following this a geometric series of stock solutions for each test concentration were prepared in BG-11 medium at twice the nominal test concentrations.

The test solution (100μL) was added to 100 μL of cyanobacteria inoculum to achieve a final cyanobacteria concentration at 2 AFU at the nominal test concentration in each well. Assays were conducted in non-transparent, 96 well plates (Greiner Bio-one item no. 650201), sealed with AMPLIseal^{TM} sealer (Greiner Bio-one item no. 676040) to prevent water loss due to evaporation over the test period. The plate layout for the incubations described is provided in Figure S.A1.

The assays were run in Multitron II incubators (Infors) under the following test conditions: light intensity = 4000 lux, temperature = 28 +/- 1°C and shaking = 140 rpm. The test lengths were optimised to best ensure toxicity testing was carried out during exponential growth for each species whilst avoiding the lag or stationary phase and as such the following exposure lengths were selected for each species: i) 24 hours for the fastest growing species, *S. leopoliensis, S. elongates* and *Synechococcus sp*; ii) 48 hours for *A. flos-aque* and *Synechocystis sp*, with the exception for the exposure of the *Synechocystis sp.* to sulfadiazine where the exposure period adopted was 72 hours (rather than 48 hours due to a slower growth rate than expected in all replicates); iii) 72 hours for the slower growing species, *A. cylindrical, C. gracile* and *G. herdmanii*.

Daily cell density determinations were obtained for each culture well via measurement of phycocyanin fluorescence (excitation = 590nm, emission = 650nm, cut-off = 635nm).

pH was measured in the stocks and in a replicate of each test concentration for each species at the end of the test using micro pH meter (Jenco 6230N; pH probe: Hanna instruments HI1083) to ensure fluctuations did not exceed the

acceptable limits of ±0.2 as defined by most standardised test guidelines (OECD 2011).

For the azithromycin exposure, dimethyl sulfoxide (DMSO) was used as a solvent carrier at a concentration of 10 µl/L. Ten solvent control replicates were employed and comparisons of the dilution water control and solvent control replicates for all species are provided in Supplementary material A. Growth rate was found not to be significantly different from the dilution water control for any cyanobacteria with exception of *A. flos-aquae* and *S. elongates* where small but significant decreases in growth rate ($p = 0.01$; Supplementary Material A, Figure S.A2) were observed in the solvent control (t.test in R, version 3.3.0; R Project for Statistical Computing, Vienna, Austria). All dose-response curves and subsequent statistical comparisons with antibiotic exposures were performed using the solvent control data.

Antibiotic	**Primary pharmacological Target** [a]	**Log Kow** [b]	**pKa** [b]	**Log Dow (pH 8)** [b]	**Solubility at pH 8.0** [b] **(g/L)**
Cefazolin	Penicillin binding protein	-1.52	2.84 (acid) 0.26 (base)	-5.04	454.5
Cefotaxime	Penicillin binding protein	-1.49	2.73 (acid) 3.58 (base)	-4.24	455.5
Ampicillin	Penicillin binding protein	-2	3.24 (acid) 7.23 (base)	-2.72	0.04
Sulfadiazine	Dihydropteroate synthetase	-0.39	6.99 (acid) 2.01 (base)	-0.33	8.91
Sulfamethazine	Dihydropteroate synthetase	0.65	6.99 (acid) 2.00 (base)	-0.06	4.72
Azithromycin	Bacterial ribosome	2.44	12.43 (acid) 9.57 (base)	-0.08	1810
Erythromycin	Bacterial ribosome	2.6	12.45 (acid) 9 (base)	1.55	43.3

Table 1 – Chemical properties of antibiotics.[a] according to drugbank (www.drugbank.ca). [b] predicted by ChemAxon (www.chemicalize.org)

Chemical analysis

The concentrations of antibiotics in the stocks and in three exposure replicates for each species at the end of the tests were measured using LC-MS (method supplied in Supplementary material A). Following the final cell density determination of the assay, microplates were centrifuged at 4000 rpm for 30 minutes. 150µL of supernatant was carefully removed and transferred to a deep well microplate (96-well, 2ml; Porvair Sciences) with acetonitrile (50% volume). Where necessary samples were further diluted to within the calibration range.

All chemical concentrations are reported as free acids and bases.

In the instances where analytical data was <LOQ or where an extraction error occurred (see Supplementary material B and Table S.B1) these samples/replicates were excluded from further analysis (detailed in Table S.B1) Limits of quantification (LOQ) for each antibiotic are given in Table S.B2.

Statistical analysis

Growth rate calculations

Growth rate of cyanobacteria was calculated according to equation 1.

Equation 1: $$\text{Growth Rate} = \frac{\ln X_j - ln X_i}{t_j - t_i}$$

where X = cell density at time *i* and *j*

t = time at time *i* and *j*

Dose-response modelling and EC_x determination

Dose-response curves were fitted in r (version 3.3.0; R Project for Statistical Computing, Vienna, Austria) using the drc package (Ritz *et al*. 2015). The known distributions; 3, 4 and 5 parameter log-logistic distributions and 4 and 5 parameter Weibull 1 and 2 distributions were fitted to the growth rate data for each antibiotic and each species and the optimal distribution selected based on log-likelihood score. From this fitted distribution estimates of the 10% and 50% effective concentrations (EC_x) and associated confidence limits were determined. The growth rate data handing and standard code used for each species is presented in Supplementary material A.

Results

The dose-response curves for growth inhibition for each antibiotic on the eight species of cyanobacteria are presented in Figure 1 based upon geometric mean measured test concentrations. The data in Figure 1 also shows the maximum measured environmental concentration (MEC; derived from the UBA 'Pharmaceuticals in the environment database', (Umwelt bundesamt 2018)). The measured concentrations of the antibiotics in each microplate assay are provided in the Supplementary material B (graphs S.B1 – S.B14). Reductions in

antibiotic concentrations due to the presence of the cyanobacteria in the replicates, determined as the difference in percentage between exposure replicates (cyanobacteria present) and blank replicates (without cyanobacteria) are also illustrated in the Supplementary material (graphs S.B15 – S.B21).

The EC_{10}s, EC_{50}s and NOECs for the experimental data are given in Table 2 (raw data are provided, and shown graphically in Supplementary material C). All dose-response analyses (EC_x's) are based upon geometric mean measured test concentrations. The pHs at the start and the end of the tests are given in Supplementary material D.

Exposure concentrations

Cefazolin: Measured cefazolin concentrations were consistently low also across all stocks (17 to 42% of nominal) indicating low stability. Mean measured concentrations (calculated using a geometric mean of the concentrations at the start (stocks) and end (exposure replicates)) of cefazolin in the exposure replicates ranged between 14 to 32 % of the nominal concentrations (Fig S.B1). The greatest losses of cefazolin (Fig S.B1, S.B2 and S.B17) occurred in the exposure replicates of *S. elongates* and *Synechococcus sp.* (that were lower than in the blank replicates (no cyanobacteria) by an additional 10 to 36% respectively). Reductions in antibiotic concentration in the presence of cyanobacteria also occurred for *A. flos-aquae* (10 to 25% further reduction) and *A. cylindrical* (between 6 and 12% further reduction S.B15).

Cefotaxime: Measured concentrations of cefotaxime in the stock solutions were between 25 and 52% of nominal in the stock solutions, indicating low stability. Mean measured concentrations of cefotaxime in the exposure replicates ranged between 18 and 44% of nominal (Fig S.B3). The presence of the cyanobacteria had variable effects on the levels of cefotaxime with the greatest reductions in the exposure replicates for the three species from the *Synechococcus* genus (particularly at the higher exposure concentrations of cefotaxime (Fig S.B16)).

Ampicillin: Measured ampicillin concentrations in the stocks were between 78 and 102% with exception of the lowest concentration (0.83 μg/L) that was 124% of nominal. Mean measured concentrations of ampicillin in the exposure replicates ranged between 44 and 95% of nominal (Fig S.B5). In the presence of all species of cyanobacteria there were between 10 and 30% reductions in

the level of measured ampicillin compared with in the blank replicates without bacteria. This was most pronounced in *A. cylindrical* exposure replicates where ampicillin was reduced by 35 and 68% compared to the blanks (S.B17).

Sulfadiazine: Stock solutions at the start the test were between 88 and 142% of nominal, with the exceptions for the nominal concentrations of 6.6 and 407 μg/L that were 41 and 71% of nominal, respectively. Mean measured concentrations of sulfadiazine in the blanks were between 101 and 142% of nominal (Fig S.B7). Additional reductions in sulfadiazine due to the presence of the cyanobacteria varied across tests concentrations (S.B18).

Sulfamethazine: Solutions at the test outset were between 81 and 118% of nominal, with exception of the highest concentration (nominal 100,000 μg/L; 136% of nominal). Mean measured concentrations of sulfamethazine in the exposure replicates ranged between 87 to and 134% of nominal (Fig S.B9). There were extraction errors for nominal 907 and 1633 μg/L in the *S. elongates* exposure and these were not included in the analyses. Additional reductions of sulfamethazine in the tests due to the presence of cyanobacteria were variable (between 13 to 23%, S.B19). The exceptions to this were for *A. flos-aquae* and *Synechocystis* sp.

Erythromycin: Stock solutions at the start of the test were between 54% and 109% of nominal. Mean measured concentrations of erythromycin in the exposure replicates were between 71 and 100% of nominal (Fig S.B11) with exception of the nominal 3.77 μg/L test concentration (53% of nominal). Erythromycin exposure concentrations were further decreased by up to 50% over the exposure period in the exposure and blank replicates and the presence of the cyanobacteria in the exposure replicates caused additional erythromycin losses of on average of 10 and 20% (but up to 60%) compared to the blank replicates (Fig S.B20).

Azithromycin: Measured stock solutions at the start the test were between 108% and 156% of nominal. Mean measured concentrations of azithromycin in the exposure replicates ranged between 23 and 79% of nominal (Fig S.B13). Azithromycin concentrations in the exposure and blank replicates at the end of the exposures were considerably lower, by up to 96%. The presence of all

species of cyanobacteria had an effect of reducing the test concentrations further compared to the banks (by between 10 and 15%; Fig S.B21).

Growth inhibition for cell membrane inhibitors:

Cefazolin: Across the different cyanobacteria species, the EC_{10}s for cefazolin ranged between 2.4 and 124 μg/L and the EC_{50}s ranged between 4.1 to 283 μg/L (Table 2). Based upon both EC_{10} and EC_{50} *A. flos-aquae* and *G. herdmanii* were the most sensitive species to cefazolin (Figure 1) and the three species in the *Synechococcus* genera (*S. leopoliensis*, *S. elongates* and *Synechococcus sp.*) along with *Synechocystis sp.* were the least sensitive. *Synechococcus sp.* was up to 70 times less sensitive than the most sensitive species based on the EC_{50}.

Cefotaxime: EC_{10}s across cyanobacteria exposed to cefotaxime ranged between 1.2 and 39.8 μg/L and the EC_{50}s ranged between 2.2 and 98 μg/L for the different bacteria species. The maximum difference in sensitivity was 45 times between the most sensitive (*A. flos-aquae*) and least sensitive species (*Synechococcus sp)*. The four least sensitive species, *S. leopoliensis*, *Synechocystis sp., S. elongates* and *Synechococcus sp.*, were also the least sensitive species to cefazolin, the other cephalosporin tested, with the same order of relative sensitivity.

Ampicillin: EC_{10}s for the different cyanobacteria exposure to ampicillin ranged between 5.9 and 44.6 μg/L and EC_{50}s ranged between 8.4 and 81.4 μg/L. Based on the EC_{50}, there was a difference in sensitivity of approximately 10-fold (9.7) between the most sensitive and least sensitive species (*C. gracile* and *A. cylindrical* respectively). *C. gracile* was particularly sensitive compared with the other species tested (3 times more sensitive than the next most sensitive species, *S. leopoliensis).* The remaining cyanobacteria all had similar sensitivities with EC_{50}s of between 52 and 81.4 μg/L.

Growth inhibition for DNA synthesis inhibitors:

Sulfadiazine: Sulfadiazine caused partial inhibition only of growth of the cyanobacteria tested. It was possible to fit log-logistic or weibul distributions to the growth data but as growth inhibition was incomplete (growth rate stopped decreasing before the point of 50% growth inhibition) EC_{10} or EC_{50} values could not be calculated.

Sufamethazine: As for sulfadiazine, sulfamethazine did not induce full growth inhibition for any of the cyanobacteria tested and it was thus not possible to calculate EC_{10} or EC_{50} values. *C. gracile* was the most sensitive species to the growth inhibition effects of sulfamethazine (a 50% reduction in growth rate was observed down to an exposure concentration of 1465 μg/L). At the highest tested sulfamethazine exposure concentration (10,000 μg/L) there was between a 30% to 40% decrease in growth rate in *A. flos-aquae*, *A. cylindrical, S. leopoliensis*, *S. elongates*, *Synechococcus sp.,* and *G. herdmanii*. *Synechocystis sp* was far less affected with only a 4% inhibition of growth at the highest tested concentration.

Growth inhibition for protein synthesis inhibitors:

Erythromycin: EC_{10}s across the different cyanobacteria species exposed to erythromycin ranged from 21.1 and 58.8 μg/L and the EC_{50}s were between 43.4 and 135.1 μg/L. Based upon the EC_{50}, there was only a small difference in sensitivity, of 3.1-fold, between the most sensitive (*A. cylindrical*) and least sensitive species (*A. flos-aquae*).

Azithromycin: EC_{10}s for the different cyanobacteria exposure to azithromycin ranged between 3.2 and 17.7 μg/L and EC_{50}s ranged between 5.4 and 33.8 μg/L. Based upon the EC_{50}, there was difference in sensitivity of 6.3 times only between the most sensitive (*A. cylindrical*) and least sensitive species (*Synechococcus sp.*).

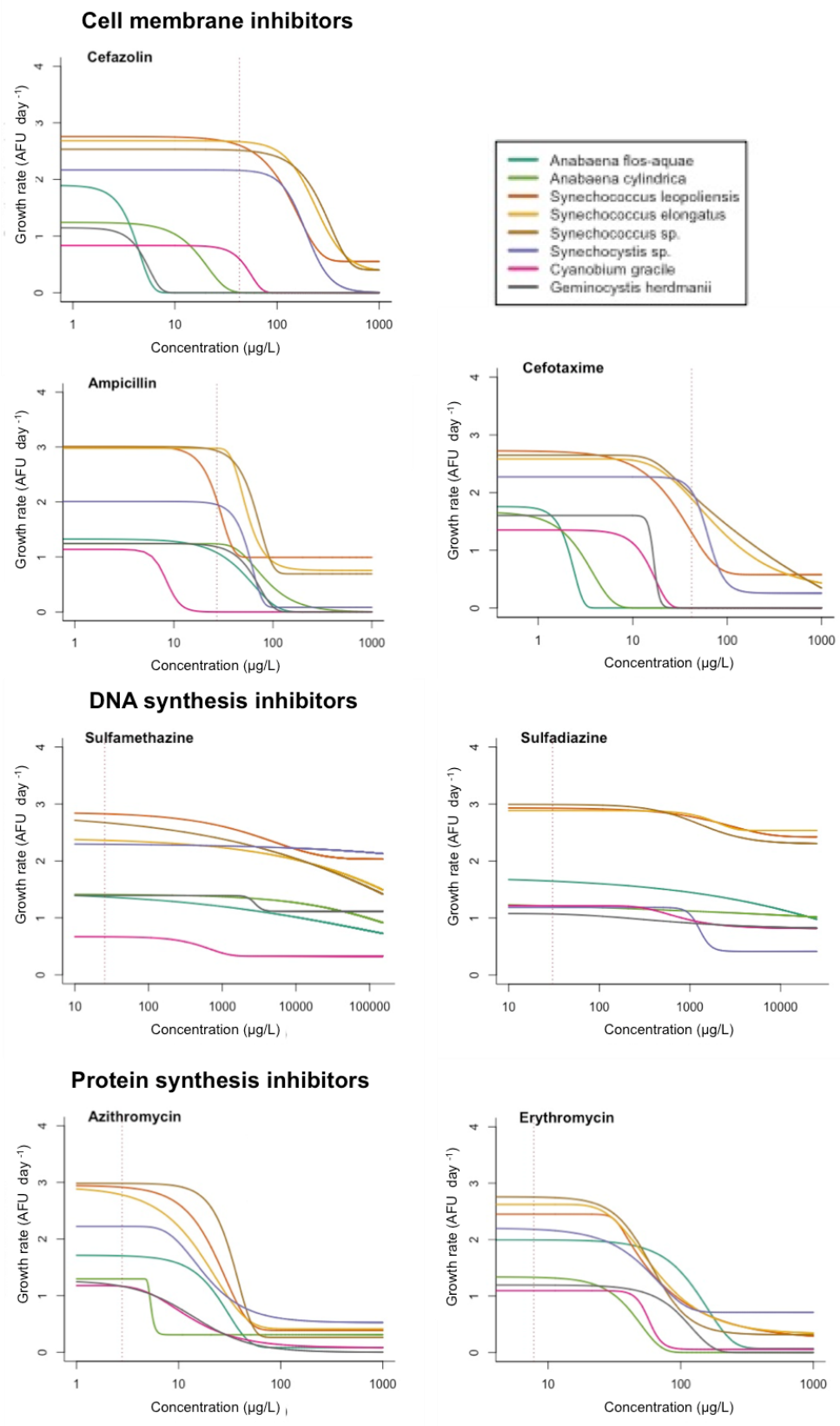

Figure 1 – Concentration- response curves showing the effects of antibiotics on the growth rate of cyanobacteria. Antibiotics are arranged vertical panels related to their mode of action. Red dotted line indicates the highest measured environmental concentration (MEC) in UBA database (Umwelt bundesamt 2018). Median MEC values sit below the lowest concentration on the x-axis. Raw data plots are presented in Supplementary material C.

Mode of action	Antibiotic	Species	EC_{10} estimate (µg/L)	EC_{10} Low CL (µg/L)	EC_{10} High CL (µg/L)	EC_{50} estimate (µg/L)	EC_{50} Low CL (µg/L)	EC_{50} High CL (µg/L)	NOEC (µg/L)	Difference in sensitivity [a]
Cell membrane synthesis inhibitor	Cefazolin	*A. flos-aquae*	2.4	1.7	3.0	4.1	3.7	4.5	1.5	70
		A. cylindrical	7.9	2.9	12.8	17.8	15.4	20.2	6.4	
		C. gracile	32.2	24.8	39.5	51.3	47.5	55.1	44.0	
		G. herdmanii	3.1	2.6	3.5	5.1	4.8	5.3	4.5	
		S. elongates	111.3	97.3	125.3	238.0	217.6	258.3	66.4	
		S. leopoliensis	51.6	41.4	61.9	134.1	122.5	145.6	45.3	
		Synechococcus sp	124.1	101.5	146.8	283.2	263.6	302.8	93.4	
		Synechocystis sp	104.5	80.9	128.1	191.3	170.0	212.5	157.0	
	Cefotaxime	*A. flos-aquae*	1.4	1.0	1.8	2.2	2.0	2.4	1.9	45
		A. cylindrical	1.2	0.3	2.2	3.1	2.2	4.1	1.7	
		C. gracile	8.3	7.5	9.2	15.4	14.8	16.0	9.6	
		G. herdmanii	15.1	8.9	21.4	17.7	14.7	20.6	9.9	
		S. elongates	20.8	16.2	25.4	75.4	56.3	94.4	12.7	
		S. leopoliensis	8.7	7.0	10.3	31.0	28.7	33.3	7.0	
		Synechococcus sp	16.2	11.5	20.8	97.9	74.2	121.6	12.1	
		Synechocystis sp	39.8	28.3	51.3	62.3	53.7	71.0	46.3	
	Ampicillin	*A. flos-aquae*	18.7	11.6	25.9	52.4	45.2	59.7	30.2	9.7
		A. cylindrical	44.6	40.0	49.3	81.4	73.5	89.2	37.1	
		C. gracile	5.9	5.1	6.7	8.4	7.4	9.4	4.9	
		G. herdmanii	34.3	27.2	41.4	64.4	60.5	68.3	12.2	
		S. elongates	38.8	35.0	42.7	54.0	50.4	57.6	36.4	
		S. leopoliensis	16.2	11.8	20.6	27.5	25.1	29.9	11.5	
		Synechococcus sp	38.0	30.1	45.9	66.6	63.0	70.1	31.5	
		Synechocystis sp	36.5	25.8	47.2	57.1	50.7	63.5	34.2	

Mode of action	Antibiotic	Species	EC_{10} estimate (µg/L)	EC_{10} Low CL (µg/L)	EC_{10} High CL (µg/L)	EC_{50} estimate (µg/L)	EC_{50} Low CL (µg/L)	EC_{50} High CL (µg/L)	NOEC (µg/L)	Difference in sensitivity [a]
DNA synthesis inhibitor - Anti-folate	Sufadiazine	*A. flos-aquae*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
		A. cylindrical	N/A	N/A	N/A	N/A	N/A	N/A	N/A	
		C. gracile	N/A	N/A	N/A	N/A	N/A	N/A	N/A	
		G. herdmanii	N/A	N/A	N/A	N/A	N/A	N/A	N/A	
		S. elongates	N/A	N/A	N/A	N/A	N/A	N/A	N/A	
		S. leopoliensis	N/A	N/A	N/A	N/A	N/A	N/A	N/A	
		Synechococcus sp	N/A	N/A	N/A	N/A	N/A	N/A	N/A	
		Synechocystis sp	N/A	N/A	N/A	1275	1058	1493	380	
	Sulfamethazine	*A. flos-aquae*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
		A. cylindrical	N/A	N/A	N/A	N/A	N/A	N/A	N/A	
		C. gracile	N/A	N/A	N/A	N/A	N/A	N/A	N/A	
		G. herdmanii	N/A	N/A	N/A	N/A	N/A	N/A	N/A	
		S. elongates	N/A	N/A	N/A	N/A	N/A	N/A	N/A	
		S. leopoliensis	N/A	N/A	N/A	N/A	N/A	N/A	N/A	
		Synechococcus sp	N/A	N/A	N/A	N/A	N/A	N/A	N/A	
		Synechocystis sp	N/A	N/A	N/A	N/A	N/A	N/A	N/A	
Protein synthesis inhibitor	Azithromycin	*A. flos-aquae*	10.5	7.1	14.0	25.8	22.4	29.3	10.2	6.3
		A. cylindrical	5.0	3.8	6.2	5.4	0.6	10.1	4.9	
		C. gracile	4.8	3.8	5.7	12.5	10.3	14.6	9.5	
		G. herdmanii	3.2	2.2	4.3	13.8	11.8	15.8	1.5	
		S. elongates	4.4	2.7	6.0	17.4	14.9	19.9	3.3	
		S. leopoliensis	8.7	6.7	10.6	23.5	21.6	25.4	1.9	
		Synechococcus sp	17.7	13.5	21.9	33.8	31.5	36.1	2.6	
		Synechocystis sp	8.6	5.7	11.4	18.1	12.9	23.4	9.6	

Mode of action	Antibiotic	Species	EC_{10} estimate (µg/L)	EC_{10} Low CL (µg/L)	EC_{10} High CL (µg/L)	EC_{50} estimate (µg/L)	EC_{50} Low CL (µg/L)	EC_{50} High CL (µg/L)	NOEC (µg/L)	Difference in sensitivity [a]
	Erythromycin	*A. flos-aquae*	58.8	41.5	76.1	135.1	121.9	148.3	28.8	3.1
		A. cylindrical	22.3	16.5	28.2	43.9	40.2	47.6	12.2	
		C. gracile	44.5	15.5	73.5	57.3	56.0	58.6	31.2	
		G. herdmanii	50.7	42.6	58.7	104.8	98.1	111.5	11.5	
		S. elongates	30.1	26.4	33.9	63.3	57.7	68.9	<6.2	
		S. leopoliensis	35.0	28.6	41.3	63.9	53.6	74.1	31	
		Synechococcus sp	29.1	23.8	34.4	59.8	55.6	64.0	13.4	
		Synechocystis sp	21.2	12.3	30.1	55.7	49.5	61.8	<7.2	

Table 2 – 10 and 50% effective concentrations (EC_x) and no observed effect concentrations (NOEC) of antibiotics for exposure to eight cyanobacteria. All concentrations reported in µg/L. CL = Confidence Limit. [a] Times difference calculated by largest EC_x/smallest EC_x – reported value is based on largest range of EC_{10} and EC_{50}.

Discussion

The data we present includes, to our knowledge, the first ecotoxicological data available on cefazolin and sulfamethazine for MoA and environmentally relevant species. We show that for the eight species of cyanobacteria, tested under equivalent culture conditions, the interspecies sensitivity based on growth inhibition following exposure to antibiotics can vary widely and is influenced strongly by the MoA of the antibiotic. For the β-lactam antibiotics in particular, the sensitivity between the different cyanobacteria species varied by up to 70-fold. This far exceeds the assessment factor of 10 applied to the NOEC for a single cyanobacteria species currently used in ERA. We show also that cyanobacteria were not sensitive to sulphonamides with neither species showing complete inhibition of growth for sulfamethazine nor sulfadiazine. This work illustrates that to be most effective, ERA requires the adoption of additional bacteria species relevant to the antibiotic MoA in order to ensure protection limits are suitable for bacteria populations and the ecosystem functions they provide.

Chemical analysis, fate and behaviour in the cultures

Our analytical results showed considerable variation in the fate of the antibiotics in our assays. Generally speaking, reductions in the measured concentrations over exposure period were high in the β-lactams and macrolides whilst the sulphonamides were more stable. Losses of these antibiotics during the cultures may be due to i) abiotic degradation by hydrolysis or photolysis, ii) absorption to the polypropylene microplate, AmpliSeal membrane and/or the bacteria (or extracellular matter) in the well, iii) biodegradation by the cyanobacteria and/or iv) uptake into the cells. Generally, the presence of the cyanobacteria resulted in reduced amount of antibiotic (measured at the end of the exposure) that may have resulted from antibiotic biodegradation by the cyanobacteria and/or adsorption to the bacterial cells. Biodegradation is considered the most likely factor as the outer membranes of cyanobacteria species are similar and therefore surface binding of the antibiotic to the bacteria is unlikely to account for the wide variation in antibiotic loss across the different cyanobacteria species.

***β-lactams*:** The loss of the cephalosporins, cefazolin and cefotaxime in our cultures may in part be explained by photodegradation. Cefazolin and cefotaxime have been shown to have half-lives of just 1.1 and 6.6 hours respectively, attributed to photolysis in synthetic river water (Wang and Lin 2012). Ampicillin too is rapidly degraded by photolysis (Arsand *et al*. 2018). Additionally, bicarbonate and nitrate, both at relatively high levels in the BG-11 media used in this study (Na_2CO_3 = 20 mg/L and $NaNO_3$ = 1500 mg/L), have been indicated to affect (reduce) the half-life of cefotaxime (Wang and Lin 2012).

In all the species of cyanobacteria tested the levels of the β lactams were considerably lower in the exposure replicates compared with the blank replicates without the bacteria, suggesting biodegradation. In activated sewage sludge the main removal mechanism for cefalexin (a 1st generation cephalosporin like cefazolin) and amoxicillin (a penicillin closely related to ampicillin) is via biodegradation (≥90%) (Andreozzi *et al*. 2004; Li and Zhang 2010). The considerable variability in the rates of biotransformation of β-lactams reported in the literature is likely attributable to the variable side chains in the chemical structures (Li and Zhang 2010).

***Sulfonamides*:** Although both sulfonamides, sulfamethazine and sulfadiazine, were relatively stable in the microplate assays, some reductions in levels due to the presence of the bacteria in our assays occurred for both antibiotics. Sulphonamides are hydrophilic with a K_{ow} of -2.09 and -2.13 for sulfamethazine and sulfadiazine, respectively (assuming a negative charge in at the tested pH of approximately 8.2) and thus are unlikely to be absorbed strongly to the bacteria. Photolysis has been reported as a key degradation mechanism for sulfamethazine and sulfadiazine (Biošić *et al*. 2017), with half-life's reported of 28-72 days and 28 to 69 days respectively depending on the season (summer or winter, at 30° latitude (Boreen *et al*. 2005) (Baran *et al*. 2006; Biošić *et al*. 2017; Pan *et al*. 2014; Yu *et al*. 2011).

***Macrolides*:** The relatively limited (20%) loss of erythromycin in our assays over the course of the test period is in accordance with its known persistent nature (including in effluents, freshwater, seawater, soils, sediments and organisms (Schafhauser *et al*. 2018)). The persistence and potential for bioaccumulation of erythromycin are major reasons for its inclusion in the

European Unions watch list of priority substances and in the United States Environmental Protection Agency contaminate candidate list 3 (Carvalho *et al*. 2015; US EPA 2009).

Azithromycin too is also considered ‘very persistent’ (also included in the European Unions watch list of priority substances (Carvalho *et al*. 2015)), but in our assays azithromycin levels were heavily reduced in both exposure and blank replicates. The partition-coefficient (LogP) and adsorption coefficient (K_d) of 4.02 and 3100 respectively (National Centre for Biotechnology Information 2018) suggests that azithromycin could have been bound to the either the microplate, membrane and/or any cellular or extracellular matter. This is supported by the higher losses in the lowest tested concentrations (i.e. more cells and thus higher surface area) and no losses in the highest concentrations.

Cyanobacteria sensitivity

β-lactams: There was a large difference in sensitivity to β-lactams (and in particular the cephalosporins) observed between the different species of cyanobacteria in our study of up to 70-fold. The reason for this difference in sensitivity is unknown but it may reflect differences in the quantity and type of porins they contain that in turn affect antibiotic uptake rtes. The bilayered outer membrane of cyanobacteria (and in Gram-negative bacteria) is comprised of a hydrophobic lipopolysaccharide and acts as an effective barrier to most drugs. Antibiotics must therefore permeate through the membrane or use porin channels that mediate a size-selective diffusion of molecules into the periplasm. Porins tend to let small and non-lipophilic molecules pass through with ease and this includes the β-lactams (as well as fluoroquinolones, tetracycline, chloramphenicol, cycloserine, and aminoglycosides antibiotics) (Delcour 2009; Li *et al*. 2015). The rate at which porin channels allow the diffusion of an antibiotic through the outer membrane may be a key determinate to the sensitivity of the species (Li *et al*. 2015; Sugawara *et al*. 2016).

In cyanobacteria and Gram-negative bacteria, β-lactams need only pass through the outer membrane into the periplasm to reach the penicillin binding protein drug target. For cefazolin and ampicillin that have relatively small chemical structures, we might expect therefore that the porin channels are likely to be the main routes through the outer membrane. Cefotaxime however, a

larger 3rd generation cephalosporin, may not pass in as easily depending on the size of the porin channels. Indeed, it has been shown that the susceptibility of the Gram-negative bacteria *K. pneumoniae* was 4-8 times higher when the strain expressed a larger porin channel (OmpK35) compared to those expressing the smaller OmpK36 (García-Sureda *et al*. 2011). If cefotaxime is too large to easily enter the cells through the porin channels, diffusion through the outer membrane, although slow, may be more important in cellular uptake.

It is possible therefore that uptake via porins is might be a driving factor in the interspecies sensitivity differences observed between our cyanobacteria, at this time we cannot directly equate porins and the relative sensitivity to β-lactams for the species cultured in this study. More is known about porins in clinically relevant Gram-negative bacteria that have a thinner peptidoglycan layer and different protein families to cyanobacteria. Indeed, porins in the outer membrane differ between bacterial clades and cyanobacteria specifically do not appear to have the same porin families as those typically found in other bacteria (Flores *et al*. 2006). Gram-negative bacteria, for example, generally have smaller outer membrane porins but with higher channel conductance than cyanobacteria and thus allow more molecules to enter into the cell (Hoiczyk and Hansel 2000). It is hypothesised cyanobacteria synthesise the large organic molecules they need due to their autotrophic nature and thus only require smaller molecules from outside the cell (Hoiczyk and Hansel 2000; Kowata *et al*. 2017). Conversely, non-autotrophic bacteria need to uptake all molecules from outside of the cell. It is therefore reasonable to hypothesise that cyanobacteria may not be as susceptible as Gram-negative bacteria to larger antibiotics (such as macrolides) that require larger porin channels. Since ERA only uses one species of cyanobacteria to represent all primary producer diversity, if sensitivity is, at least in part, driven by uptake due to their outer membrane porins, other bacterial clades such as Gram-negative bacteria that differ in their membrane structure and porins may show even more variability as compared to cyanobacteria and thus may not be well represented.

In addition to uptake, efflux and β-lactamase enzymes may have key roles in determining the sensitivity of bacteria to antibiotics. Efflux rates of the antibiotics in cyanobacteria studied are not known, but our data do indicate biodegradation for all the β-lactams tested. For cefazolin this (potential) biodegradation was

greatest for *S. elongates* and *Synechococcus sp*. These species were also the least sensitive species tested based on growth inhibition. There was no apparent relationship observed however between the level of biodegradation and sensitivity rankings for either cefotaxime or ampicillin. Further investigation quantifying β-lactamase in the different bacteria might help determine if biodegradation might, at least partly, explain the differences in sensitivity observed between cyanobacteria exposed to β-lactams. Studies on the Gram-negative bacteria *Kluyvera ascorbata* and *Kluyvera cryocrescens* (from the family *Enterobacteriaceae*), Stock (2005) have shown natural variability to β-lactams within species (for 58 strains of *K. ascorbata* and 24 strains of *K. cryocrescens)* ranged by between one and two orders of magnitude (based upon minimum inhibitory concentrations (MIC)) and these differences were attributed to differences in chromosomal β-lactamases.

Reductions of porins in the outer membrane over the exposure period could also help explain the differences in variability observed between the cyanobacteria cultures. This is a well reported β-lactam resistance mechanism in Gram-negative bacteria (Delcour 2009). Charrel *et al*. (1996) found that the MIC in 80 Enterobacteriaceae increased after exposure to four β-lactams, including for exposure to cefotaxime, and this was associated with a decrease in porins. Whether this resistance mechanism is present and could explain the high interspecies variability in cyanobacteria is currently unknown.

It should also be noted that the photolysis product of cefotaxime, attributed to its 5-methyl-1,3,4-thiadiazole-2-thiol moiety may have increased toxicity (Wang and Lin 2012) and has been predicted to have potential chronic toxicity to algae and daphnids in the high μg/L range (Fabbri *et al*. 2015). It is therefore possible that the toxicity observed could be due to this rather than the parent compound and further investigation is required for clarification.

Sulphonamides: Growth inhibition of cyanobacteria for exposure to the sulphonamides was generally limited and in some species the inhibitory effect stabilised with increasing antibiotic concentration suggesting the development or initiation of a possible resistance mechanism. The results are in accordance with a recent meta-analysis where cyanobacteria were found to be less sensitive to sulphonamides compared to microalgae and macrophytes (Le Page

et al. 2017). A possible explanation for the insensitivity could be that cyanobacteria contain a protein (*slr0642* identified in *Synechocystis*) that may act as a folate transporter and which allows the uptake of folates from the environment. This in turn overcomes the effect of the targeting of this drug on the folate synthesis pathway (de Crécy-Lagard *et al*. 2007; Klaus *et al*. 2005). The small differences in sensitivity across the species observed in our assays may include differences in the ability to activate the folate transporter resistance mechanism. It should be highlighted that the growth rate was lower than the controls and thus there appears to be some fitness consequence to this resistance mechanism.

Macrolides: Responses to the macrolides were more consistent across the cyanobacteria compared with the β-lactam antibiotics; the EC_{10}s and EC_{50}s for the eight species differed by less than an order of magnitude (6.3 and 3.1 times difference between the most and least sensitive species for azithromycin and erythromycin respectively). The similar levels of efficacy of the macrolides across the different cyanobacteria may, in part, be explained by the highly conserved ribosome drug target (Lecompte *et al*. 2002; Yutin *et al*. 2012). R-proteins however, which make up the ribosome, do vary between broader bacterial taxonomic clades and because the MoA of macrolide antibiotics is highly dependent on the positioning and interaction with the ribosome, differences in r-proteins between bacterial taxa could feasibly affect antibiotic efficacy/action.

The uptake of macrolides may be a key driver of the small difference in cyanobacteria sensitivity. Due to the large size of macrolides their uptake is generally thought to be restricted by the outer membrane (Delcour 2009; Stock 2005). Although there is some evidence that porin-like uptake maybe present (Hahn *et al*. 2012), erythromycin is both large and hydrophobic and is likely to permeate slowly via diffusion through the outer membrane. Azithromycin on the other hand, is dicationic and less hydrophobic, and will therefore pass through the outer membrane more easily (Farmer *et al*. 1992; Stock 2005). Indeed, Stock (2005) hypothesized that Gram-negative bacteria species specific differences are at least partially, driven by outer membrane hydrophobicity differences. Because little is understood regarding the outer membranes of

cyanobacteria, we can only hypothesise that differences in uptake could explain the relatively small interspecies sensitivity observed in our results.

The molecular mechanisms for protein secretion in cyanobacteria are not well understood, but there is evidence to suggest cyanobacteria do have some porin-like proteins that are associated with the efflux of antibiotics and secondary metabolites, although they could also be part of an uptake mechanism. For example, HgdD, a TolC-like protein (an outer membrane efflux protein found in Gram-negative bacteria) is responsible for the efflux of erythromycinin in *Anabaena* sp. PCC 7120 and may have a multidrug resistance function more generally (Hahn *et al*. 2013; Hahn *et al*. 2012). Another TolC-like protein, Slr1270, has been identified in *Synechocystis* sp. PCC 6803 which also provides resistance to antibiotics (Oliveira *et al*. 2016). Based on the literature therefore, the differences in in cyanobacterial sensitivity to macrolides are more likely to driven by differences in uptake or efflux than differences in the drug target given that their ribosomes are likely evolutionarily well conserved. In addition to decreased uptake/increased efflux, other mechanisms of resistance to macrolides in Gram-negative bacteria comprise of target mutations, methylation, pseudouridylation and modification of the macrolide (Gomes *et al*. 2017), but such resistance mechanisms have not yet been considered in cyanobacteria.

In our assays azithromycin had a greater potency than erythromycin across all cyanobacteria. Interestingly, azithromycin is reported to have modes of action in addition to the ribosomal drug target that may help to explain this enhanced potency. It is dicationic and in a similar MoA as aminoglycosides, may disrupt the outer bacterial membrane through the displacement of divalent cations from their binding sites on adjacent lipopolysaccharide molecules in Gram-negative bacteria (Farmer *et al*. 1992; Imamura *et al*. 2005). Azithromycin has also been shown to reduce the ability of *P. aeruginosa* to form fully polymerised alginate biofilms making them cells more susceptible to other chemicals (Hoffmann *et al*. 2007).

Sensitivity comparisons with other bacteria

When comparing our β-lactam effects with the MICs of clinically relevant bacteria in the EUCAST database, the most sensitive cyanobacteria in our assay study were 3-6 times more sensitive to cefozolin than *Streptococcus pneumoniae* and *Staphylococc*us *aureus* (most sensitive pathogens with MICs of 32 and 64 μg/L respectively (EUCAST)). Several of the clinically relevant bacteria appeared to be more sensitive to cefotaxime than our cyanobacteria with MICs at the lowest tested concentration of 2 μg/L (EUCAST). The effects of ampicillin upon the cyanobacteria were similar to those observed by Ando *et al*. (2007) and within the ranges seen in clinically relevant bacteria in the EUCAST database (EUCAST). It should be noted that due to the limited data available we have compared the MIC for clinically relevant bacteria with the data obtained in our assays, but these values represent different parts of the dose-response curve (the MIC represents the concentration with complete inhibition and the EC_{10} the concentration that inhibits growth rate by 10%) (Bengtsson-Palme and Larsson 2018; Le Page *et al*. 2018).

There are limited data available in the literature for sulfadiazine and sulfamethazine and being veterinary antibiotics neither have EUCAST data, thus their ecotoxicological profile is rather less well understood than that of their fate and behaviour. Investigators have found however that sulfamethazine MICs tend to be relatively high in comparison to other antibiotics, for example, an MIC of >512 mg/L for both Gram-negative and gram positive bacterial strains (Salmon and Watts 2000; Salmon *et al*. 1995). The MIC reflects only the concentration with complete inhibition of growth and thus it is not known whether the incomplete growth inhibition observed in our results is mirrored and further research is required to establish the effects of sulphonamides on a more diverse range of bacterial taxa. A 7 day EC_{50} of 0.14 mg/L was reported for the effects of sulfadiazine on the cyanobacteria *Microcystis aeruginosa* (Lützhøft *et al*. 1999), which is lower than in this study, although it may be a reflection of the longer exposure length and it is unknown if there was complete inhibition and a full dose-response curve obtained.

There is also only limited data on azithromycin in the literature for traditional ecotoxicologically relevant species and methods, but our results are in accordance with those reported in Vestel *et al*. (2015) where cyanobacteria have a EC_{50} of 1.8 μg/L (species not provided). The MICs of clinically relevant bacteria in the EUCAST database suggest the most sensitive bacteria have complete growth inhibition at 16 μg/L, which is consistent the more sensitive cyanobacteria in this study potentially indicating limited sensitivity differences across bacterial clades due to drug target conservation. For the second macrolide, erythromycin, EC_{50}s were generally similar to those obtained by Ando *et al*. (2007) for eight species of cyanobacteria and although the most sensitive species they tested was the same as ours, *A. cylindrical*, they calculated it to be over an order of magnitude lower than in this study (3.5 compared to 44 μg/L respectively), possibly due to differences in test period (3 days in this study compared to 6 days in (Ando *et al*. 2007)). The most sensitive clinically relevant bacteria in the EUCAST database to erythromycin have MICs from 8 μg/L (EUCAST), suggesting that for this antibiotic they may be more sensitive than cyanobacteria.

It should be noted that the MIC is equivalent to the EC_{100}, at which there is 100% growth inhibition and thus isn't directly comparable to the EC_{10} used in this study (Bengtsson-Palme and Larsson 2018; Le Page *et al*. 2018). Additionally, the EUCAST data is not based upon measured concentrations and is determined in the dark and we might thus expect less degradation from photolysis.

Implications for ERA

Our data show that for some antibiotics, especially β-lactams, there may be differences in sensitivity that exceed an order of magnitude, thus indicating that a PNEC in some instances will not be protective of all cyanobacteria populations. Comparing our results, with the two species of cyanobacteria recommended in the OECD 201 guideline (*A. flos-aquae* and *S. leopoliensis*), we found *S. leopoliensis* was between 1.6 to 21.8 times less sensitive (based upon EC_{10}) than the most sensitive species of the eight we tested. Thus a PNEC for cefazolin based upon *S. leopoliensis* would be not be protective of *A. flos-aquae* nor *G. herdmanii*, even with the given assessment factor of 10. *A. flos-aquae* was overall the most sensitive to the β-lactam antibiotics tested. A

PNEC based upon *A. flos-aquae* would have been protective of all the other species tested for cephalosporin and macrolide antibiotics, even though it was one of the least sensitive species to the macrolide antibiotics. Careful selection of a cyanobacteria species is therefore of importance to ensure ERA remains protective of bacterial species more widely and the MoA can be an important factor in this consideration.

Our results indicate that we require a more comprehensive understanding of the effects of antibiotics upon prokaryotic diversity. We argue that this should include consideration of microbes that are known to play key roles in ecosystems function/services, such as nitrifying bacteria or sulphate-reducing bacteria as some of the organisms we may wish most to protect. Additionally, the effects on community structure and diversity should also be considered given that if a specific group of bacteria in a community increased or decreased in abundance due to antibiotic exposure, there may be significant consequences for the normal functioning of that community.

The limited sensitivity of cyanobacteria tested here to sulphonamide antibiotics confirmed the conclusions from the previous meta-analysis (Le Page *et al*. 2017) that cyanobacteria may not be suitable for the estimating protection limits. Furthermore, in some cases microalgae and macrophytes may be more sensitive than cyanobacteria to this class of antibiotics (Le Page *et al*. 2017) but under current ERA framework for pharmaceuticals neither microalgae nor macrophytes would be tested.

Finally, there is some evidence in the literature that the degradation products of at least some β-lactams may have increased toxicity (Fabbri *et al*. 2015; Wang and Lin 2012). It is therefore possible that these products could be influencing, or driving, the toxicity observed in these results. Further research is thus required to identify the risk such degradation products pose to environmental bacteria.

Conclusions

In this study we have used a microplate assay to assess the relative interspecies sensitivity of a range of cyanobacteria to the effects of seven antibiotics spanning three different MoAs. Our experimental data experimentally verify the findings of a meta-analysis of published literature (Le Page *et al*. 2017) where interspecies sensitivity spanned by more than an order of magnitude depending on the MoA; including that not all species in the meta-analysis were able to be tested in our assays, including the most sensitive species in the meta-analysis, *Microcystis aeruginosa*. To our knowledge, we present the first environmentally relevant bacterial data for cefazolin, cefotaxime and sulfamethazine and we show that for the β-lactam antibiotics, the interspecies sensitivity exceeds the assessment factor of 10 currently used in ERA to account for such variability. These results therefore support previous recommendations by Le Page *et al*. (2017) and Brandt *et al* (2016) to widen the number of bacterial and cyanobacteria species tested. We show also that cyanobacteria may not be a suitable group of bacteria for determining environmental risk to sulphonamides due to their insensitivity relative to other environmentally important taxa. Furthermore, when comparing these data with clinically relevant bacteria MICs there was evidence that an ERA based on just one cyanobacteria may not be protective of other clades of environmental bacteria. We conclude that the ERA of antibiotics should include additional bacterial assays that cover a wider range of bacterial diversity.

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Supplementary material A

Material and methods

Analytical method

Details of chromatographic separation and mass spectrometry analysis for each antibiotic are summarised below:

Analytical Chemistry: LC-MSMS Methods

Analyses of exposure media samples were performed using a TSQ Vantage triple quadrupole mass spectrometer. The mass spectrometer was equipped with a heated electrospray (HESI II) source (ThermoFisher Scientific, Hemel Hempstead, UK). The HESI probe was operating in positive mode; an ion-spray voltage of 4.0 kV, heated capillary temperature was set at 270 °C and the vaporizer temperature was 350 °C. Nitrogen was employed as a sheath and auxiliary gas at a pressure of 60 and 2 arbitrary units, respectively.

The argon CID gas was used at a pressure of 1.5 mTorr and the optimum collision energy (CE) for each transition was selected. Quantification of the target compounds was performed by monitoring two characteristic multiple reaction monitoring (MRM) transitions (Table below).

Analyte	Parent ion (m/z)	Product ion (m/z)	CE (eV)
Cefazolin	455.040	323.104	11
		156.046	11
Erythromycin	734.471	158.126	30
		576.341	30
Ampicillin	350.120	106.080	19
		114.043	19
Sulfadiazine	251.062	156.091	15
		92.116	15
Azithromycin	749.519	591.357	27
		116.071	27
Sulfamethazine	279.094	186.118	17
		124.141	17
Cefotaxime	456.070	125.021	47

Chromatographic separation was achieved using a reversed-phase, 3 µm particle size, C18 Hypersil GOLD column (50 mm × 2.1 mm i.d., Thermo Scientific, San Jose CA, USA).

All analytes were separated using a linear gradient with a flow rate was 500µL/min. The autosampler temperature was maintained at 6˚C, while column was kept at a room temperature. Several variations of mobile phase and gradient were required and these are summarised in the following tables:

Analyte	Gradient	(A) 0.1% Formic Acid in Water	(B) 0.1% Formic Acid in Methanol
Cefazolin	0	80%	20%
Erythromycin	3.0 Min		100%
Ampicillin	3.01 min	80%	20%
Cefotaxime	3.5 Min	80%	20%

Analyte	Gradient	(A) 0.1% Formic Acid in Water	(B) 0.1% Formic Acid in Methanol
Sulfadiazine	0	95%	5%
Sulfamethazine	3.0 Min		100%
	4.0 Min		100%
	4.01 Min	95%	5%
	5.0 Min	95%	5%
Sulfamethazine (B)	0	95%	5%
	1.5 Min		100%
	3.0 Min		100%
	3.01 Min	95%	5%
	3.5 Min	95%	5%

Analyte	Gradient	(A) 0.1% Ammonium Hydroxyde in Water	(B) 0.1% Ammonium Hydroxyde in Methanol	(B) 0.1% Formic Acid in Methanol
Azithromycin	0	50%	30%	20%
	1.5 Min		80%	20%
	3.5 Min		80%	20%
	3.51 min	50%	30%	20%
	4.0	50%	30%	20%
Azithromycin (B)	0	70%	20%	10%
	1.5 Min		90%	10%
	3.5 Min		90%	10%
	3.51 min	70%	20%	10%
	4.0	70%	20%	10%

Microplate layout

Control BL	T1 BL	T2 BL	T3 BL	T4 BL	T5 BL	T6 BL	T7 BL	T8 BL	T9 BL	T10 BL	Control BL
Control BL	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	Control BL
Control BL	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	Control BL
Control BL	Control	Control	Control	Control	Control	Control	Control	Control	Control	Control	Control BL
Control BL	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	Control BL
Control BL	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	Control BL
Control BL	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	Control BL
Control BL	T1 BL	T2 BL	T3 BL	T4 BL	T5 BL	T6 BL	T7 BL	T8 BL	T9 BL	T10 BL	Control BL

Figure S.A1. A schematic plate map of the layout of replicates in a mirotitre plate. BL = blank (sterile medium only)

The concentration gradient runs across the length of the plate from columns 2 to 11, with cyanobacteria blanks in columns 1 and 12. The 10 control replicates also run across the length of the plate from D2 to D11 so that any effect in growth across the plate, caused by uneven environmental conditions in the incubator (e.g. light intensity) or by the contamination from surrounding wells (e.g. volatile compounds) can be identified and render the test invalid.

Five replicates of each antibiotic test concentration run in columns 2 to 11. The outer cells of the 96 well microplate had no cyanobacteria inoculum, creating sixteen control blanks and two blanks per concentration.

If a solvent control was required this was run in the blank wells in row H.

Dilution water control and solvent control comparisons

The growth rates of the bacteria in the dilution water control (DWC) and solvent control (SC) replicates (10 replicates each) were compared using a t test in R (version 3.3.0; R Project for Statistical Computing, Vienna, Austria; (Figure S.A1)).

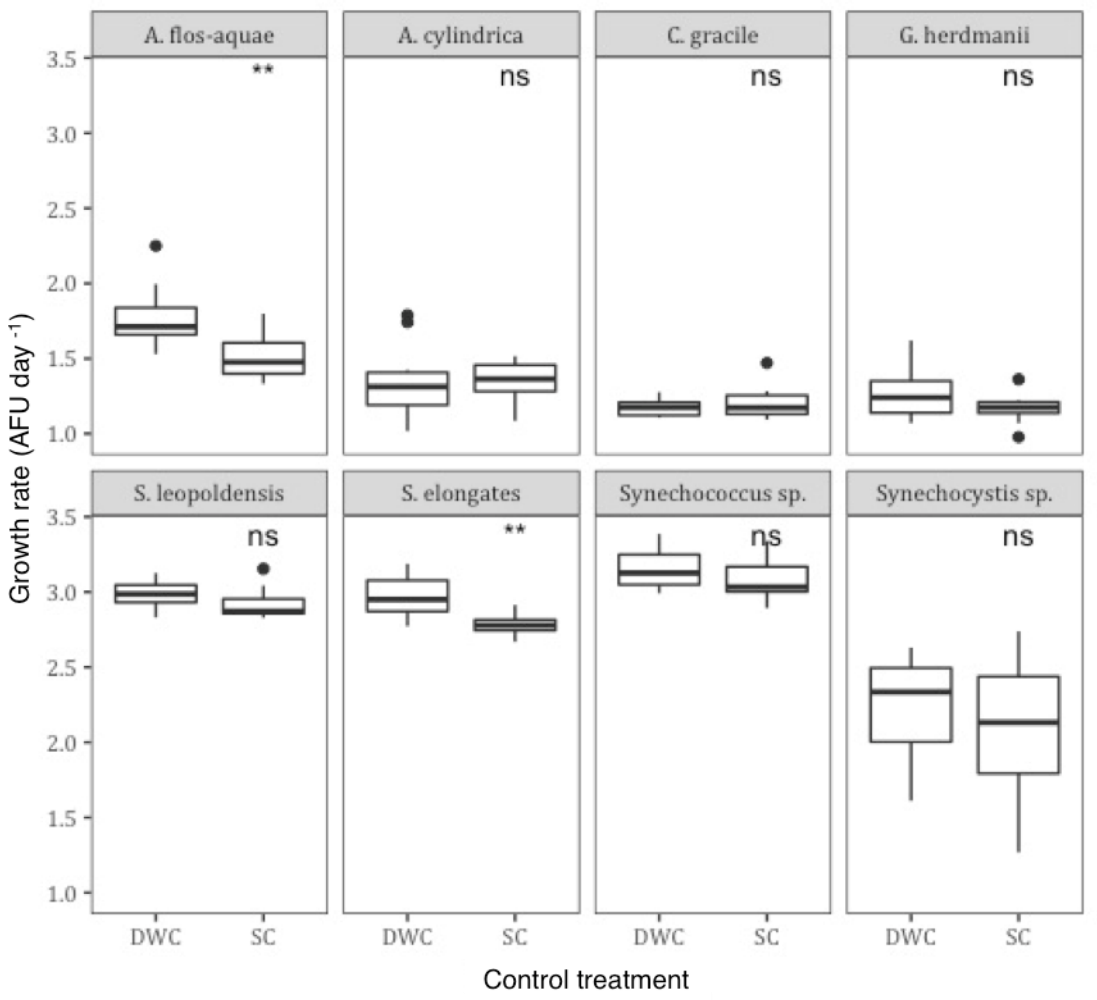

Figure S.A2 – Cyanobacteria growth rates in dilution water control (DWC) and solvent control (SC) medium. SC comprised of BG-11 with 10 µl/L of dimethyl sulfoxide (DMSO). ns = not significant. ** = $p < 0.01$.

Data handling and dose-response modelling

Dose response models were established using the using the drc package (Ritz *et al*. 2015) in r (version 3.3.0; R Project for Statistical Computing, Vienna, Austria).

Before establishing the model, the data was examined to identify poor replicates and exclude data where necessary according to the following criteria:

1. Where the growth rate cannot be calculated because the fluorescent signal at the start of the test (day 0) was a negative number due to the background/blank value being higher than the exposure well value; the replicates were excluded from further analysis.

2. Where the growth rate is negative because the fluorescence at the end of the test is less than that at the beginning; the growth rate was fixed at 0. N.B. Although the population size decreased (grew negatively) it is unknown from this fluorescence data if cells were alive or dead (with phycocyanin still fluorescing).

3. Where the growth rate cannot be calculated because the fluorescent signal at the end of the test was a negative number due to the background/blank value being higher than the exposure well value; the replicate was considered to have zero growth.

The optimal distributions for the data were selected based on log-likelihood score (distributions examined were 3, 4 or 5 parameter log-logistic distributions and 4, or 5 parameter weibull 1 or 2 distributions). Model residuals were examined for normality and heteroscedasticity and residuals with a disproportionate effect on the model were identified using cooks distance (>0.5 is considered to have a disproportionate effect). Any outliers were individually examined and excluded if biologically justifiable and the dose response model was then re-run. Replicates that did have a disproportionate effect on the model but without any reason to suggest an experimental error (e.g. not inoculated correctly) were included in the analysis rather than excluded as an outlier, even if statistically justifiable. This approach may result in wider confidence intervals and/or less statistical power but ensured inclusion of the inherent variability of the species and test designs

Example of the code used for each dose response curve:

```
library(drc)
library(dplyr)

# Make starter model
SpeciesX.m1<-drm(GR~Conc,
 data=Data,
 fct=LL.3(),
 na.action = na.omit)

# Identify best distribution of data for dose response curve:
mselect(CYAN.m1, list(LL.3(), LL.4(), LL.5(), W1.3(), W1.4(), W2.4()))

# Make best fitting model
SpeciesX.m1<-drm(GR~Conc,
 data= Data,
 fct=LL.4(),
 na.action = na.omit)

# Plot model
plot(SpeciesX.m1, xlab = bquote('Concentration ('*mu~'g/L)'), ylab = "Growth rate (per day)",
 main = "SpeciesX",
 broken = TRUE, type = "all",
 col = TRUE, legendPos = c(10,2), cex.legend = 0.5)

# Estimate EC50, EC20 and EC10

ED(SpeciesX.m1, c(10, 20, 50), interval = "delta")

# Summarise the model parameters:

summary(CYAN.m1)

# Graphical analysis of residuals – check for heteroscedasticity

plot(residuals(CYAN.m1) ~ fitted(CYAN.m1), main="Residuals vs Fitted") +
 abline(h=0)

# Graphical analysis of residuals – check for heteroscedasticity

qqnorm(residuals(CYAN.m1))
qqline(residuals(CYAN.m1))

# Identification of outliers – cooks distance of > 0.5 indicates data had a disproportionate
 effect on model

plot(cooks.distance(CYAN.m1))
```

Supplementary material B

Analytical results

Analytical results are presented as i) geometric means as a percentage of nominal and ii) the measured concentrations at the end of the exposure as a percentage of the stock solutions. Species are represented as follows: ANA CCAP = *A. flos-aquae*, ANA PCC = *A. cylindrical,* CYAN = *C. gracile*, GEM = *G. herdmanii*, SYNch CCAP = *S. leopoliensis*, SYNch PCC1 = *S. elongates*, SYNch PCC2 = *Synechococcus sp.* and SYNcy = *Synechocystis sp.*

Antibiotics measured in the dilution water control solutions at the end of the test:
Antibiotics were measured at >LOQ in some of the control solutions sampled at the end of the exposures (both cyanobacteria blank and exposure replicates). Since the stock solutions from which all microplates were prepared did not have measurable quantities of antibiotics this contamination is considered to be caused by operator error in the extraction process or from carryover in the mass spectrometer.

The numbers of replicates excluded across all eight species were as follows:

- For azithromycin one blank replicate
- For erythromycin one stock; five blank and six exposure replicates
- For ampicillin seven blank and seven exposure replicates

Analytical comments
In some cases the analytical data did not allow the accurate determination of the exposure concentrations and as such these concentrations were excluded from further analysis in order not to adversely affect the dose response curves. The details of these instances are given the table below.

Antibiotic	Species	Nominal concentration (µg/L)	Comment
Azithromycin	ANA PCC	0.43 and 0.77	Stock preparation error - removed from further dose response analysis
Azithromycin	ANA PCC, CYAN, GEM	1.4	Measured concentrations at the end of the exposure were below the LOQ - removed from further dose response analysis
Azithromycin	SYNch CCAP, SYNch PCC2	2.5	Measured concentrations at the end of the exposure were below the LOQ - removed from further dose response analysis
Azithromycin	SYNch CCAP, SYNch PCC1, SYNch PCC2	4.5	Measured concentrations at the end of the exposure were below the LOQ - removed from further dose response analysis
Erythromycin	All	1.7	Sampling/extraction error - removed from further dose response analysis
Erythromycin	All	6.6 and 407	Sampling/extraction error - removed from further dose response analysis
Sulfamethazine	SYNch PCC1	55556 and 100000	Sampling/extraction error - removed from further dose response analysis
Ampicillin	All	Across all concentrations	Many replicates measured as <LOQ - Where this occurred in blanks or in all 3 exposure replicates they removed from further dose response analysis - Where only 1 replicate of 3 was <LOQ the average of the remaining 2 replicates was used for dose response analysis. - For SYNcy nominal concentration of 165 µg/L there was high variability replicates was used for dose response analysis
Cefotaxime	All	0.46, 0.82 and 1.5	Measured concentrations at the end of the exposure were below the LOQ - removed from further dose response analysis

Table S.B1 - Analytical comments and concentrations excluded from further analysis. LOQ = limit of quantification

Antibiotic	LOQ (µg/L)
Cefazolin	0.12
Cefotaxime	0.2
Ampicillin	0.12
Sulfadiazine	1.0
Sulfamethazine	0.2
Azithromycin	0.25
Erythromycin	0.12

Table S.B2 – Antibiotic limits of quantification (LOQ)

Cefazolin

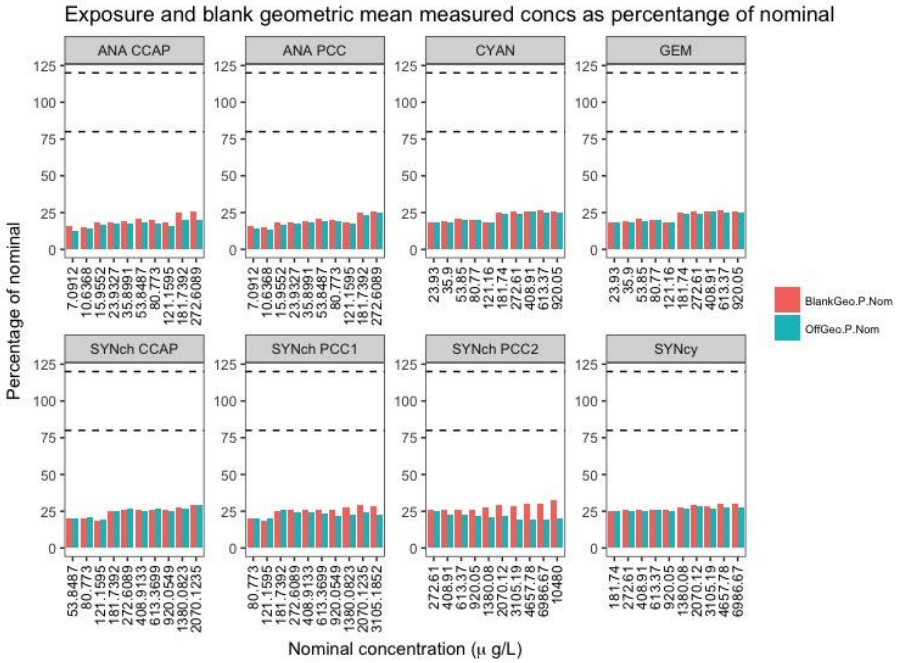

Figure S.B1 – Geometric mean measured concentrations of cefazolin as a percentage of nominal. Exposure (blue - with cyanobacteria) calculated by median average of three replicates. Blanks (red – without cyanobacteria) calculated by median average of up to three replicates. Dotted lines indicate 100 ± 20% of nominal.

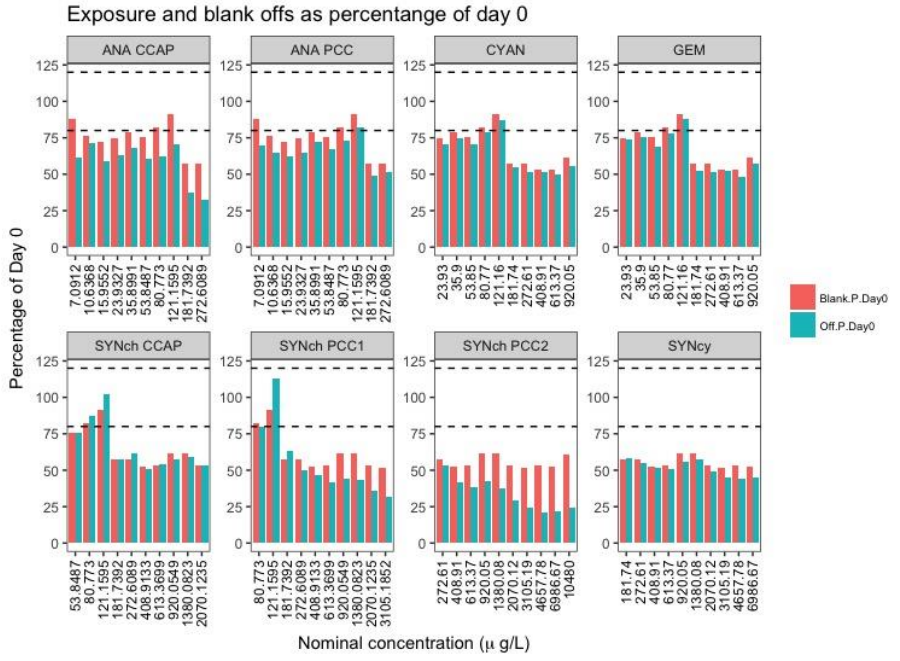

Figure S.B2 – Measured concentrations at the end of the exposure of cefazolin as a percentage of the stock solutions. Exposure (blue - with cyanobacteria) calculated by median average of three replicates. Blanks (red – without cyanobacteria) calculated by median average of up to three replicates. Dotted lines indicate 100 ± 20% of nominal.

Cefotaxime

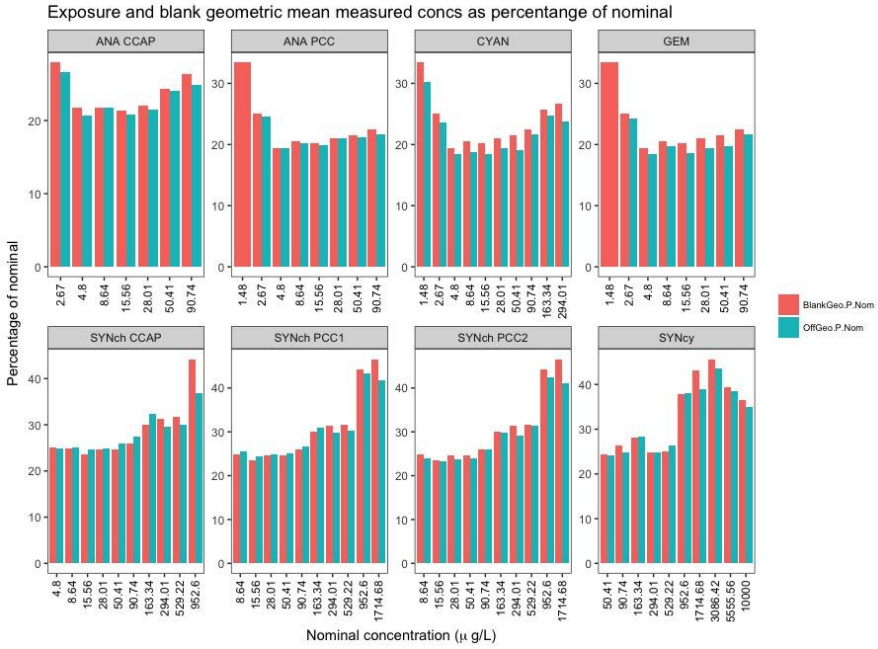

Figure S.B3 – Geometric mean measured concentrations of cefotaxime as a percentage of nominal. Exposure (blue - with cyanobacteria) calculated by median average of three replicates. Blanks (red – without cyanobacteria) calculated by median average of up to three replicates. Dotted lines indicate 100 ± 20% of nominal. Missing bars indicate replicates that were removed (see table S.B2).

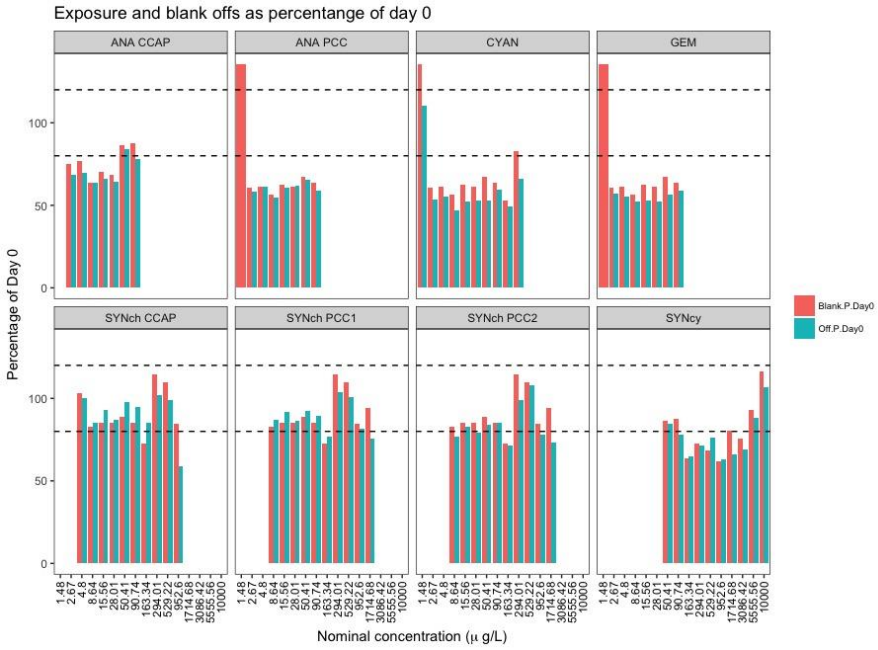

Figure S.B4 – Measured concentrations at the end of the exposure of cefotaxime as a percentage of the stock solutions. Exposure (blue - with cyanobacteria) calculated by median average of three replicates. Blanks (red – without cyanobacteria) calculated by median average of up to three replicates. Dotted lines indicate 100 ± 20% of nominal. Missing bars indicate replicates that were removed (see table S.B2).

Ampicillin

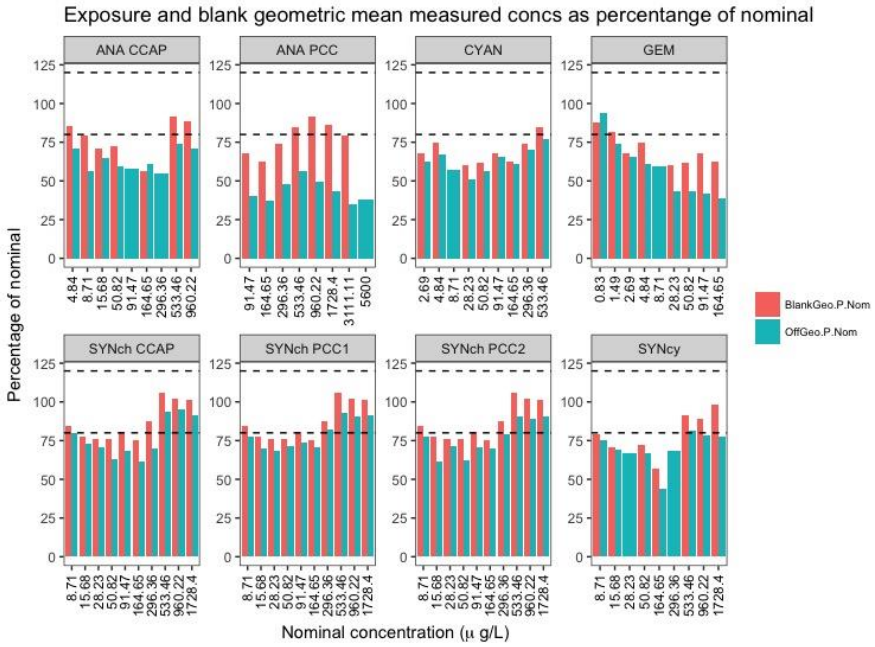

Figure S.B5 – Geometric mean measured concentrations of ampicillin as a percentage of nominal. Exposure (blue - with cyanobacteria) calculated by median average of three replicates. Blanks (red – without cyanobacteria) calculated by median average of up to three replicates. Dotted lines indicate 100 ± 20% of nominal. Missing bars indicate replicates that were removed (see table S.B2).

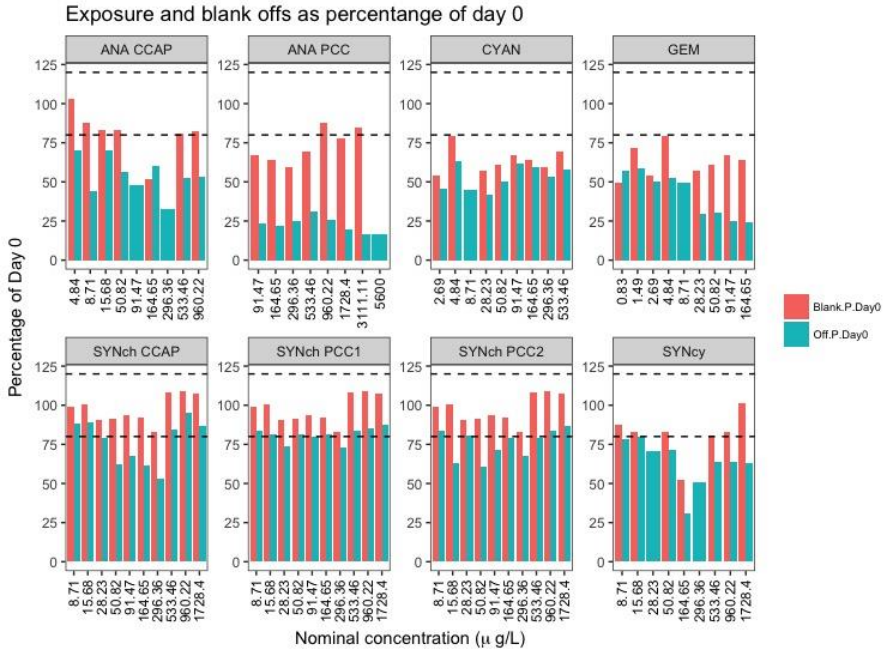

Figure S.B6 – Measured concentrations at the end of the exposure of ampicillin as a percentage of the stock solutions. Exposure (blue - with cyanobacteria) calculated by median average of three replicates. Blanks (red – without cyanobacteria) calculated by median average of up to three replicates. Dotted lines indicate 100 ± 20% of nominal. Missing bars indicate replicates that were removed (see table S.B2).

Sulfadiazine

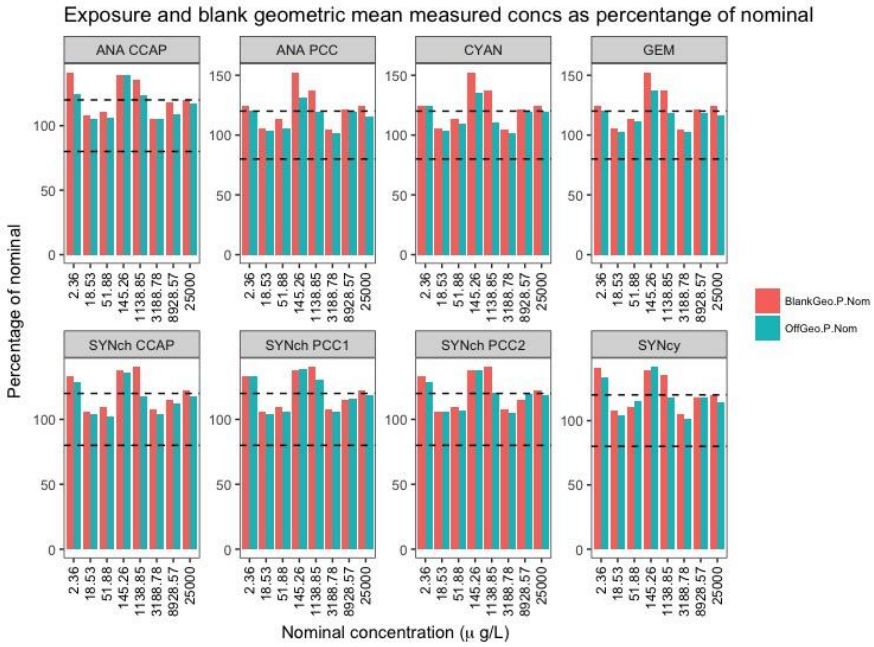

Figure S.B7 – Geometric mean measured concentrations of sulfadiazine as a percentage of nominal. Exposure (blue - with cyanobacteria) calculated by median average of three replicates. Blanks (red – without cyanobacteria) calculated by median average of up to three replicates. Dotted lines indicate 100 ± 20% of nominal.

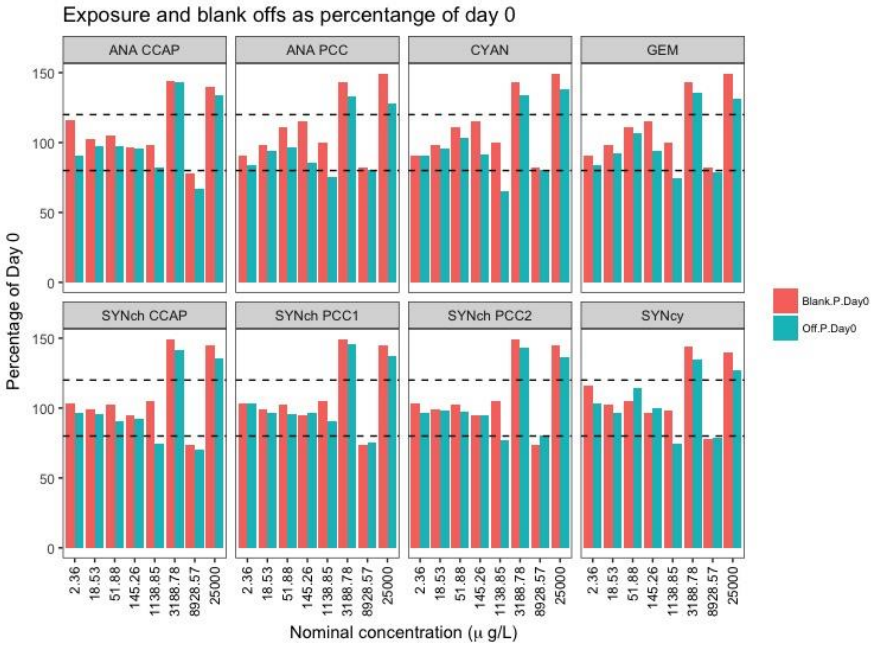

Figure S.B8 – Measured concentrations at the end of the exposure of sulfadiazine as a percentage of the stock solutions. Exposure (blue - with cyanobacteria) calculated by median average of three replicates. Blanks (red – without cyanobacteria) calculated by median average of up to three replicates. Dotted lines indicate 100 ± 20% of nominal.

Sulfamethazine

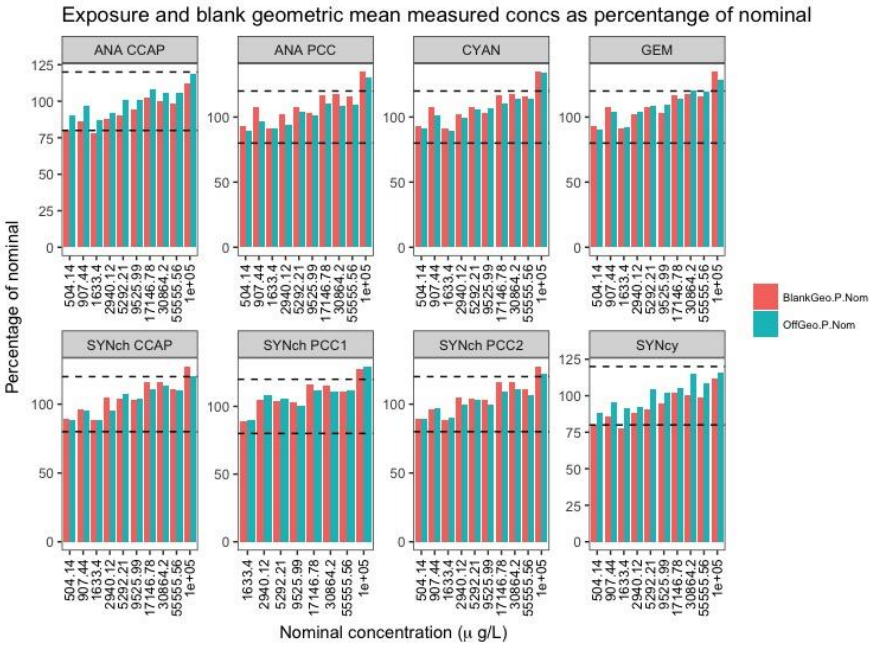

Figure S.B9 – Geometric mean measured concentrations of sulfamethazine as a percentage of nominal. Exposure (blue - with cyanobacteria) calculated by median average of three replicates. Blanks (red – without cyanobacteria) calculated by median average of up to three replicates. Dotted lines indicate 100 ± 20% of nominal.

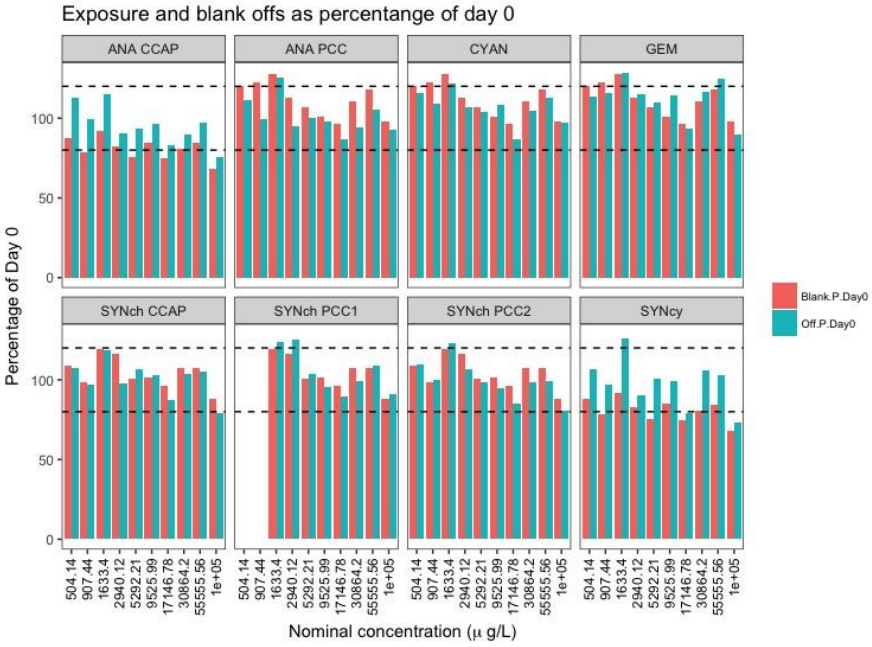

Figure S.B10 – Measured concentrations at the end of the exposure of sulfamethazine as a percentage of the stock solutions. Exposure (blue - with cyanobacteria) calculated by median average of three replicates. Blanks (red – without cyanobacteria) calculated by median average of up to three replicates. Dotted lines indicate 100 ± 20% of nominal. Missing bars indicate replicates that were removed (see table S.B2).

Erythromycin

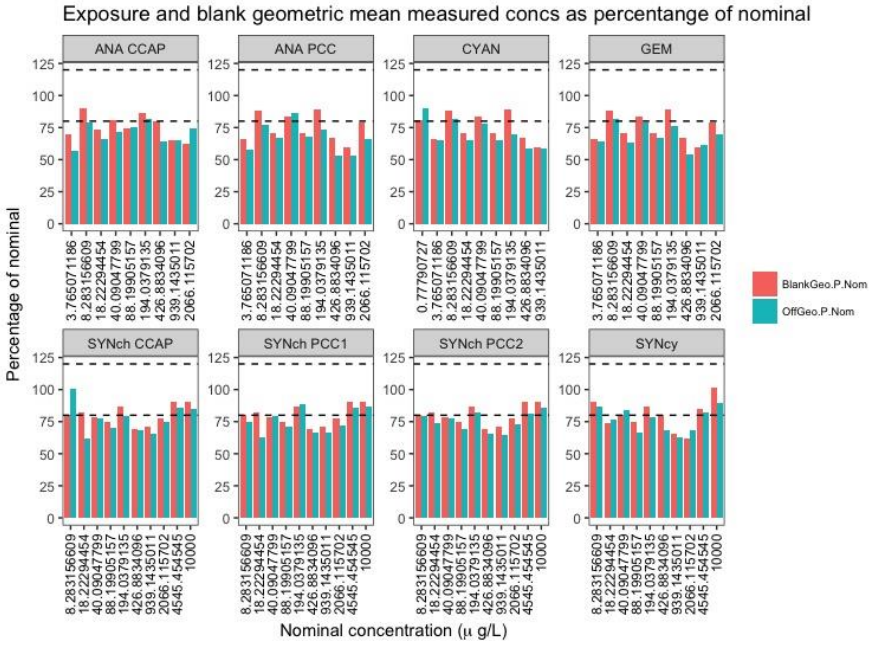

Figure S.B11 – Geometric mean measured concentrations of erythromycin as a percentage of nominal. Exposure (blue - with cyanobacteria) calculated by median average of three replicates. Blanks (red – without cyanobacteria) calculated by median average of up to three replicates. Dotted lines indicate 100 ± 20% of nominal.

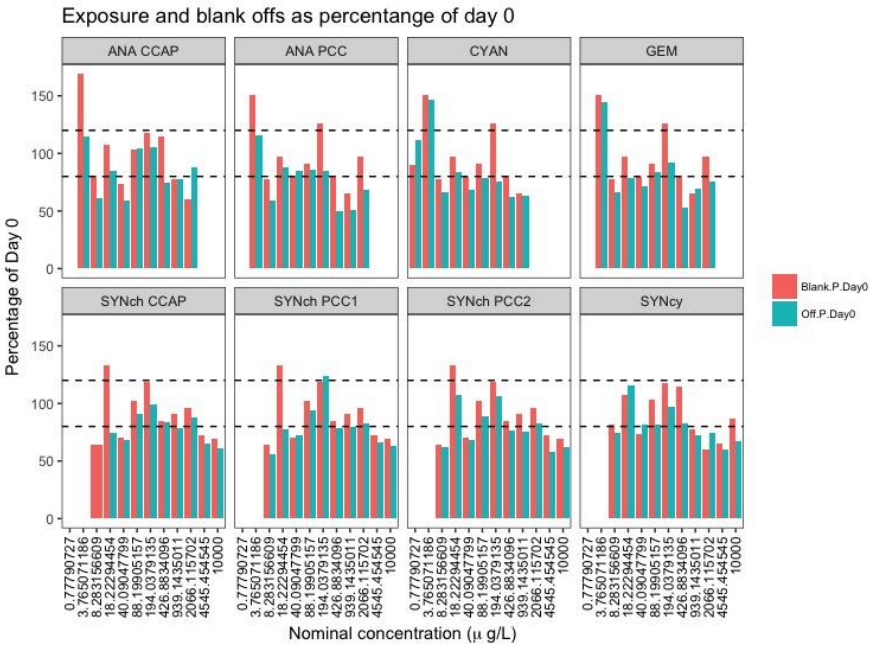

Figure S.B12 – Measured concentrations at the end of the exposure of erythromycin as a percentage of the stock solutions. Exposure (blue - with cyanobacteria) calculated by median average of three replicates. Blanks (red – without cyanobacteria) calculated by median average of up to three replicates. Dotted lines indicate 100 ± 20% of nominal.

Azithromycin

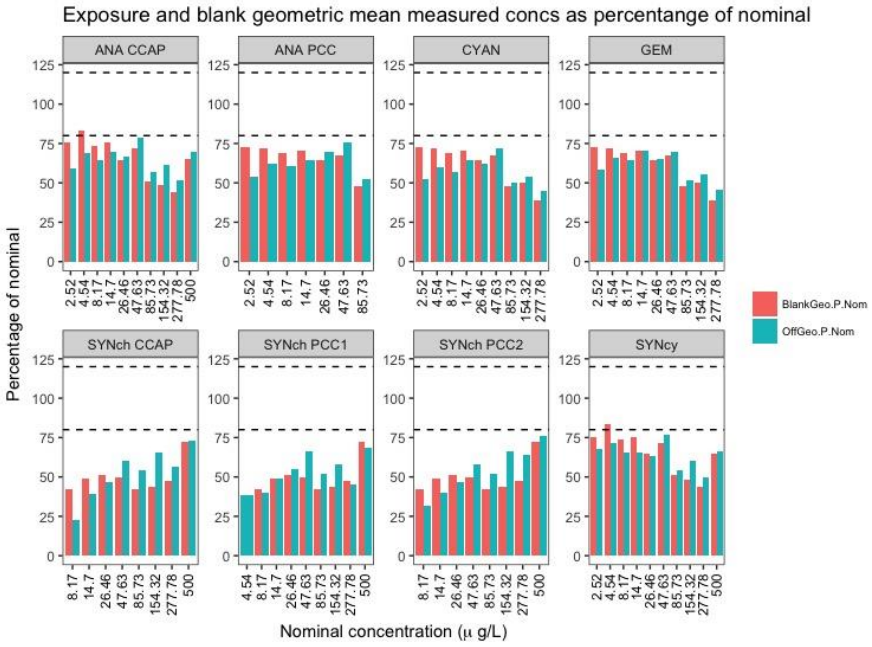

Figure S.B13 – Geometric mean measured concentrations of azithromycin as a percentage of nominal. Exposure (blue - with cyanobacteria) calculated by median average of three replicates. Blanks (red – without cyanobacteria) calculated by median average of up to three replicates. Dotted lines indicate 100 ± 20% of nominal. Missing bars indicate replicates that were removed (see table S.B2).

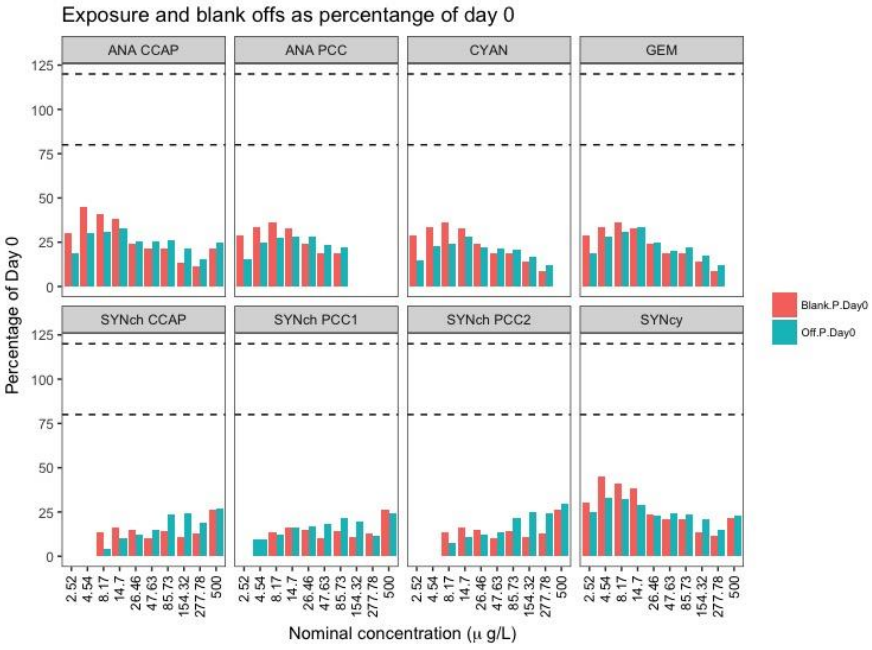

Figure S.B14 – Measured concentrations at the end of the exposure of azithromycin as a percentage of the stock solutions. Exposure (blue - with cyanobacteria) calculated by median average of three replicates. Blanks (red – without cyanobacteria) calculated by median average of up to three replicates. Dotted lines indicate 100 ± 20% of nominal. Missing bars indicate replicates that were removed (see table S.B2).

Graphs showing the percentage loss due to the presence of cyanobacteria

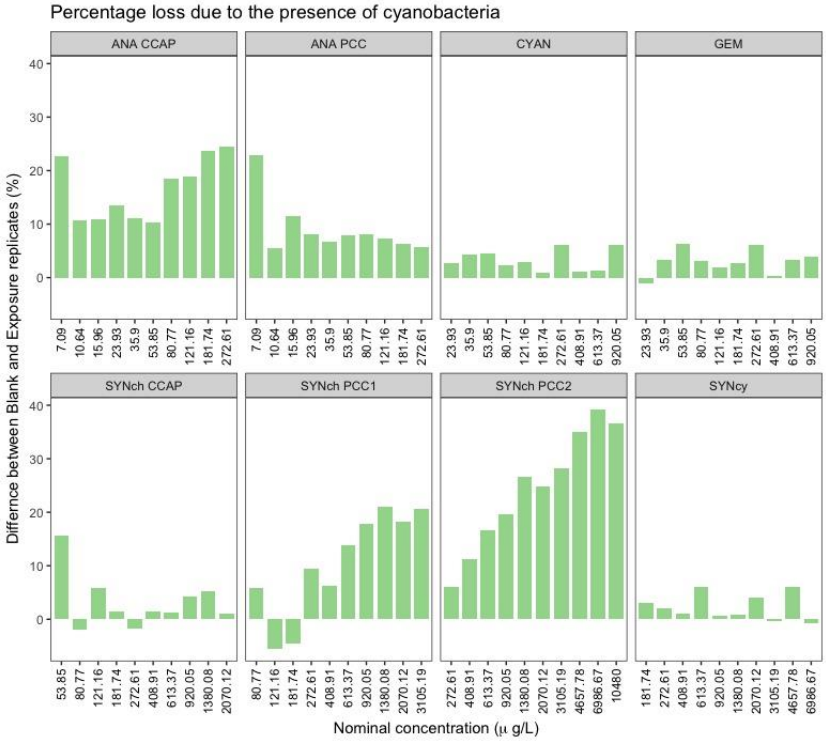

Figure S.B15 – Percentage loss of cefazolin due the presence of cyanobacteria. Y-axis calculated as the difference in percentages of the blank replicates (sterile culture medium with no cyanobacteria) and exposure replicates (with cyanobacteria).

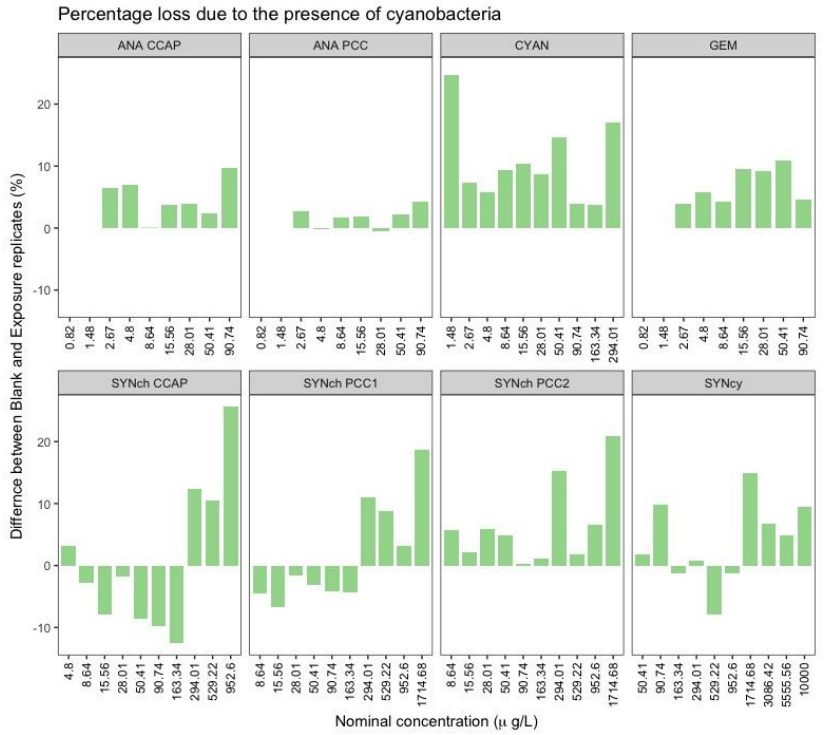

Figure S.B16 –Percentage loss of cefotaxime due the presence of cyanobacteria. Y-axis calculated as the difference in percentages of the blank replicates (sterile culture medium with no cyanobacteria) and exposure replicates (with cyanobacteria).

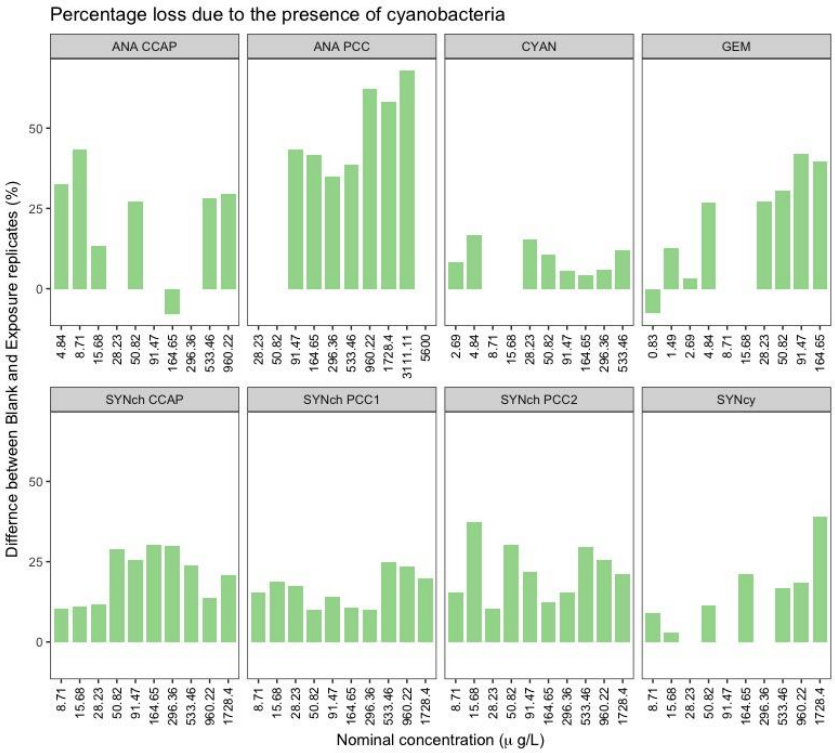

Figure S.B17 –Percentage loss of ampicillin due the presence of cyanobacteria. Y-axis calculated as the difference in percentages of the blank replicates (sterile culture medium with no cyanobacteria) and exposure replicates (with cyanobacteria).

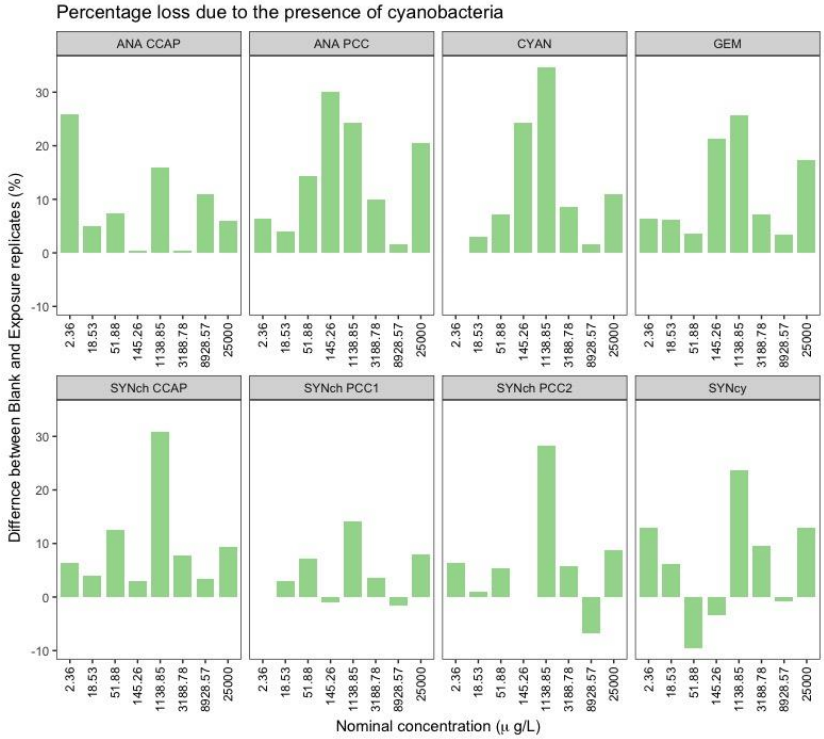

Figure S.B18 –Percentage loss of sulfadiazine due the presence of cyanobacteria. Y-axis calculated as the difference in percentages of the blank replicates (sterile culture medium with no cyanobacteria) and exposure replicates (with cyanobacteria).

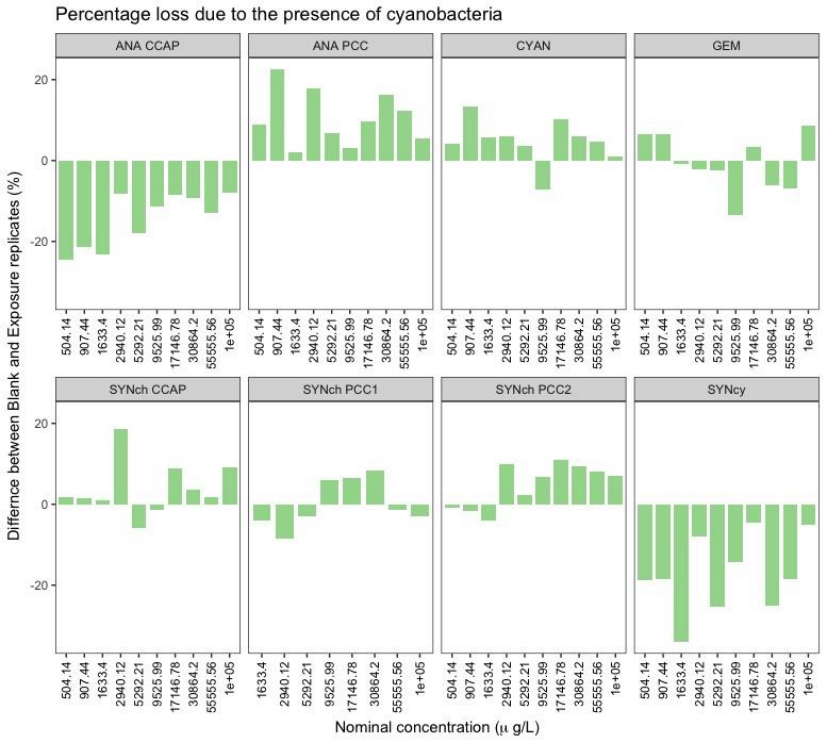

Figure S.B19 – Percentage loss of sulfamethazine due the presence of cyanobacteria. Y-axis calculated as the difference in percentages of the blank replicates (sterile culture medium with no cyanobacteria) and exposure replicates (with cyanobacteria).

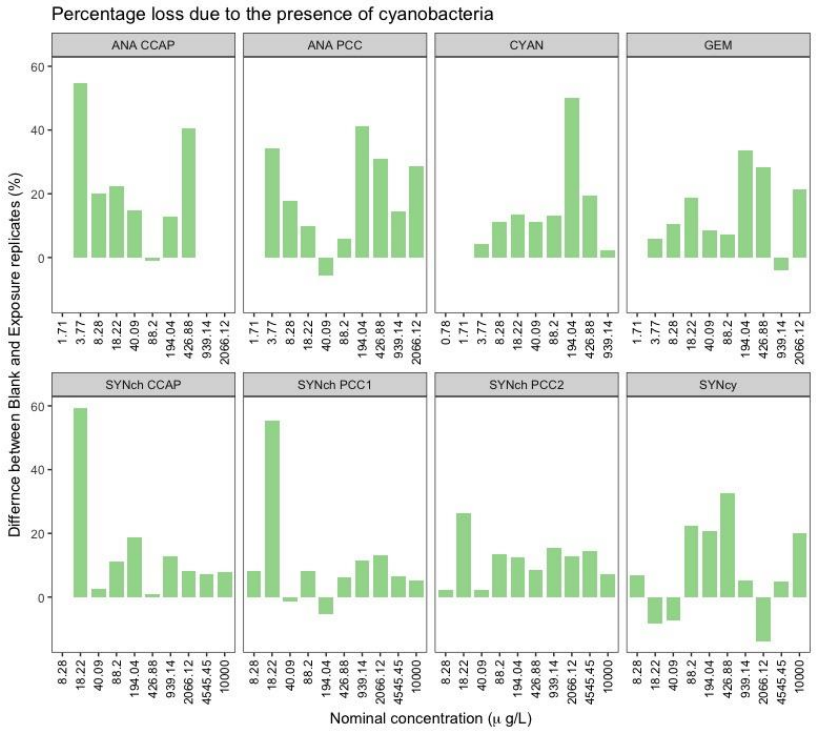

Figure S.B20 –Percentage loss of erythromycin due the presence of cyanobacteria. Y-axis calculated as the difference in percentages of the blank replicates (sterile culture medium with no cyanobacteria) and exposure replicates (with cyanobacteria).

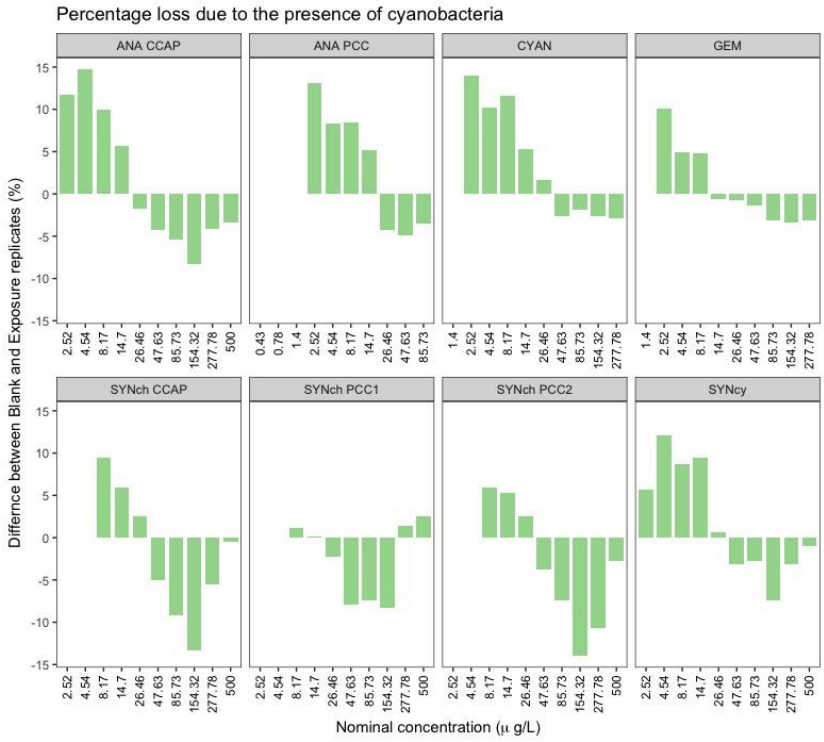

Figure S.B21 –Percentage loss of azithromycin due the presence of cyanobacteria. Y-axis calculated as the difference in percentages of the blank replicates (sterile culture medium with no cyanobacteria) and exposure replicates (with cyanobacteria).

Supplementary material C

Growth inhibition raw data

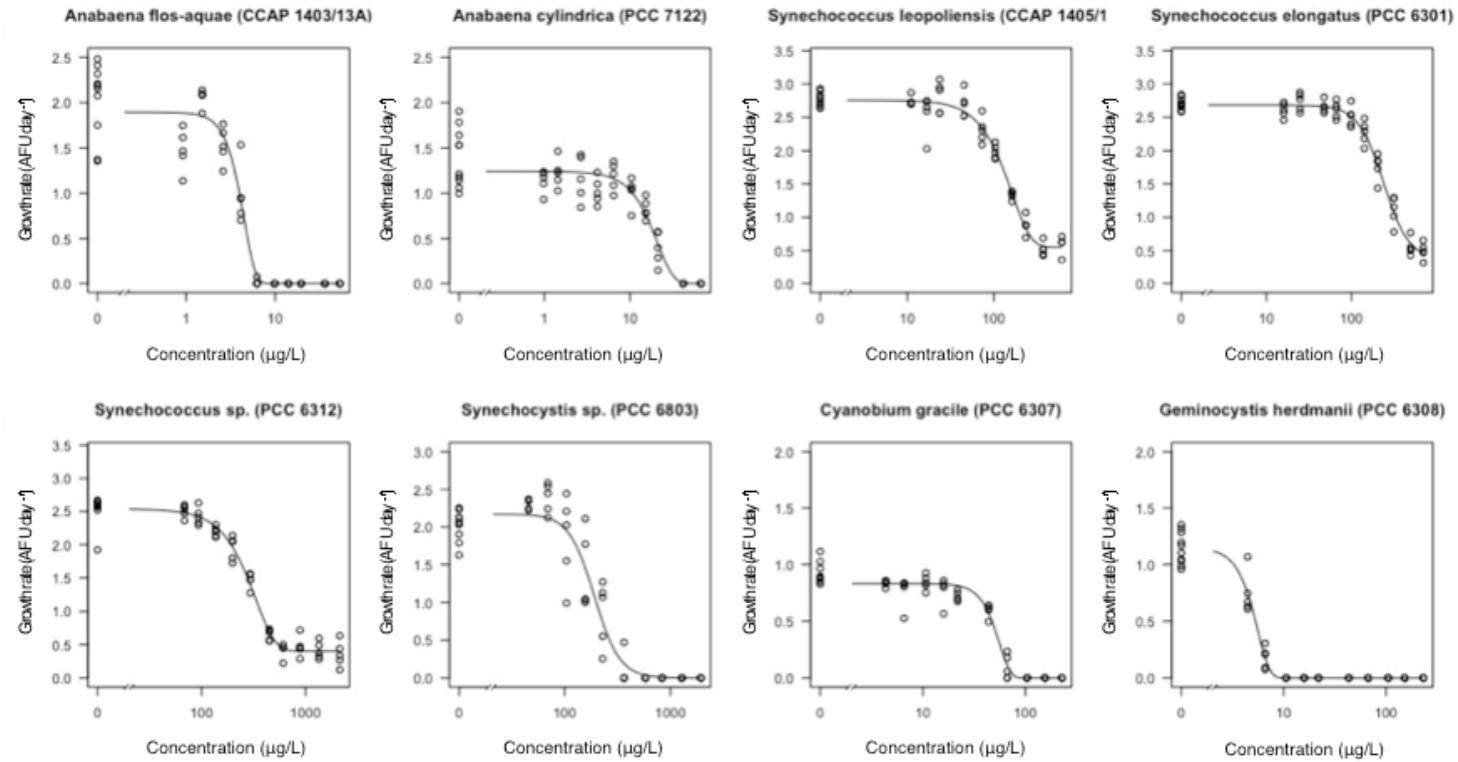

Figure S.C1 - Growth inhibition of cyanobacteria exposed to cefazolin including the raw data for each replicate.

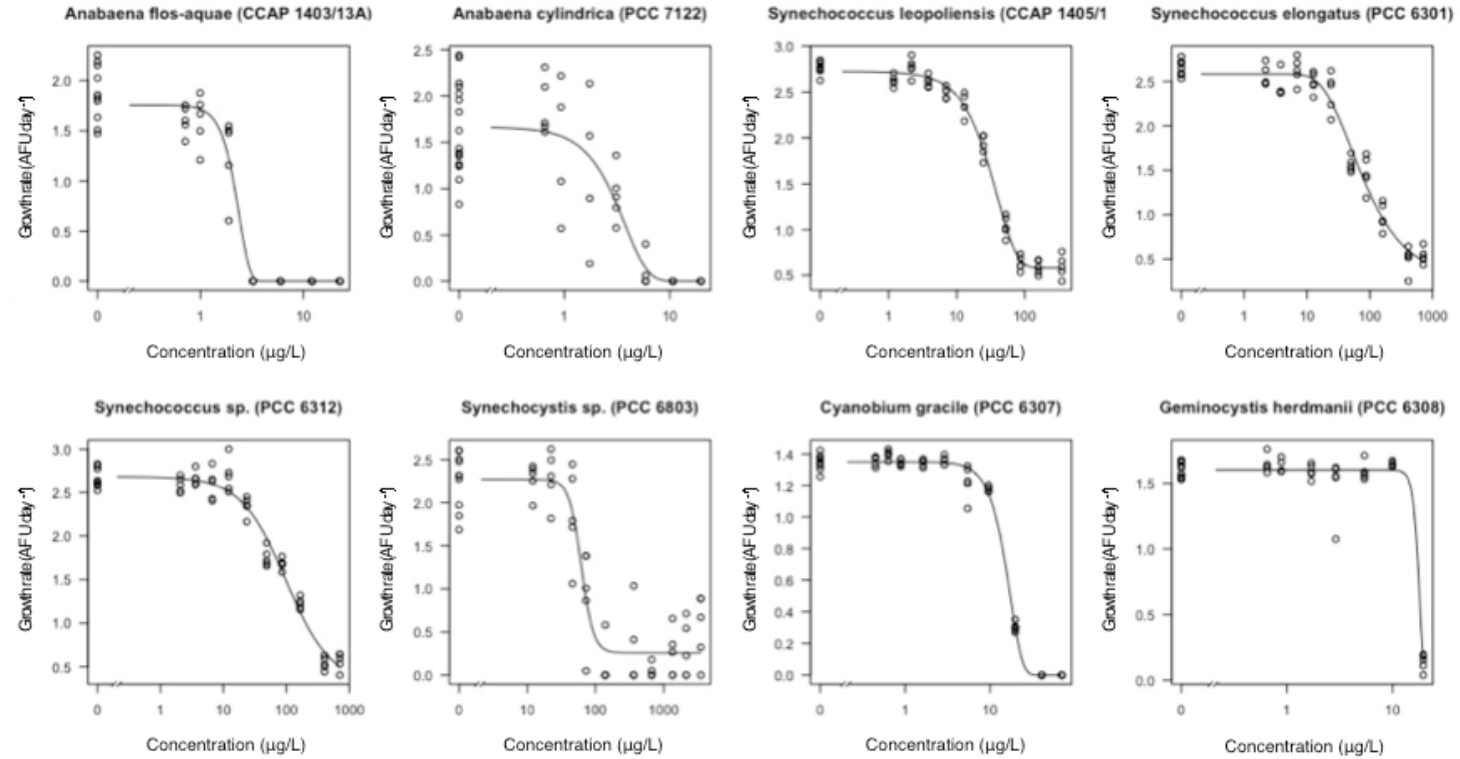

Figure S.C2 - Growth inhibition of cyanobacteria exposed to cefotaxime including the raw data for each replicate.

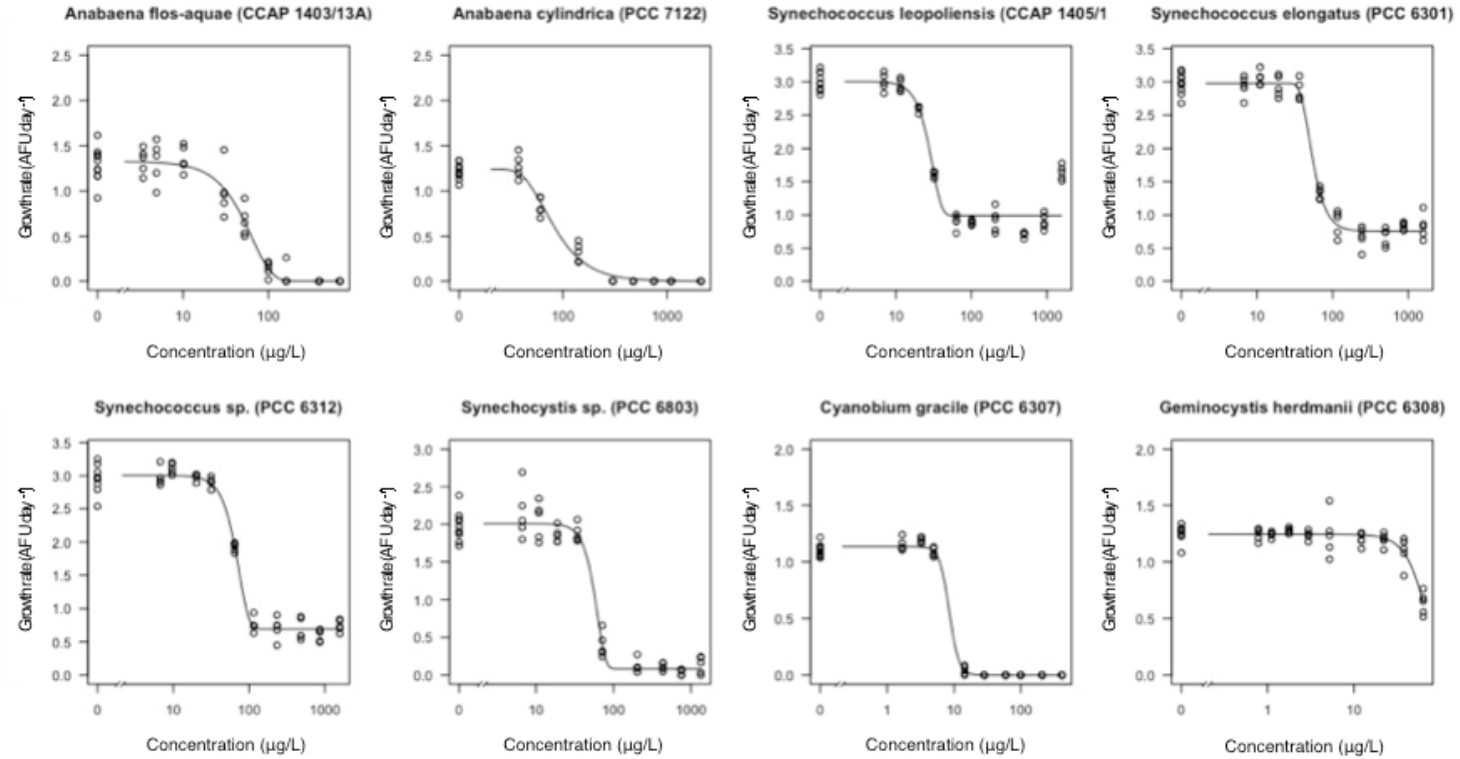

Figure S.C3 - Growth inhibition of cyanobacteria exposed to ampicillin including the raw data for each replicate.

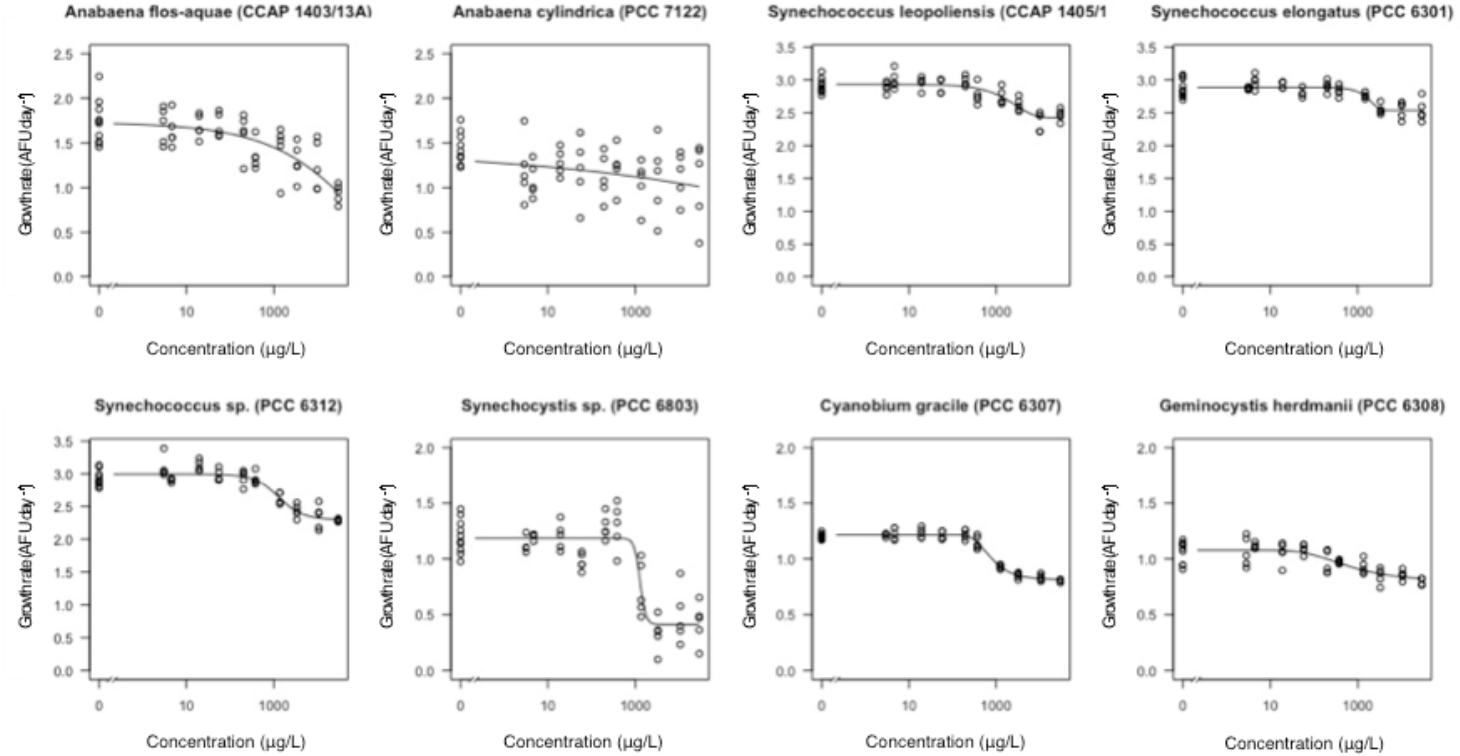

Figure S.C4 - Growth inhibition of cyanobacteria exposed to sulfadiazine including the raw data for each replicate.

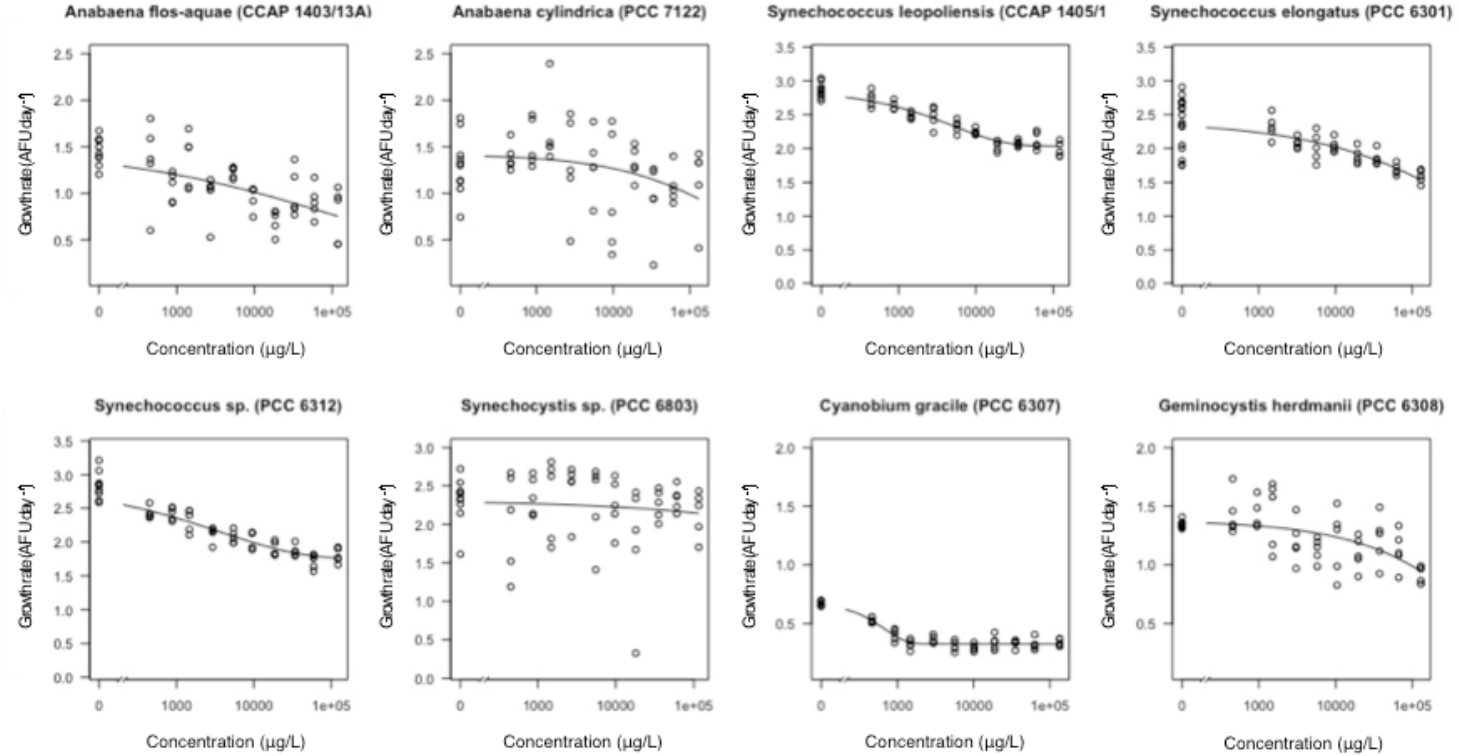

Figure S.C5 - Growth inhibition of cyanobacteria exposed to sulfamethazine including the raw data for each replicate.

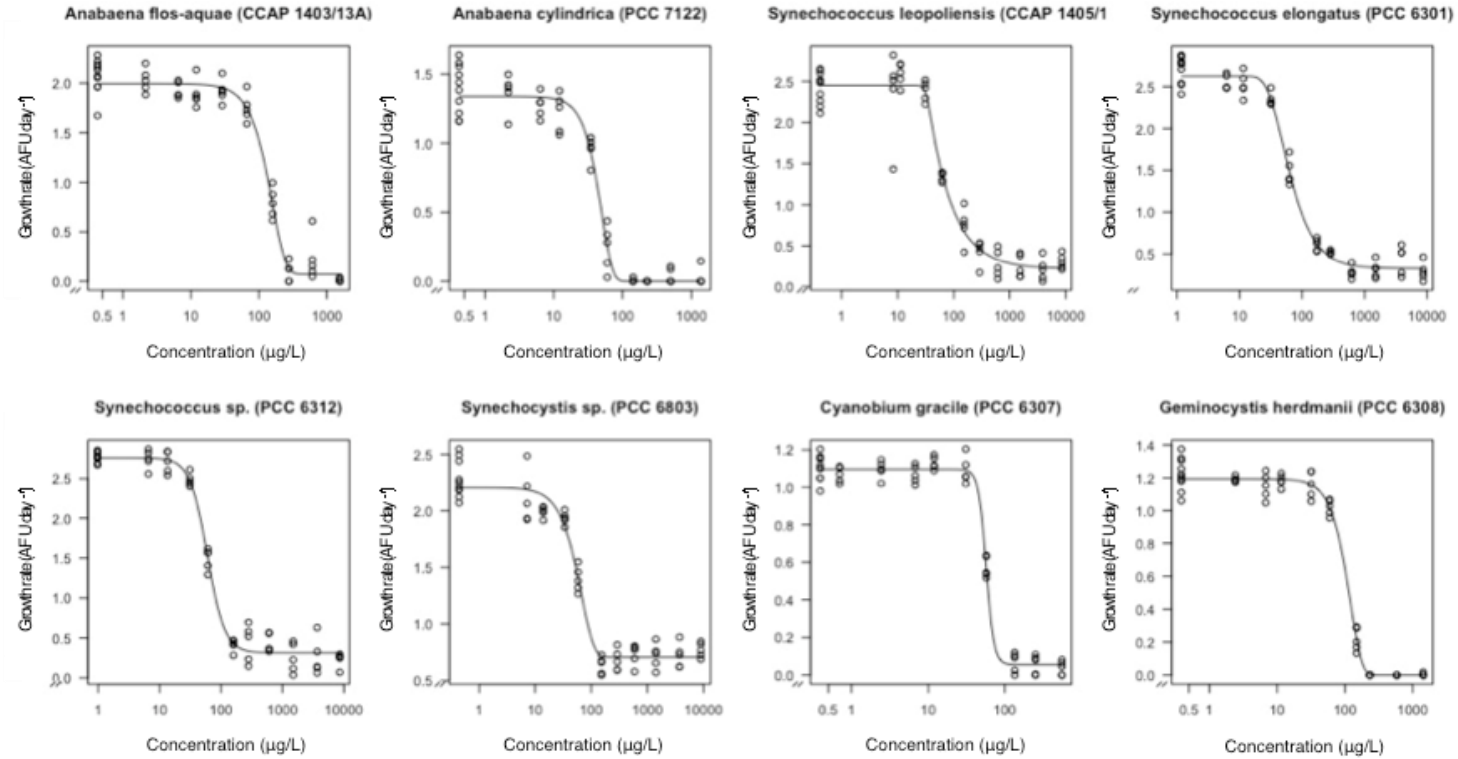

Figure S.C6 - Growth inhibition of cyanobacteria exposed to erythromycin including the raw data for each replicate.

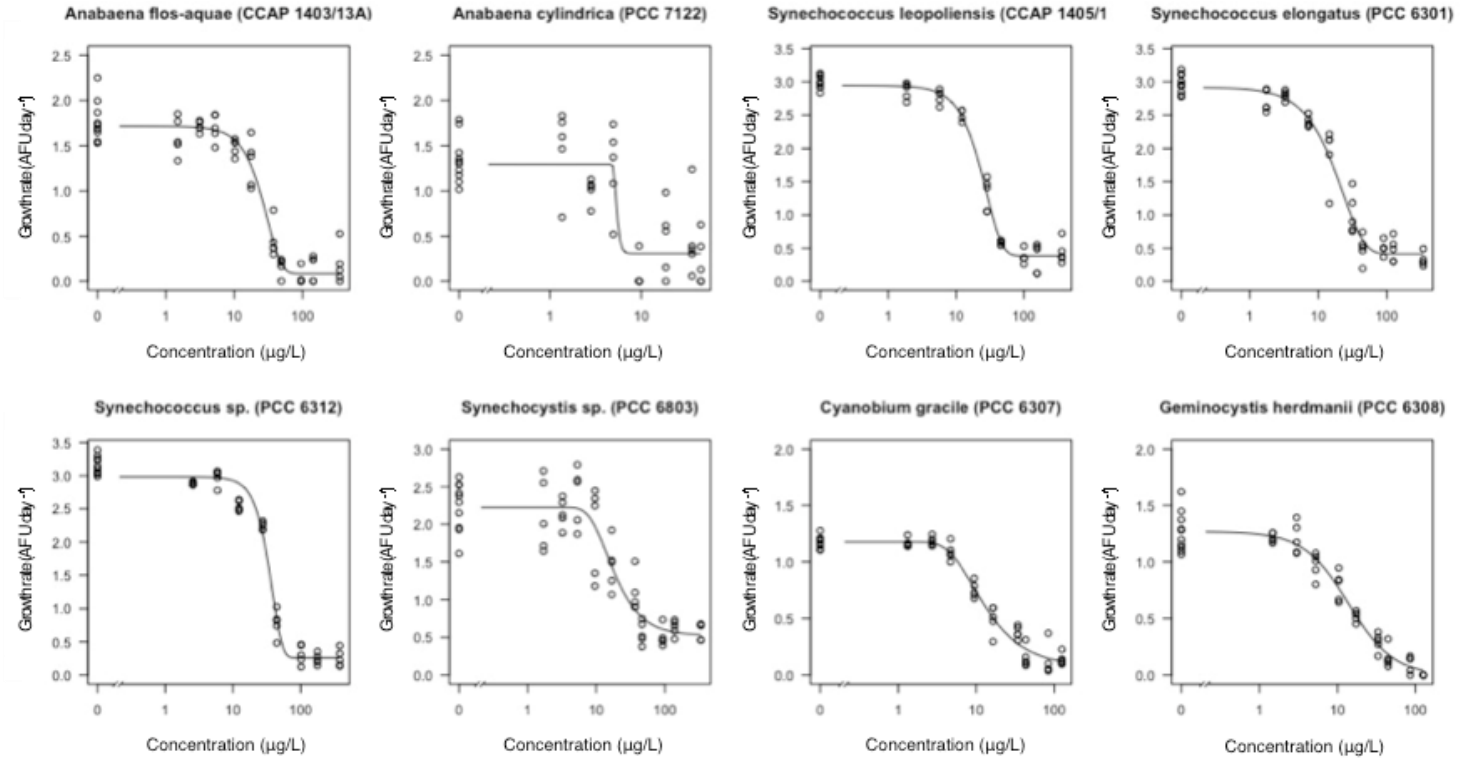

Figure S.C7 - Growth inhibition of cyanobacteria exposed to azithromycin including the raw data for each replicate.

Supplementary material D

pH measurements

Antibiotic	Species	Test concentration (mg/L)	Starting pH	Final pH	pH change
Ampicillin	*A. flos-aquae*	DWC	8.0	8.0	0.0
		0.96	8.0	7.9	-0.1
		0.53	8.0	7.9	-0.1
		0.30	8.0	7.9	-0.1
		0.16	8.0	7.9	-0.1
		0.091	8.0	7.9	-0.1
		0.051	8.0	7.9	-0.1
		0.028	8.0	8.0	0.0
		0.016	8.0	8.0	0.0
		0.0087	8.0	8.0	0.0
		0.0048	8.0	8.0	0.0
	A. cylindrica	DWC	8.0	8.1	0.1
		5.60	8.0	8.0	0.0
		3.11	8.0	7.9	-0.1
		1.73	8.0	7.9	-0.1
		0.96	8.0	7.9	-0.1
		0.533	8.0	8.0	0.0
		0.296	8.0	8.0	0.0
		0.165	8.0	8.0	0.0
		0.091	8.0	8.1	0.1
		0.0508	8.0	8.1	0.1
		0.0282	8.0	8.1	0.1
	S. leopoliensis	DWC	8.0	8.1	0.1
		1.73	8.0	8.0	0.0
		0.96	8.0	8.0	0.0
		0.53	8.0	8.0	0.0
		0.30	8.0	8.0	0.0
		0.165	8.0	8.0	0.0
		0.091	8.0	8.0	0.0
		0.051	8.0	8.0	0.0
		0.028	8.0	8.0	0.0
		0.0157	8.0	8.0	0.0
		0.0087	8.0	8.0	0.0
	S. elongates	DWC	8.0	8.1	0.1
		1.73	8.0	8.0	0.0
		0.96	8.0	8.0	0.0
		0.53	8.0	8.0	0.0
		0.30	8.0	8.0	0.0
		0.165	8.0	8.0	0.0
		0.091	8.0	8.0	0.0
		0.051	8.0	8.0	0.0
		0.028	8.0	8.0	0.0
		0.0157	8.0	8.0	0.0
		0.0087	8.0	8.0	0.0

Antibiotic	Species	Test concentration (mg/L)	Starting pH	Final pH	pH change
Ampicillin	*Synechococcus sp.*	DWC	8.0	8.1	0.1
		1.73	8.0	8.0	0.0
		0.96	8.0	8.0	0.0
		0.53	8.0	8.0	0.0
		0.30	8.0	8.0	0.0
		0.165	8.0	8.0	0.0
		0.091	8.0	8.0	0.0
		0.051	8.0	8.0	0.0
		0.028	8.0	8.0	0.0
		0.0157	8.0	8.0	0.0
		0.0087	8.0	8.0	0.0
	Synechocystis sp.	DWC	8.0	8.1	0.1
		1.73	8.0	7.9	-0.1
		0.96	8.0	7.9	-0.1
		0.53	8.0	7.9	-0.1
		0.30	8.0	7.9	-0.1
		0.165	8.0	7.9	-0.1
		0.091	8.0	7.9	-0.1
		0.051	8.0	8.0	0.0
		0.028	8.0	8.1	0.1
		0.0157	8.0	8.1	0.1
		0.0087	8.0	8.1	0.1
	C. gracile	DWC	8.0	8.2	0.2
		0.53	8.0	8.0	0.0
		0.30	8.0	8.0	0.0
		0.16	8.0	8.0	0.0
		0.09	8.0	8.0	0.0
		0.051	8.0	8.0	0.0
		0.028	8.0	8.0	0.0
		0.016	8.0	8.0	0.0
		0.009	8.0	8.1	0.1
		0.0048	8.0	8.1	0.1
		0.0027	8.0	8.1	0.1
	G. herdmanii	DWC	8.0	8.1	0.1
		0.16	8.0	8.0	0.0
		0.09	8.0	8.0	0.0
		0.05	8.0	8.1	0.1
		0.03	8.0	8.1	0.1
		0.016	8.0	8.1	0.1
		0.009	8.0	8.1	0.1
		0.005	8.0	8.1	0.1
		0.003	8.0	8.1	0.1
		0.0015	8.0	8.1	0.1
		0.0008	8.0	8.1	0.1

Antibiotic	Species	Test concentration (mg/L)	Starting pH	Final pH	pH change
Cefotaxime	*A. flos-aquae*	DWC	8.3	8.3	0.0
		0.09	8.3	8.1	-0.2
		0.05	8.3	8.0	-0.3
		0.03	8.3	8.1	-0.2
		0.02	8.3	8.1	-0.2
		0.009	8.3	8.2	-0.1
		0.005	8.3	8.3	0.0
		0.003	8.3	8.3	0.0
		0.001	8.3	8.3	0.0
		0.0008	8.3	8.3	0.0
		0.0005	8.3	8.3	0.0
	A. cylindrica	DWC	8.3	8.2	-0.1
		0.09	8.3	8.1	-0.2
		0.05	8.3	8.1	-0.2
		0.03	8.3	8.1	-0.2
		0.02	8.3	8.1	-0.2
		0.009	8.3	8.1	-0.2
		0.005	8.3	8.2	-0.1
		0.003	8.3	8.2	-0.1
		0.001	8.3	8.1	-0.2
		0.0008	8.3	8.1	-0.2
		0.0005	8.3	8.2	-0.1
	S. leopoliensis	DWC	8.3	8.4	0.1
		0.95	8.3	8.2	-0.1
		0.53	8.3	8.2	-0.1
		0.29	8.3	8.3	0.0
		0.16	8.3	8.2	-0.1
		0.091	8.3	8.2	-0.1
		0.050	8.3	8.3	0.0
		0.028	8.3	8.4	0.1
		0.016	8.3	8.5	0.2
		0.0086	8.3	8.4	0.1
		0.0048	8.3	8.5	0.2
	S. elongates	DWC	8.3	8.4	0.1
		1.71	8.3	8.0	-0.3
		0.95	8.3	8.1	-0.2
		0.53	8.3	8.1	-0.2
		0.29	8.3	8.1	-0.2
		0.163	8.3	8.0	-0.3
		0.091	8.3	8.1	-0.2
		0.050	8.3	8.3	0.0
		0.028	8.3	8.4	0.1
		0.0156	8.3	8.4	0.1
		0.0086	8.3	8.4	0.1

Antibiotic	Species	Test concentration (mg/L)	Starting pH	Final pH	pH change
Cefotaxime	*Synechococcus sp.*	DWC	8.3	8.5	0.2
		1.71	8.3	8.0	-0.3
		0.95	8.3	8.1	-0.2
		0.53	8.3	8.2	-0.1
		0.29	8.3	8.2	-0.1
		0.163	8.3	8.2	-0.1
		0.091	8.3	8.2	-0.1
		0.050	8.3	8.4	0.1
		0.028	8.3	8.4	0.1
		0.0156	8.3	8.5	0.2
		0.0086	8.3	8.5	0.2
	Synechocystis sp.	DWC	8.3	8.4	0.1
		10.00	8.3	8.1	-0.2
		5.56	8.3	8.2	-0.1
		3.09	8.3	8.2	-0.1
		1.71	8.3	8.1	-0.2
		0.953	8.3	8.1	-0.2
		0.529	8.3	8.1	-0.2
		0.294	8.3	8.1	-0.2
		0.163	8.3	8.2	-0.1
		0.0907	8.3	8.3	0.0
		0.0504	8.3	8.4	0.1
	C. gracile	DWC	8.3	8.5	0.2
		0.29	8.3	8.1	-0.2
		0.16	8.3	8.1	-0.2
		0.09	8.3	8.2	-0.1
		0.05	8.3	8.4	0.1
		0.028	8.3	8.2	-0.1
		0.016	8.3	8.5	0.2
		0.009	8.3	8.5	0.2
		0.005	8.3	8.5	0.2
		0.0027	8.3	8.6	0.3
		0.0015	8.3	8.5	0.2
	G. herdmanii	DWC	8.3	8.4	0.1
		0.09	8.3	8.1	-0.2
		0.05	8.3	8.5	0.2
		0.03	8.3	8.5	0.2
		0.02	8.3	8.5	0.2
		0.009	8.3	8.5	0.2
		0.005	8.3	8.5	0.2
		0.003	8.3	8.5	0.2
		0.001	8.3	8.5	0.2
		0.0008	8.3	8.5	0.2
		0.0005	8.3	8.5	0.2

Antibiotic	Species	Test concentration (mg/L)	Starting pH	Final pH	pH change
Cefazolin	*A. flos-aquae*	DWC	8.1	8.1	0.0
		0.27	8.1	8.1	0.0
		0.18	8.1	8.2	0.1
		0.12	8.1	8.1	0.0
		0.08	8.1	8.2	0.1
		0.054	8.1	8.2	0.1
		0.036	8.1	8.2	0.1
		0.024	8.1	8.2	0.1
		0.016	8.1	8.2	0.1
		0.0106	8.1	8.2	0.1
		0.0071	8.1	8.1	0.0
	A. cylindrica	DWC	8.1	8.1	0.0
		0.27	8.1	8.2	0.1
		0.18	8.1	8.2	0.1
		0.12	8.1	8.2	0.1
		0.08	8.1	8.2	0.1
		0.054	8.1	8.2	0.1
		0.036	8.1	8.1	0.0
		0.024	8.1	8.1	0.0
		0.016	8.1	8.1	0.0
		0.0106	8.1	8.1	0.0
		0.0071	8.1	8.1	0.0
	S. leopoliensis	DWC	8.1	8.4	0.3
		2.07	8.1	8.4	0.3
		1.38	8.1	8.4	0.3
		0.92	8.1	8.4	0.3
		0.61	8.1	8.4	0.3
		0.409	8.1	8.4	0.3
		0.273	8.1	8.4	0.3
		0.182	8.1	8.4	0.3
		0.121	8.1	8.4	0.3
		0.0808	8.1	8.4	0.3
		0.0538	8.1	8.4	0.3
	S. elongates	DWC	8.1	8.3	0.2
		3.11	8.1	8.3	0.2
		2.07	8.1	8.3	0.2
		1.38	8.1	8.3	0.2
		0.92	8.1	8.3	0.2
		0.613	8.1	8.3	0.2
		0.409	8.1	8.3	0.2
		0.273	8.1	8.3	0.2
		0.182	8.1	8.3	0.2
		0.1212	8.1	8.3	0.2
		0.0808	8.1	8.3	0.2

Antibiotic	Species	Test concentration (mg/L)	Starting pH	Final pH	pH change
Cefazolin	*Synechococcus sp.*	DWC	8.1	8.3	0.2
		10.48	8.1	8.3	0.2
		6.99	8.1	8.2	0.1
		4.66	8.1	8.3	0.2
		3.11	8.1	8.3	0.2
		2.070	8.1	8.3	0.2
		1.380	8.1	8.3	0.2
		0.920	8.1	8.3	0.2
		0.613	8.1	8.3	0.2
		0.4089	8.1	8.3	0.2
		0.2726	8.1	8.3	0.2
	Synechocystis sp.	DWC	8.1	8.3	0.2
		6.99	8.1	8.3	0.2
		4.66	8.1	8.3	0.2
		3.11	8.1	8.3	0.2
		2.07	8.1	8.3	0.2
		1.380	8.1	8.3	0.2
		0.920	8.1	8.3	0.2
		0.613	8.1	8.3	0.2
		0.409	8.1	8.3	0.2
		0.2726	8.1	8.3	0.2
		0.1817	8.1	8.3	0.2
	C. gracile	DWC	8.1	8.4	0.3
		0.92	8.1	8.1	0.0
		0.61	8.1	8.2	0.1
		0.41	8.1	8.2	0.1
		0.27	8.1	8.2	0.1
		0.182	8.1	8.2	0.1
		0.121	8.1	8.3	0.2
		0.081	8.1	8.3	0.2
		0.054	8.1	8.3	0.2
		0.0359	8.1	8.4	0.3
		0.0239	8.1	8.4	0.3
	G. herdmanii	DWC	8.1	8.3	0.2
		0.92	8.1	8.2	0.1
		0.61	8.1	8.2	0.1
		0.41	8.1	8.2	0.1
		0.27	8.1	8.2	0.1
		0.182	8.1	8.2	0.1
		0.121	8.1	8.3	0.2
		0.081	8.1	8.2	0.1
		0.054	8.1	8.3	0.2
		0.0359	8.1	8.3	0.2
		0.0239	8.1	8.3	0.2

Antibiotic	Species	Test concentration (mg/L)	Starting pH	Final pH	pH change
Sufadiazine	*A. flos-aquae*	DWC	8.2	8.3	0.1
		25.00	8.2	8.3	0.1
		8.93	8.2	8.3	0.1
		3.19	8.2	8.3	0.1
		1.14	8.2	8.3	0.1
		0.407	8.2	8.3	0.1
		0.145	8.2	8.3	0.1
		0.052	8.2	8.3	0.1
		0.019	8.2	8.3	0.1
		0.0066	8.2	8.3	0.1
		0.0024	8.2	8.3	0.1
	A. cylindrica	DWC	8.2	8.3	0.1
		25.00	8.2	8.3	0.1
		8.93	8.2	8.3	0.1
		3.19	8.2	8.3	0.1
		1.14	8.2	8.3	0.1
		0.407	8.2	8.3	0.1
		0.145	8.2	8.3	0.1
		0.052	8.2	8.3	0.1
		0.019	8.2	8.3	0.1
		0.0066	8.2	8.3	0.1
		0.0024	8.2	8.3	0.1
	S. leopoliensis	DWC	8.2	8.3	0.1
		25.00	8.2	8.3	0.1
		8.93	8.2	8.3	0.1
		3.19	8.2	8.3	0.1
		1.14	8.2	8.3	0.1
		0.407	8.2	8.3	0.1
		0.145	8.2	8.3	0.1
		0.052	8.2	8.3	0.1
		0.019	8.2	8.3	0.1
		0.0066	8.2	8.3	0.1
		0.0024	8.2	8.3	0.1
	S. elongates	DWC	8.2	8.3	0.1
		25.00	8.2	8.3	0.1
		8.93	8.2	8.3	0.1
		3.19	8.2	8.3	0.1
		1.14	8.2	8.3	0.1
		0.407	8.2	8.3	0.1
		0.145	8.2	8.3	0.1
		0.052	8.2	8.3	0.1
		0.019	8.2	8.3	0.1
		0.0066	8.2	8.3	0.1
		0.0024	8.2	8.3	0.1

Antibiotic	Species	Test concentration (mg/L)	Starting pH	Final pH	pH change
Sulfadiazine	*Synechococcus sp.*	DWC	8.2	8.3	0.1
		25.00	8.2	8.3	0.1
		8.93	8.2	8.3	0.1
		3.19	8.2	8.3	0.1
		1.14	8.2	8.3	0.1
		0.407	8.2	8.3	0.1
		0.145	8.2	8.3	0.1
		0.052	8.2	8.3	0.1
		0.019	8.2	8.3	0.1
		0.0066	8.2	8.3	0.1
		0.0024	8.2	8.3	0.1
	Synechocystis sp.	DWC	8.2	8.3	0.1
		25.00	8.2	8.2	0.0
		8.93	8.2	8.2	0.0
		3.19	8.2	8.2	0.0
		1.14	8.2	8.2	0.0
		0.407	8.2	8.2	0.0
		0.145	8.2	8.3	0.1
		0.052	8.2	8.3	0.1
		0.019	8.2	8.3	0.1
		0.0066	8.2	8.3	0.1
		0.0024	8.2	8.3	0.1
	C. gracile	DWC	8.2	8.3	0.1
		25.00	8.2	8.2	0.0
		8.93	8.2	8.2	0.0
		3.19	8.2	8.2	0.0
		1.14	8.2	8.2	0.0
		0.407	8.2	8.3	0.1
		0.145	8.2	8.3	0.1
		0.052	8.2	8.3	0.1
		0.019	8.2	8.3	0.1
		0.0066	8.2	8.3	0.1
		0.0024	8.2	8.3	0.1
	G. herdmanii	DWC	8.2	8.3	0.1
		25.00	8.2	8.2	0.0
		8.93	8.2	8.2	0.0
		3.19	8.2	8.2	0.0
		1.14	8.2	8.2	0.0
		0.407	8.2	8.2	0.0
		0.145	8.2	8.2	0.0
		0.052	8.2	8.2	0.0
		0.019	8.2	8.2	0.0
		0.0066	8.2	8.2	0.0
		0.0024	8.2	8.2	0.0

Antibiotic	Species	Test concentration (mg/L)	Starting pH	Final pH	pH change
Sufamethazine	*A. flos-aquae*	DWC	8.2	8.1	-0.1
		100.00	8.2	8.1	-0.1
		55.56	8.2	8.1	-0.1
		30.86	8.2	8.2	0.0
		17.15	8.2	8.1	-0.1
		9.526	8.2	8.1	-0.1
		5.292	8.2	8.1	-0.1
		2.940	8.2	8.2	0.0
		1.633	8.2	8.1	-0.1
		0.9074	8.2	8.1	-0.1
		0.5041	8.2	8.1	-0.1
	A. cylindrica	DWC	8.2	8.4	0.2
		100.00	8.2	8.2	0.0
		55.56	8.2	8.3	0.1
		30.86	8.2	8.2	0.0
		17.15	8.2	8.3	0.1
		9.526	8.2	8.3	0.1
		5.292	8.2	8.3	0.1
		2.940	8.2	8.2	0.0
		1.633	8.2	8.3	0.1
		0.9074	8.2	8.3	0.1
		0.5041	8.2	8.3	0.1
	S. leopoliensis	DWC	8.2	8.3	0.1
		100.00	8.2	8.2	0.0
		55.56	8.2	8.2	0.0
		30.86	8.2	8.2	0.0
		17.15	8.2	8.2	0.0
		9.526	8.2	8.2	0.0
		5.292	8.2	8.2	0.0
		2.940	8.2	8.2	0.0
		1.633	8.2	8.2	0.0
		0.9074	8.2	8.2	0.0
		0.5041	8.2	8.2	0.0
	S. elongates	DWC	8.2	8.3	0.1
		100.00	8.2	8.1	-0.1
		55.56	8.2	8.1	-0.1
		30.86	8.2	8.1	-0.1
		17.15	8.2	8.1	-0.1
		9.526	8.2	8.1	-0.1
		5.292	8.2	8.2	0.0
		2.940	8.2	8.2	0.0
		1.633	8.2	8.2	0.0
		0.9074	8.2	8.2	0.0
		0.5041	8.2	8.2	0.0

Antibiotic	Species	Test concentration (mg/L)	Starting pH	Final pH	pH change
Sulfamethazine	*Synechococcus sp.*	DWC	8.2	8.2	0.0
		100.00	8.2	8.2	0.0
		55.56	8.2	8.2	0.0
		30.86	8.2	8.2	0.0
		17.15	8.2	8.2	0.0
		9.526	8.2	8.2	0.0
		5.292	8.2	8.2	0.0
		2.940	8.2	8.2	0.0
		1.633	8.2	8.2	0.0
		0.9074	8.2	8.2	0.0
		0.5041	8.2	8.2	0.0
	Synechocystis sp.	DWC	8.2	8.4	0.2
		100.00	8.2	8.3	0.1
		55.56	8.2	8.3	0.1
		30.86	8.2	8.3	0.1
		17.15	8.2	8.3	0.1
		9.526	8.2	8.4	0.2
		5.292	8.2	8.4	0.2
		2.940	8.2	8.4	0.2
		1.633	8.2	8.4	0.2
		0.9074	8.2	8.4	0.2
		0.5041	8.2	8.4	0.2
	C. gracile	DWC	8.2	8.3	0.1
		100.00	8.2	8.1	-0.1
		55.56	8.2	8.2	0.0
		30.86	8.2	8.2	0.0
		17.15	8.2	8.2	0.0
		9.526	8.2	8.2	0.0
		5.292	8.2	8.2	0.0
		2.940	8.2	8.2	0.0
		1.633	8.2	8.2	0.0
		0.9074	8.2	8.2	0.0
		0.5041	8.2	8.2	0.0
	G. herdmanii	DWC	8.2	8.3	0.1
		100.00	8.2	8.3	0.1
		55.56	8.2	8.3	0.1
		30.86	8.2	8.3	0.1
		17.15	8.2	8.4	0.2
		9.526	8.2	8.4	0.2
		5.292	8.2	8.4	0.2
		2.940	8.2	8.4	0.2
		1.633	8.2	8.4	0.2
		0.9074	8.2	8.4	0.2
		0.5041	8.2	8.4	0.2

Antibiotic	Species	Test concentration (mg/L)	Starting pH	Final pH	pH change
Azithromycin	*A. flos-aquae*	DWC	8.2	8.3	0.1
		SC	8.2	8.3	0.1
		0.50	8.2	8.1	-0.1
		0.28	8.2	8.1	-0.1
		0.15	8.2	8.1	-0.1
		0.09	8.2	8.1	-0.1
		0.048	8.2	8.1	-0.1
		0.026	8.2	8.1	-0.1
		0.015	8.2	8.1	-0.1
		0.008	8.2	8.1	-0.1
		0.0045	8.2	8.1	-0.1
		0.0025	8.2	8.1	-0.1
	A. cylindrica	DWC	8.2	8.3	0.1
		SC	8.2	8.3	0.1
		0.09	8.2	8.1	-0.1
		0.05	8.2	8.1	-0.1
		0.03	8.2	8.1	-0.1
		0.01	8.2	8.1	-0.1
		0.008	8.2	8.1	-0.1
		0.005	8.2	8.1	-0.1
		0.003	8.2	8.3	0.1
		0.001	8.2	8.2	0.0
		0.0008	8.2	8.3	0.1
		0.0004	8.2	8.3	0.1
	S. leopoliensis	DWC	8.2	8.3	0.1
		SC	8.2	8.3	0.1
		0.50	8.2	8.2	0.0
		0.28	8.2	8.2	0.0
		0.15	8.2	8.3	0.1
		0.09	8.2	8.2	0.0
		0.048	8.2	8.2	0.0
		0.026	8.2	8.2	0.0
		0.015	8.2	8.3	0.1
		0.008	8.2	8.3	0.1
		0.0045	8.2	8.3	0.1
		0.0025	8.2	8.3	0.1

Antibiotic	Species	Test concentration (mg/L)	Starting pH	Final pH	pH change
Azithromycin	*S. elongates*	DWC	8.2	8.3	0.1
		SC	8.2	8.3	0.1
		0.50	8.2	8.3	0.1
		0.28	8.2	8.3	0.1
		0.15	8.2	8.2	0.0
		0.09	8.2	8.2	0.0
		0.048	8.2	8.2	0.0
		0.026	8.2	8.2	0.0
		0.015	8.2	8.3	0.1
		0.008	8.2	8.3	0.1
		0.0045	8.2	8.3	0.1
		0.0025	8.2	8.3	0.1
	Synechococcus sp.	DWC	8.2	8.2	0.0
		SC	8.2	8.2	0.0
		0.50	8.2	8.3	0.1
		0.28	8.2	8.2	0.0
		0.15	8.2	8.2	0.0
		0.09	8.2	8.2	0.0
		0.048	8.2	8.2	0.0
		0.026	8.2	8.2	0.0
		0.015	8.2	8.2	0.0
		0.008	8.2	8.2	0.0
		0.0045	8.2	8.3	0.1
		0.0025	8.2	8.3	0.1
	Synechocystis sp.	DWC	8.2	8.3	0.1
		SC	8.2	8.3	0.1
		0.50	8.2	8.2	0.0
		0.28	8.2	8.1	-0.1
		0.15	8.2	8.1	-0.1
		0.09	8.2	8.1	-0.1
		0.048	8.2	8.2	0.0
		0.026	8.2	8.2	0.0
		0.015	8.2	8.2	0.0
		0.008	8.2	8.2	0.0
		0.0045	8.2	8.3	0.1
		0.0025	8.2	8.2	0.0

Antibiotic	Species	Test concentration (mg/L)	Starting pH	Final pH	pH change
Azithromycin	*C. gracile*	DWC	8.2	8.3	0.1
		SC	8.2	8.3	0.1
		0.28	8.2	8.1	-0.1
		0.15	8.2	8.1	-0.1
		0.09	8.2	8.1	-0.1
		0.05	8.2	8.1	-0.1
		0.026	8.2	8.1	-0.1
		0.015	8.2	8.1	-0.1
		0.008	8.2	8.2	0.0
		0.005	8.2	8.3	0.1
		0.0025	8.2	8.3	0.1
		0.0014	8.2	8.3	0.1
	G. herdmanii	DWC	8.2	8.3	0.1
		SC	8.2	8.2	0.0
		0.28	8.2	8.2	0.0
		0.15	8.2	8.1	-0.1
		0.09	8.2	8.1	-0.1
		0.05	8.2	8.1	-0.1
		0.026	8.2	8.1	-0.1
		0.015	8.2	8.1	-0.1
		0.008	8.2	8.2	0.0
		0.005	8.2	8.2	0.0
		0.0025	8.2	8.3	0.1
		0.0014	8.2	8.3	0.1

Antibiotic	Species	Test concentration (mg/L)	Starting pH	Final pH	pH change
Erythromycin	*A. flos-aquae*	DWC	8.1	8.1	0.0
		0.50	8.1	7.8	-0.3
		0.28	8.1	7.8	-0.3
		0.15	8.1	7.9	-0.2
		0.09	8.1	7.9	-0.2
		0.048	8.1	8.0	-0.1
		0.026	8.1	8.0	-0.1
		0.015	8.1	8.0	-0.1
		0.008	8.1	8.0	-0.1
		0.0045	8.1	8.0	-0.1
		0.0025	8.1	8.0	-0.1
	A. cylindrica	DWC	8.1	8.0	-0.1
		0.09	8.1	7.9	-0.2
		0.05	8.1	7.9	-0.2
		0.03	8.1	7.9	-0.2
		0.01	8.1	7.9	-0.2
		0.008	8.1	7.9	-0.2
		0.005	8.1	7.9	-0.2
		0.003	8.1	8.0	-0.1
		0.001	8.1	8.0	-0.1
		0.0008	8.1	8.0	-0.1
		0.0004	8.1	8.0	-0.1
	S. leopoliensis	DWC	8.1	8.0	-0.1
		0.50	8.1	7.9	-0.2
		0.28	8.1	7.9	-0.2
		0.15	8.1	7.9	-0.2
		0.09	8.1	7.9	-0.2
		0.048	8.1	8.0	-0.1
		0.026	8.1	7.9	-0.2
		0.015	8.1	7.9	-0.2
		0.008	8.1	7.9	-0.2
		0.0045	8.1	8.0	-0.1
		0.0025	8.1	8.0	-0.1
	S. elongates	DWC	8.1	8.0	-0.1
		0.50	8.1	8.0	-0.1
		0.28	8.1	7.9	-0.2
		0.15	8.1	7.9	-0.2
		0.09	8.1	8.0	-0.1
		0.048	8.1	8.0	-0.1
		0.026	8.1	7.9	-0.2
		0.015	8.1	7.9	-0.2
		0.008	8.1	8.0	-0.1
		0.0045	8.1	8.0	-0.1
		0.0025	8.1	8.0	-0.1

Antibiotic	Species	Test concentration (mg/L)	Starting pH	Final pH	pH change
Erythromycin	*Synechococcus sp.*	DWC	8.1	8.0	-0.1
		0.50	8.1	8.0	-0.1
		0.28	8.1	7.9	-0.2
		0.15	8.1	7.9	-0.2
		0.09	8.1	8.0	-0.1
		0.048	8.1	8.0	-0.1
		0.026	8.1	7.9	-0.2
		0.015	8.1	7.9	-0.2
		0.008	8.1	8.0	-0.1
		0.0045	8.1	8.0	-0.1
		0.0025	8.1	8.0	-0.1
	Synechocystis sp.	DWC	8.1	8.1	0.0
		0.50	8.1	7.9	-0.2
		0.28	8.1	7.8	-0.3
		0.15	8.1	7.9	-0.2
		0.09	8.1	7.9	-0.2
		0.048	8.1	7.9	-0.2
		0.026	8.1	7.8	-0.3
		0.015	8.1	7.9	-0.2
		0.008	8.1	8.0	-0.1
		0.0045	8.1	8.1	0.0
		0.0025	8.1	8.1	0.0
	C. gracile	DWC	8.1	8.0	-0.1
		0.28	8.1	7.9	-0.2
		0.15	8.1	7.9	-0.2
		0.09	8.1	7.9	-0.2
		0.05	8.1	7.9	-0.2
		0.026	8.1	8.0	-0.1
		0.015	8.1	8.0	-0.1
		0.008	8.1	8.0	-0.1
		0.005	8.1	8.0	-0.1
		0.0025	8.1	8.0	-0.1
		0.0014	8.1	8.0	-0.1
	G. herdmanii	DWC	8.1	8.0	-0.1
		0.28	8.1	7.9	-0.2
		0.15	8.1	7.9	-0.2
		0.09	8.1	7.9	-0.2
		0.05	8.1	7.9	-0.2
		0.026	8.1	8.0	-0.1
		0.015	8.1	8.0	-0.1
		0.008	8.1	8.0	-0.1
		0.005	8.1	8.0	-0.1
		0.0025	8.1	8.0	-0.1
		0.0014	8.1	8.0	-0.1

Chapter 6

Protection limits for antibiotics are enhanced via the incorporation of species sensitivity distributions in bacterial assays

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This article is in preparation for submission as a short communication for publication at the time of submitting this thesis. It is a paper that uses the dose response curves from chapter 5 to construct species sensitivity distributions and from these evaluate the protection limits derived under current environmental risk assessment guidelines.

I performed all statistical analysis, modelling and first drafting of the paper. All authors were actively involved in later discussions on the work and in the preparation of the manuscript.

Protection limits for antibiotics are enhanced via the incorporation of species sensitivity distributions in bacterial assays

Abstract

Limits established for the protection of the environment from the adverse effects of antibiotics have been criticised due to the lack of bacterial representation. Probabilistic analysis using species sensitivity distributions (SSD) for a wider and more diverse selection of bacteria may provide for more accurate and statistically robust protection limits compared with the single cyanobacteria species growth inhibition test currently used in environmental risk assessment. Here we used the dose-response data for eight cyanobacteria species to create SSDs for cefazolin, cefotaxime, ampicillin, erythromycin and azithromycin. From this we determined the 5% hazardous concentrations (concentration at which 5% of species will be effected) to compare with the predicted no effect concentration (PNEC) used in the traditional approach for determining the protection limit. We show that the current PNEC may be either over or under protective of cyanobacteria species dependent on the species choice and mode of action of the antibiotic. For instance, the PNEC for macrolides was over protective but generally under protective for β-lactams. We also used measured environmental concentrations of selected antibiotics to conduct an expected total risk assessment and this indicates that in some locations of high antibiotic concentrations there is a significant risk to cyanobacteria populations. We conclude that protection limits using SSDs and a wider range of bacteria would result in improved confidence in the Environmental risk assessment of antibiotics.

Introduction

The environmental risk assessment (ERA) of antibiotics aims to establish protection limits that prevent “risk of undesirable effects on the environment” (EC 2001). But how effective the current approach to ERA is for antibiotics has recently been questioned (Brandt *et al*. 2015; Le Page *et al*. 2017). Thus, there is an urgent need to identify if the protection limits currently derived by ERA are able to protect against adverse effects upon environmental populations and ecosystem functions and how they compare with protection limits for antimicrobial resistance (AMR).

The approach currently taken to determine a protection limit for pharmaceuticals in surface water, including for antibiotics, is to calculate a predicted no effect concentration (PNEC) by applying an assessment factor of 10 to the lowest no observed effect concentration (NOEC) following testing upon a cyanobacteria (green algae when not an antibiotic), an invertebrate, a fish and an activated sludge respiration inhibition test. The assessment factor is applied to account for uncertainty caused by interspecies variability and the extrapolation from controlled laboratory studies to the field. But a factor of 10 is unsupported by experimental data and recent evidence shows that in some cases interspecies sensitivity may exceed this by several orders of magnitude (Chapman *et al*. 1998; Le Page *et al*. 2017) (Chapter 5). This suggests that there may be cases where the PNEC is likely not to be protective of the most sensitive species. Moreover, a PNEC uses a NOEC, which has two potential drawbacks i) the NOEC has been heavily criticised due to its dependence on experimental design (both for replication for statistical power to detect differences from the controls and for the choice in test concentration range) (Green *et al*. 2013) and ii) because it uses only a single effect value (e.g. the NOEC) so that there can be no quantification of the uncertainty around the PNEC (Chapman *et al*. 1998).

A second approach for establishing protection limits is to construct a species sensitivity distribution (SSD), which is a probability model of interspecies variability across a toxicity endpoint following chemical exposure (e.g. NOEC or EC_x) allowing the prediction of the proportion of species affected at any concentration for the species group modelled (Aldenberg *et al*. 2001; Belanger *et al*. 2017; Wheeler *et al*. 2002). SSDs are more commonly used for higher tier ERA such as in plant protection product regulations (EFSA 2013) and in the

Water Framework Directive (European Commission Joint Research Centre 2003). The protection limit most often derived from a SSD is the hazardous concentration that affects 5% of species (HC5), although it has been suggested that the lower 95% confidence limit of the HC5 ($HC5_{2.5\%}$) should be used to ensure a truly protective limit (Verdonck *et al*. 2001; Wheeler *et al*. 2002). An assessment factor of less than 10 is sometimes applied depending on the specific regulations and quality/quantity of the data on which the SSD is based (EFSA 2013).

Although SSDs have been criticised in the past for being ecologically unrealistic and for lack of statistical robustness (equally, no more so than single species testing with an assessment factor), there have been recent advances that allow for the mitigation of some of these concerns (Forbes and Calow 2002; Kon Kam King *et al*. 2015; Kon Kam King *et al*. 2014). Importantly, SSDs are influenced by the quality and number of data included. Where previously a sample size of 10-15 species was required for a robust analysis (TGD 2003), newer protocols that use bootstrap regression and the incorporation of censored data now allow for the reliable use of a more limited dataset (<10 data points) (Kon Kam King *et al*. 2014; Wheeler *et al*. 2002). Furthermore, less species are arguably required if they are all known to be sensitive to the mode of action (MoA) of the chemical. This is because data from non-sensitive species or taxonomic clades will not be impacting the distribution and thus allowing a SSD that focuses upon the lower tail of the SSD from which a protection limit is derived and allowing for a more reliable estimate (Schmitt-Jansen *et al*. 2008; Segner 2011).

It is not uncommon for the measured environmental concentrations (MECs) of antibiotics to surpass the PNEC in the environment, especially in sewerage treatment plants, hospital and manufacturing effluents (Batt *et al*. 2007; Brown *et al*. 2006; Jaimes-Correa *et al*. 2015; Larsson 2014; Larsson *et al*. 2007; Li *et al*. 2008; Watkinson *et al*. 2009). In these cases it is likely that there is a significant risk to bacterial communities and the ecosystem functions that they provide. By considering these MECs in relation to the SSD it is possible to obtain an indication of the potential fraction of species that may be at risk under that toxic pressure. Moreover, by integrating the frequency probability distribution of MECs with the SSD it is possible to perform probabilistic ecological risk assessments (PERA) to determine the likelihood of adverse

effects. One such PERA is the expected total risk (ETR), which gives the probability that a randomly selected species will be affected given the distribution of measured concentrations and is equivalent to the risk determined by the area under the curve of a joint probability plot (Aldenberg *et al*. 2001).

Interspecies differences in sensitivity to antibiotics, ranging by several orders of magnitude, mean that current ERA procedures that rely upon a single species of cyanobacteria to represent all bacterial diversity could underestimate the PNEC (Le Page *et al*. 2017) (Chapter 5). Thus, in order to improve the ERA for antibiotics there have been calls to increase the bacterial diversity tested (Brandt *et al*. 2015; Le Page *et al*. 2017). In Chapter 5 we attempted to address this by performing growth inhibition assays on eight species of cyanobacteria and found up to two orders of magnitude difference in interspecies sensitivity following exposure to β-lactams and approximately one order of magnitude difference to macrolides. This supported the findings of the meta-analysis (Le Page *et al*. 2017) where large differences in sensitivity were shown between cyanobacteria species. We therefore hypothesise that when calculating protection limits for antibiotics using the current approach to ERA there may be cases where the PNEC for surface water ($PNEC_{SW}$) is not fully protective of all cyanobacteria populations. Using the data for eight species of cyanobacteria exposed to five antibiotics in Chapter 5, SSDs were established and how protective a PNEC derived using a NOEC and assessment factor of 10 for cyanobacteria populations was explored. We then establish the fraction of cyanobacteria species affected (based on our SSDs) by published MECs and consider the risk posed to cyanobacteria in the environment through the determination of the ETR.

Methods

Data collection

For this analysis two datasets were used. The first was the effect data from for eight species of cyanobacteria exposed to the antibiotics cefazolin, cefotaxime, ampicillin, azithromycin and erythromycin in Chapter 5. The cyanobacteria were *Anabaena flos-aquae* (CCAP 1403/13A), *Synechococcus leopoliensis* (CCAP 1405/1), *Anabaena cylindrica* (PCC 7122), *Synechococcus elongates* (PCC 6301), *Synechococcus sp* (PCC 6312), *Synechocystis sp* (PCC 6803), *Cyanobium gracile* (PCC 6307) and *Geminocystis herdmanii* (PCC 6308).

Further information on the choice of species, experimental design and statistical analysis can be found in Chapter 5. The second dataset used for these analyses were the MECs for each antibiotic in Umweltbundesamt's (UBA) 'Pharmacuiticals in the environment' database (Umwelt bundesamt 2018). MECs from all matrices that were measured in, or able to be converted into μg/L were extracted for use. Results that were recorded as 0 μg/L were removed, as they represent either no antibiotic presence or below the limit of detection and their inclusion would prevent the fitting of a parametric distribution.

SSDs

SSDs were constructed in r (version 3.3.0; R Project for Statistical Computing, Vienna, Austria) using the fitdistrplus package (Delignette-Muller and Dutang 2015) following procedure outlined in the MOSAIC SSD platform (Kon Kam King *et al*. 2014). Briefly, the 95% confidence intervals of the EC_{10} for each species (Chapter 5) were used as interval-censored data (i.e. not a single fixed value but a range between the 95% confidence limits). This allowed for the incorporation of the uncertainty around the EC_{10} into the SSD and thus increase confidence in the SSD output (Kon Kam King *et al*. 2014). Six parametric distributions were fitted to the data; i) normal, ii) log-normal, iii) weibull, iv) log-logistic, v) gamma and vi) exponential. The best fitting distribution was selected based upon a combination of the Akaike Information Criterion (AIC) score and the goodness of fit tests; Kolmogorov-Smirnov statistic, Cramer von Mises statistic and Anderson-Darling statistic. It has been shown that the distribution selection can have large effects on the SSD outputs and that this is a key factor in the construction of the SSD (Obiakor *et al*. 2017).

The HC5 and associated confidence intervals were determined from bootstrapping the data (5000 iterations) based on the parameters of the fitted distribution. A similar protocol was followed to derive the SSD, HC5 and confidence intervals from the NOEC data (Chapter 5) but for this the NOEC values were used as non-censored data.

ETR

The MEC data were fitted to the selection of distributions as for the SSD analysis and the best fitting selected using the AIC score. The ETR was calculated in r by integrating the product of the MEC probability distribution

function and the SSD in respect to concentration. Due to the limited number of MECs available all matrices from all locations globally were analysed together.

An example script of code for the construction of the SSD and calculation of the ETR can be found in the supplementary material.

Results

The SSDs, based upon cyanobacteria EC_{10}'s for each antibiotic, are presented in figure 1 together with the PNECs, based upon the NOEC of the most sensitive species tested in the microplate assays ($PNEC_{lowest}$), and the PNECs, based upon the two Organisation for Economic Co-operation and Development (OECD) test guideline recommended species (from which all PNECs derived for regulatory purposes are likely derived from) *A. flos-aquae* ($PNEC_{A.\ flos\text{-}aquae}$) and *S. leopoldensis* ($PNEC_{S.leopoldensis}$). Figure 1 also illustrates the PNECs for AMR ($PNEC_R$) as calculated by (Bengtsson-Palme and Larsson 2016). SSDs based upon cyanobacteria NOECs are presented in supplementary material (figure S1).

Table 1 provides values for the HC5, PNECs and the fraction of cyanobacteria affected predicted from the SSD (based on the EC_{10}s) for each antibiotic whilst Table 2 gives the ETR for each antibiotic, the highest and median MECs and the fraction of cyanobacteria affected predicted from the SSD (based on EC_{10}s). Tables S1 and S2 in the supplementary material provide the same information as tables 1 and 2 but using the SSD based upon NOEC data. Table S3 in the supplementary material provides the best fitting distributions used for establishing the SSD and for the MECs used to the calculate the ETR.

Cefazolin

The HC5 for cefazolin, based upon EC_{10}s, was 1.13 µg/L, which was 7.5 times higher than the lowest PNEC (for *A. flos-aquae*) but four times lower than that based upon *S. leopoldensis* (Figure 1 and Table 1).

The fraction of cyanobacteria affected at the PNECs ranged between 0.95 and 13.3% depending on which species was used to derive the PNEC (Table 1). The HC5 based upon the NOEC data was twice this when based on the EC_{10} (Table S1). The HC5 was approximately the same concentration as the PNEC for resistance (1.1 and 1.0 µg/L, respectively) (Table 1).

An ETR of 6.75% was determined for cefazolin and whilst the median MEC showed little effect upon cyanobacteria (<1%) based on the SSD, the highest recorded MEC of 42.9 µg/L was predicted to affect 60.2% of cyanobacteria (Table 2 and Figure S1).

Cefotaxime

The HC5 for cefotaxime, based upon EC_{10}s, was 0.67 µg/L, which was four times higher than the lowest PNEC (for *A. cylindrica*) and approximately the same value as for the PNEC based upon *S. leopoldensis* (Figure 1 and Table 1).

The fraction of cyanobacteria affected at the PNECs ranged between 1.3 and 5.2% depending on which species was used to derive the PNEC (Table 1). The HC5 based upon the NOEC data was approximately the same as when based on the EC_{10} (Table S1). The PNEC for resistance was lower than the HC5 and all of the PNECs for ecotoxicity based on the EC_{10}s (Table 1).

There was an ETR of 2.2% for cefotaxime and whilst the median MEC showed little effect upon cyanobacteria based on the SSD, the highest recorded MEC of 41.9 µg/L was predicted to affect 95.9% of cyanobacteria (Table 2 and Figure S1).

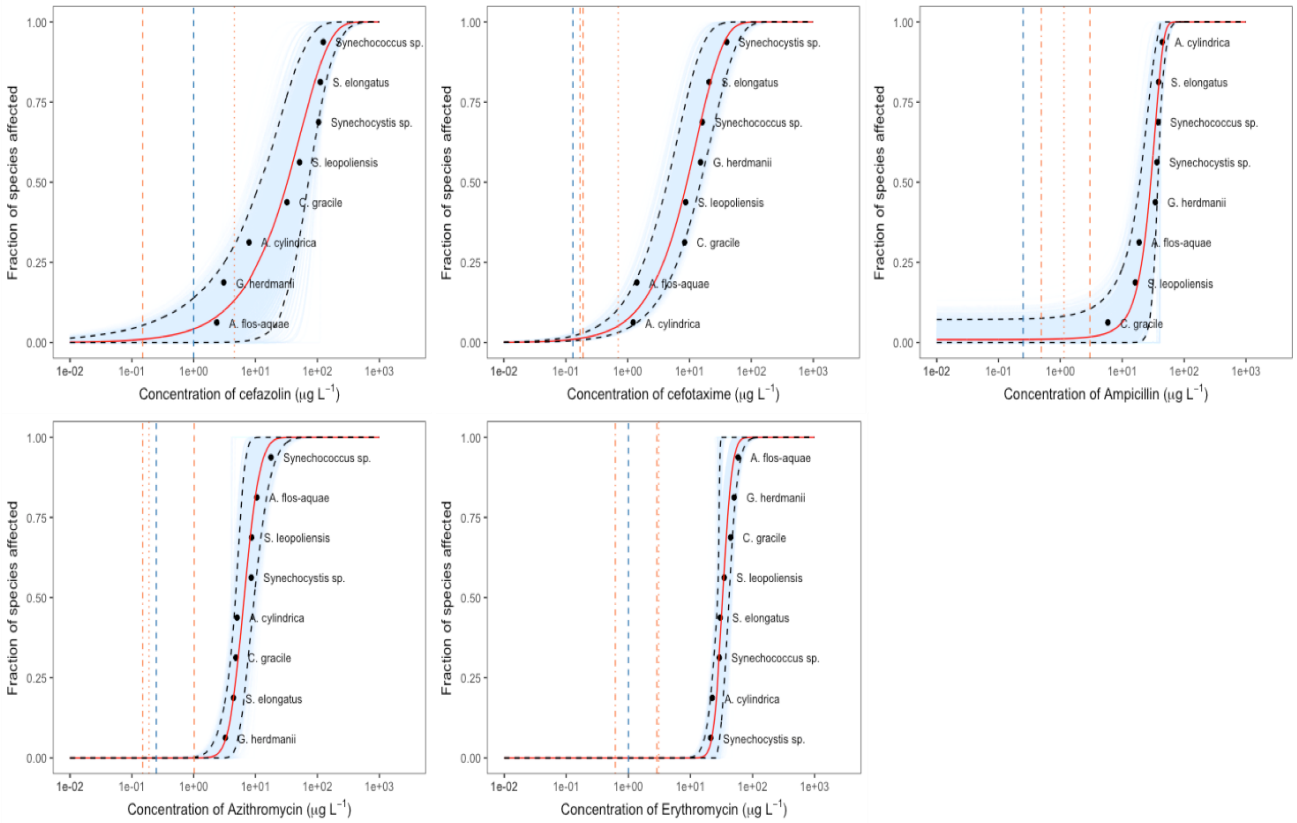

Figure 1. Species sensitivity distributions of cyanobacteria exposed to five antibiotics. Cefazolin and cefotaxime (cephalosporin), ampicillin (penicillin), azithromycin and erythromycin (macrolide). Red line indicates the modelled species sensitivity distribution. Dashed black lines represent upper and lower 95% confidence limits. Blue shaded area indicates results of bootstrapped distributions. Orange vertical lines indicate predicted no effect concentrations (PNEC): Dot-dash orange line= $PNEC_{lowest}$; Dashed orange line = $PNEC_{A.\ flos\text{-}aquae}$; Dotted orange line = $PNEC_{S.\ leopoldensis}$; Solid blue line = $PNEC_R$ as described by the Antimicrobial Resistance Industry Alliance.

Ampicillin

The HC5 for ampicillin, based upon EC_{10}s, was 8.6 µg/L, which was 17.5 times higher than the lowest PNEC (for *C. gracile*) and 2.9 and 7.4 times higher than the PNECs based upon *A. flos-aquae* and *S. leopoldensis,* respectively (Figure 1 and Table 1).

The fraction of cyanobacteria affected at the PNECs ranged between 0.9 and 1.6% depending on which species was used to derive the PNEC (Table 1). The HC5 based upon the NOEC data was approximately the same as when based on the EC_{10} (Table S1). The PNEC for resistance was lower than the HC5 and all PNECs for ecotoxicity based on the EC_{10}s (Table 1).

The ETR for ampicillin was 6.3% and whilst the median MEC indicated little effect upon cyanobacteria based on the SSD, the highest recorded MEC of 263 µg/L was predicted to affect 100% of the cyanobacteria (Table 2 and Figure S1).

Azithromycin

The HC5 for azithromycin, based upon EC_{10}s, was 3.2 µg/L, which was 21 times higher than the lowest PNEC (for *G. herdmanii*) and 3.1 and 16.6 times higher than the PNECs based upon *A. flos-aquae* and *S. leopoldensis,* respectively (Figure 1 and Table 1).

The proportion of cyanobacteria affected at the PNECs was <1% for all PNECs irrespective of which species was used to derive it (Table 1). The HC5 based upon the NOEC data was approximately half as much as when based on the EC_{10}. The PNEC for resistance was lower than the HC5. This was not the case however for the PNEC for ecotoxicity based on the EC_{10}s of the most sensitive species and *S. leopoldensis* (Table S1). $PNEC_R$ was however lower than the PNEC based on *A. flos-aquae* (Table 1).

An ETR of 1.5% was determined for azithromycin and whilst the median MEC showed no effect upon cyanobacteria based on the SSD, the highest recorded MEC of 9.7 µg/L was predicted to affect 80% of cyanobacteria (Table 2 and Figure S1).

Erythromycin

The HC5 for erythromycin, based upon EC_{10}s, was 21.3 µg/L, which was 34.4 times higher than the lowest PNEC (for S. *elongatus*) and 7.3 and 6.9 times higher than the PNECs based upon *A. flos-aquae* and *S. leopoldensis* respectively (Figure 1 and Table 1).

The fraction of cyanobacteria affected at the PNECs was <1% for all PNECs irrespective of which species was used to derive it (Table 1). The HC5 based upon the NOEC data was 3.5 times lower than when based on the EC_{10}. The PNEC for resistance was lower than the HC5 but not for the PNEC for ecotoxicity based on the EC_{10}s of the most sensitive species (Table S1). $PNEC_R$ was however lower than the PNEC based on both OECD recommended species *A. flos-aquae* and *S. leopoldensis* (Table 1).

An ETR of 0.1% was determined for erythromycin and no species of cyanobacteria are predicted to be affected by the MECs based on the SSD (highest MEC was 7.8 µg/L) (Table 2 and Figure S1).

Antibiotic	Protection limit	Concentration (µg/L)	Lower 95% CI	Higher 95% CI	Fraction of cyanobacteria affected (%)
Cefazolin	HC5	1.13	0.13	19.88	5.00
	$PNEC_{Lowest}$	0.15	-	-	0.95
	$PNEC_{A.\ flos\text{-}aquae}$	0.15	-	-	0.95
	$PNEC_{S.\ leopoldensis}$	4.53	-	-	13.26
	$PNEC_R$	1.00	-	-	4.16
Cefotaxime	HC5	0.67	0.32	1.13	5.00
	$PNEC_{Lowest}$	0.17	-	-	1.29
	$PNEC_{A.\ flos\text{-}aquae}$	0.19	-	-	1.44
	$PNEC_{S.\ leopoldensis}$	0.70	-	-	5.20
	$PNEC_R$	0.13	-	-	0.99
Ampicillin	HC5	8.56	0**	26.47	5.00
	$PNEC_{Lowest}$	0.49	-	-	0.91
	$PNEC_{A.\ flos\text{-}aquae}$	3.00	-	-	1.56
	$PNEC_{S.\ leopoldensis}$	1.15	-	-	1.05
	$PNEC_R$	0.25	-	-	0.86
Erythromycin	HC5	21.30	16.18	28.76	5.00
	$PNEC_{Lowest}$	0.62 *	-	-	0.00
	$PNEC_{A.\ flos\text{-}aquae}$	2.90	-	-	0.00
	$PNEC_{S.\ leopoldensis}$	3.10	-	-	0.00
	$PNEC_R$	1.00	-	-	0.00
Azithromycin	HC5	3.15	2.11	5.03	5.00
	$PNEC_{Lowest}$	0.15 *	-	-	0.00
	$PNEC_{A.\ flos\text{-}aquae}$	1.02	-	-	0.00
	$PNEC_{S.\ leopoldensis}$	0.19	-	-	0.00
	$PNEC_R$	0.25	-	-	0.00

Table 1. Protection limits; 5% hazardous concentration (HC5) based upon a species sensitivity distribution (SSD) using 10% effective concentrations (EC_{10}), predicted no effect concentrations (PNECs) and the fraction of cyanobacteria affected based upon the cyanobacteria SSD. PNECs determined as specified in current environmental risk assessment. $PNEC_{Lowest}$ represents the PNEC based on the most sensitive cyanobacteria in the microplate assays. $PNEC_{A.\ flos\text{-}aquae}$ and $PNEC_{S.\ leopoldensis}$ are based on the data of species recommended in the OECD 201 test guideline (OECD 2011). $PNEC_R$ is the PNEC for resistance as described by the Antimicrobial Resistance Industry Alliance. * $PNEC_{Lowest}$ for erythromycin is < 0.62 and < 0.15 for azithromycin. ** CI was determined to be <0.

Antibiotic	Expected total risk (%)	Measured concentration	Concentration (µg/L)	Fraction of cyanobacteria affected (%)
Cefazolin	6.75	Median	0.15	0.95
		Highest	42.93	60.23
Cefotaxime	2.21	Median	0.04	0.29
		Highest	41.90	95.91
Ampicillin	6.33	Median	0.10	0.83
		Highest	263.30	100.00
Erythromycin	0.13	Median	0.06	0.00
		Highest	7.84	0.00
Azithromycin	1.53	Median	0.09	0.00
		Highest	9.70	80.06

Table 2. Expected total risk, measured environmental concentrations (MEC) and the fraction of cyanobacteria affected based upon the cyanobacteria species sensitivity distributions using 10% effective concentrations and MECs obtained from Umweltbundesamt's 'Pharmacuiticals in the environment' database (Umwelt bundesamt 2018).

Discussion

The analysis conducted shows that the PNEC, as determined under the current approach to ERA using a NOEC and assessment factor of 10, was protective of >98% of cyanobacteria populations but with the exceptions of the PNECs for cefazolin and cefotaxime, based upon *S. leopoldensis*, which would adversely affect the growth of 13% and 5% of cyanobacteria respectively. We also show that based on published MECs the ETR for cyanobacteria was 6.8, 2.2 and 6.3% for cefazolin, cefotaxime and ampicillin respectively whilst there was a <1% and 1.5% risk from erythromycin and azithromycin, respectively.

PNECs with an assessment factor of 10 are not always protective

Examining how protective a PNEC was, calculated using a NOEC and assessment factor of 10, when based on the two OECD recommended species *A. flos-aquae* and *S. leopoldensis* it was found that whilst $PNEC_{A.\ flos\text{-}aquae}$ provided adequate protection for >98% of cyanobacteria, the EC_{10}s, $PNEC_{S.\ leopoldensis}$ was not always protective and failed to protect >95% cyanobacteria EC_{10}s for the two cephalosporins; cefazolin and cefotaxime. These data indicate that under current ERA procedures (of using a single test species) the choice of species is critical in establishing a protection limit. For the two macrolides however, the PNEC was protective of all cyanobacteria

regardless of the species from which the PNEC was derived. This can be explained by the MoA of the antibiotic classes.

Due to the larger interspecies variability observed between cyanobacteria exposed to cell membrane synthesis inhibitors, the PNEC has a higher probability of being under protective compared with other MoAs (Chapter 2 and 5). This relates to the fact that the assessment factor of 10 is inadequate to account for the large differences in sensitivity between the tested species (on which the PNEC is based) and the more sensitive species. For other MoAs, such as the macrolides, the smaller interspecies variability (Chapter 5) means an assessment factor of 10 is sufficient to cover the entire SSD no matter which species is selected on which to base the PNEC. Indeed an assessment factor of 10 arguably may be seen as somewhat over protective for these antibiotics. This is clearly observed by the slopes of the SSDs (Figure 1) that have a shallower gradient for the cell membrane synthesis inhibiting antibiotics compared with that for the protein synthesis inhibitors.

In addition, the variability observed between cells following exposure to cell membrane synthesis inhibiting antibiotics (reflected by the larger confidence limits around the EC_xs compared to other MoAs) is propagated in the SSDs that show higher uncertainty (larger confidence limits) compared with that for the macrolides. In Chapter 5 we concluded that the magnitude of species sensitivity might, at least in part, be explained by the conservation of the drug target between species and by the bioavailability/uptake of the antibiotic. As such, it may be possible to extrapolate these results to hypothesise that PNECs using an assessment factor of 10 may be protective for other classes of antibiotics that have a relatively well conserved drug target across bacteria and less difference in interspecies sensitivity (such as some DNA synthesis inhibitors (e.g. fluoroquinolones)) (Chapter 5).

These results are in accordance with the conclusions of other investigators that have found that the parameters of SSDs are consistent across MoAs. For example, SSDs were comparable across primary producers exposed to herbicides (Chèvre *et al*. 2006; Nagai and Taya 2015) and invertebrates and fish exposed to organophosphorus insecticides (Sala *et al*. 2012). It has been suggested that this consistent relationship between MoA and SSD parameters may allow for the extrapolation between chemicals of the same MoA (Nagai and

Taya 2015). But this assumes that the relative species sensitivity is also consistent across MoAs. This was not the case for the cyanobacteria in our assays, where there was an order of species sensitivity difference between antibiotics with the same MoA (Chapter 5). Relative species sensitivity did however, show a general trend; although the order of sensitivity varied, individual species did not show large differences in relative sensitivity between antibiotics of the same class. Thus, with further investigation, it may be possible to extrapolate SSDs across antibiotic classes to help inform prioritisation schemes for antibiotics. It is of importance to note that although SSD parameters are consistent across MoAs in the literature, our SSDs for the cephalosporins and for ampicillin (a penicillin) indicate somewhat different shapes in the line slopes. This suggests that for antibiotics it may be possible to extrapolate within an antibiotic class but not between classes, even if the MoA is similar.

The analysis conducted here shows that the $PNEC_R$ was lower than the $HC5_{(2.5)}$ for the two macrolides and cefotaxime whilst the $HC5_{(2.5)}$ was lower than the $PNEC_R$ for cefazolin (the negative $HC5_{(2.5)}$ prevents interpretation for ampicillin). This indicates that a protection limit derived for environmental health based upon a HC5 or $HC5_{(2.5)}$ would not be protective of resistance for 80% of these antibiotics, but that a $PNEC_R$ maybe protective of 95% of cyanobacteria EC_{10}'s. In the case of cefotaxime, a recently experimentally derived $PNEC_R$ was derived (0.4 μg/L; Murray *et al*. 2018), which compares to the theoretically derived $PNEC_R$ of 0.13 μg/L, obtained from the AMR Industry Alliance (AMR Industry alliance 2018; Bengtsson-Palme and Larsson 2016). In this case the $HC5_{(2.5)}$ would have been 1.2 times lower than the $PNEC_R$. The HC5 (i.e. not the lower confidence limit) however, is still a slightly higher value. These results add support to the conclusions in Chapter 2 and 3 (Le Page *et al*. 2017; Le Page *et al*. 2018) that using current methodologies neither the protection limit for environmental health nor for AMR is protective of each other and that both should be determined for use in ERA.

The HC5 may provide a better protection limit than the traditional PNEC

Results from this analysis show that for the cephalosporins, the HC5 was generally 4 - 8 times higher than the $PNEC_{lowest}$ but that the $HC5_{(2.5\%)}$ was more similar at 0.9 - 2 times higher. For the macrolides the HC5 far exceeded this and was up to 34 times higher than the $PNEC_{lowest}$, further highlighting how the PNEC and assessment factor of 10 might be highly conservative as a protective factor for this antibiotic class/MoA. The HC5s were more similar to the NOECs than the PNECs, with the $HC5_{(2.5\%)}$ being just 0.1 – 2.6 times the value for the lowest NOECs for all antibiotics. These results therefore support the suggestion that the $HC5_{(2.5\%)}$ could be used to ensure an empirically based protection limit that is a more accurate and is truly protective of 95% of species (Wheeler *et al*. 2002) without being over protective in for some MoAs as appears to be the case for the PNECs for macrolides.

The 95% confidence intervals of the HC5s for cefotaxime, azithromycin and erythromycin suggest some uncertainty (although this is relatively small) surrounding the HC5 for these antibiotics. But given that these estimates include the error around the original EC_{10} via the use of the 95% confidence limits as censored data, this might be expected. The wider confidence limits around the HC5 for cefazolin shows that there is more uncertainty in this estimate and this may be due to higher variability observed between replicates in the microplate assay.

The HC5 has been suggested as a protection limit under the premise that functional redundancy (where multiple species are capable of performing the same ecological functions) in the ecosystem will compensate for some small effects on the most sensitive species (Solomon and Sibley 2002). However, the magnitude of functional redundancy is not clear, especially in bacterial communities (Antwis *et al*. 2017). Although our results suggest that the $HC5_{(2.5\%)}$ (based upon the EC_{10}) may be suitable for setting protection limits, further investigation is required to explore the hypothesis that 5% of species can be affected beyond their EC_{10} without adverse effects upon environmental communities and ecosystem function.

The use of SSDs and the $HC5_{(2.5\%)}$ may be a suitable way to address uncertainties in interspecies differences in sensitivity and the extrapolation from

a few single species studies to reflect all species within a taxonomic group. But the selection of species is of clear importance (Verdonck *et al*. 2003). Our analysis may be a suitable reflection of cyanobacteria sensitivity, but in the case of antibiotics a more diverse range of bacteria are required since sensitivity differences between taxonomic clades could be large, even spanning several orders of magnitude (Chapters 2 and 5). Furthermore, a protection limit also needs to consider the extrapolation from the laboratory to the field. Previous authors have concluded that large safety factors are not considered necessary for extrapolation between the laboratory and field (Chapman *et al*. 1998). Indeed, bacteria associated with biofilms in the field may be more resilient to chemical toxicity than cells in laboratory testing due to the protective nature of complex biofilm communities and extracellular substances (Harrison *et al*. 2007). On the other hand, there may still be considerable unknowns regarding the effect of antibiotics in the environment that may significantly increase the sensitivity of bacteria to antibiotics. These include an increase in sensitivity in chemical mixtures or as a result of different biotic and abiotic factors (e.g. competition, predation, temperature, pH (Rohr *et al*. 2016)). As such, in the absence of conclusive evidence demonstrating the safe concentrations in mixtures or in a variety of environmental conditions, it may be prudent to take a protective approach and continue to include an assessment factor to compensate for this, as is required in some regulatory guidance (EFSA 2013; TGD 2003). Further investigation is required to determine the suitable size of such an assessment factor, as these are still largely arbitrary. Using an assessment factor with the HC5 or $HC5_{(2.5)}$ to establish a protection limit may appear to undermine the benefits of conducting a more accurate, reliable and robust SSD, which requires multiple experiments and will thus be more costly and experimentally time consuming compared to a single species test (as is currently used in antibiotic ERA). However, more confidence can be applied to an empirically derived HC5/ $HC5_{(2.5)}$ with a small assessment factor (of less than 10) and for which error can be quantified. Furthermore, a HC5 has less chance of underestimating the PNEC where interspecies variability is high and overestimating the PNEC where interspecies variability is low. Additionally, the SSD based on an EC_x avoids the criticisms of the NOEC that is flawed and dependent of experimental design.

Measured environmental concentrations and expected total risk

The SSDs highlight that for the majority of MECs there is a very limited effect on cyanobacteria in the field (potentially affected fractions of <1%) from all antibiotics based on the median MEC. However, with exception of erythromycin, between 60 and 100% of cyanobacteria may be affected when exposed to the highest antibiotic MECs recorded in the UBA database (Umwelt bundesamt 2018) for all antibiotics tested here. Our analysis therefore suggests that bacterial populations in these communities for the higher measured concentrations may be severely affected with potential consequences on the ecosystem functions that they provide.

The expected total risk (ETR) determined for the antibiotics indicated a relatively high risk (of up to 6%) for the cell membrane synthesis inhibiting antibiotic classes compared with the macrolides that had an ETR of 0.1 and 0.5% for erythromycin and azithromycin, respectively. This was not expected given that the macrolides are on the priority contaminant watch list, in part because of their persistent nature (Carvalho *et al*. 2015). One explanation for this may be that there were considerably more MEC data for the macrolides, reflecting their priority status as chemicals of concern. This additional MEC data may have resulted in a more environmentally realistic distribution with more samples consisting of low levels in surface waters and effluents. The cephalosporins and ampicillin on the other hand, have far fewer MECs (<30) of which sampling bias may have resulted in higher concentrations having a disproportionate effect on the distribution. This was unavoidable given the limited MEC data available.

The interpretation of the ETR therefore requires careful consideration of what the MEC and SSD distributions represent (Verdonck *et al*. 2003). The ETRs calculated here use the limited MEC data available on the literature, but whether each individual sample is truly representative of the concentrations found in the environment is unknown. For example, does it reflect a temporal or spatial average concentration? Or could it represent a peak (or low point) in concentration? As such, with a limited dataset such as those available for this analysis caution must be employed in the interpretation of the calculated risk. Additionally, interpretation of the risk from a PERA also needs to consider the data on which the species sensitive distribution was based. In this instance it

reflects the EC_{10} and thus the expected risk here is that of a random measured concentration exceeding a random species' EC_{10}, which may or may not lead to adverse ecological consequences. Furthermore, the effect level modelled needs to be considered in relation to the MEC data used to ensure it is relevant (Verdonck *et al*. 2003). For example, effect data from a chronic 72 hour cyanobacteria study as utilised in this analysis, would not be suitable in a PERA using MEC data that represent short <24h peaks in environmental concentrations such as following irregular surface runoff of antibiotics following agricultural use.

Our analysis represents the expected risk that the growth rate of a random species of cyanobacteria will be adversely affected by 10% in the presence of the antibiotic based upon MECs globally in a wide range of matrices. It is thus a useful indication of which antibiotics may be of a higher concern than others (based on the available MECs) but is not suitable for drawing specific conclusions about the risk of each individual antibiotic in the environment. Further investigation of antibiotic MECs that are spatially or temporally discrete in a single matrix is required to perform precise and reliable ETR assessments.

Conclusion

Our analysis shows that the choice of species from which a PNEC is derived, as currently determined in ERA, is of critical importance in order to ensure a suitable protection limit. We also show that an assessment factor of 10 may be protective of other cyanobacteria populations in some cases but may be over conservative for the protection in others and that this depends on the MoA of the antibiotic and the interspecies differences in sensitivity to it. The ETR analysis suggests that the risk to cyanobacteria populations in the environment is currently low, although the highest recorded MECs in the literature pose a significant threat to cyanobacteria populations. Finally, the data used in the analyses undertaken do not allow for the extrapolation to other bacterial taxa and thus the examination of additional bacterial diversity is required to fully assess the effectiveness of the PNEC to protect against the detrimental effects of antibiotics on the environment.

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Supplementary material A

Data handling and distribution modelling

Example code for modelling MEC and SSDs and integration of the curves to determine the ETR

 # Create distribution of MEC

```
MEC_CFZ <- read.csv("")
MEC_CFZ # show data

MEC_Fit_CFZ_Gamma <- fitdist(MEC_CFZ$MEC, "gamma") # fit gamma distribution
                                                    over MEC data
plot(MEC_Fit_CFZ_Gamma) # plot distribution

MEC_Fit_CFZ_lnorm <- b(MEC_CFZ$MEC, "lnorm") # fit log normal distribution
                                              over MEC data
plot(MEC_Fit_CFZ_lnorm) # plot distribution

MEC_Fit_CFZ_norm <- fitdist(MEC_CFZ$MEC, "norm") # fit normal distribution
                                                  over MEC data
plot(MEC_Fit_CFZ_norm) # plot distribution

MEC_Fit_CFZ_exp <- fitdist(MEC_CFZ$MEC, "exp") # fit exponential distribution
                                                over MEC data
plot(MEC_Fit_CFZ_exp) # plot distribution

MEC_Fit_CFZ_logis <- fitdist(MEC_CFZ$MEC, "logis") # fit logistic distribution
                                                    over MEC data
plot(MEC_Fit_CFZ_logis) # plot distribution

MEC_Fit_CFZ_weibull <- b(MEC_CFZ$MEC, "weibull") # fit weibull distribution
                                                  over MEC data
plot(MEC_Fit_CFZ_weibull) # plot distribution
```

 # Establish best fitting distribution

```
cdfcomp(list(MEC_Fit_CFZ_Gamma, MEC_Fit_CFZ_lnorm, MEC_Fit_CFZ_norm, MEC_Fit_CF
Z_exp, MEC_Fit_CFZ_logis, MEC_Fit_CFZ_weibull), legendtext=c("Gamma", "Lognorm", "nor
m", "exponential", "logis", "Weibull")) ") # compare empirical cumulative distributions
                                            against fitted distributions

denscomp(list(MEC_Fit_CFZ_Gamma, MEC_Fit_CFZ_lnorm, MEC_Fit_CFZ_norm, MEC_Fit_
CFZ_exp, MEC_Fit_CFZ_logis, MEC_Fit_CFZ_weibull), legendtext=c("Gamma", "Lognorm", "n
orm", "exponential", "logis", "Weibull # compare histograms against fitted distributions

qqcomp(list(MEC_Fit_CFZ_Gamma, MEC_Fit_CFZ_lnorm, MEC_Fit_CFZ_norm, MEC_Fit_CF
Z_exp, MEC_Fit_CFZ_logis, MEC_Fit_CFZ_weibull), legendtext=c("Gamma", "Lognorm", "nor
m", "exponential", "logis", "Weibull")) # compare empirical and theoretical quantiles

ppcomp(list(MEC_Fit_CFZ_Gamma, MEC_Fit_CFZ_lnorm, MEC_Fit_CFZ_norm, MEC_Fit_CF
Z_exp, MEC_Fit_CFZ_logis, MEC_Fit_CFZ_weibull), legendtext=c("Gamma", "Lognorm", "nor
m", "exponential", "logis", "Weibull"))")) # compare empirical and theoretical probabilities

gofstat(list(MEC_Fit_CFZ_Gamma, MEC_Fit_CFZ_lnorm, MEC_Fit_CFZ_norm, MEC_Fit_CFZ
_exp, MEC_Fit_CFZ_logis, MEC_Fit_CFZ_weibull), fitnames=c("Gamma", "Lognorm", "norm", "
exponential", "logis", "Weibull"))# compare model fitting scores
```

 # Create modelled distribution and data frame for plotting

```
x_vector <- seq(0.01,1000, length.out = 100000) # vector for concentrations
MEC_CFZ <- dlnorm(x_vector, MEC_Fit_CFZ_lnorm$estimate[1], MEC_Fit_CFZ_lnorm$estima
te[2]) # Create density distribution
plot(x_vector, MEC_CFZ, type = "l", log="x", ylim = c(0, 1)) # Plot distributions to x vector

MECdata_CFZ <- data.frame(MEC_CFZ) # make into a dataframe
MECdata_CFZ$x_vector <- x_vector # add x-values
```

 # Make SSD using EC10

Use 95% CI as censored data to create a cumulative distribution for SSD

```
df_CFZ <- read.csv("")

df_CFZ <- df_CFZ[order(df_CFZ$Conc), ] # Order EC10s
df_CFZ$frac <- ppoints(df_CFZ$Conc, 0.5) # Probability
df_CFZ_cens <- data.frame(left = df_CFZ$Low.CL, right = df_CFZ$High.CL)
# create dataframe of censored data

fit2_CFZ_lnorm <- fitdistcens(df_CFZ_cens, 'lnorm') # fit the censored data
                                                    using log normal distribution

fit2_CFZ_norm <- fitdistcens(df_CFZ_cens, 'norm') # fit the censored data
                                                  using normal distribution

fit2_CFZ_exp <- fitdistcens(df_CFZ_cens, 'exp') # fit the censored data
                                                using exponential distribution

fit2_CFZ_logis <- fitdistcens(df_CFZ_cens, 'logis') # fit the censored data using
                                                    logistic distribution

fit2_CFZ_weibull <- fitdistcens(df_CFZ_cens, 'weibull') # fit the censored data using
                                                        weibull distribution

fit2_CFZ_Gamma <- fitdistcens(df_CFZ_cens, 'gamma') # fit the censored data using
                                                    gamma distribution
```

 # compare models

```
cdfcompcens(list(fit2_CFZ_lnorm, fit2_CFZ_norm, fit2_CFZ_exp,
            fit2_CFZ_logis,fit2_CFZ_weibull, fit2_CFZ_Gamma),
          legendtext=c("Lognorm", "norm", "exponential", "logis", "Weibull", "Gamma"))
```

 # create AIC and LogLik tables for distribution comparison

```
AICtable <- matrix(c(fit2_CFZ_lnorm$aic,fit2_CFZ_norm$aic,fit2_CFZ_exp$aic,
                fit2_CFZ_logis$aic,fit2_CFZ_weibull$aic, fit2_CFZ_Gamma$aic),ncol=6,byrow=FA
LSE)
colnames(AICtable) <- c("Lognorm", "norm", "exponential", "logis", "Weibull", "Gamma")
rownames(AICtable) <- c("AIC")
AICtable <- as.table(AICtable)
AICtable

Logliktable <- matrix(c(fit2_CFZ_lnorm$loglik,fit2_CFZ_norm$loglik,fit2_CFZ_exp$loglik,
                fit2_CFZ_logis$loglik,fit2_CFZ_weibull$loglik,fit2_CFZ_Gamma$loglik),ncol=6,byr
ow=FALSE)
colnames(Logliktable) <- c("Lognorm", "norm", "exponential", "logis", "Weibull", "Gamma")
rownames(Logliktable) <- c("Loglik")
Logliktable <- as.table(Logliktable)
Logliktable
```

 # Run bootstraps and obtain new distribution parameters

```
fit2_boot_CFZ <- bootdistcens(fit2_CFZ_Gamma, niter = 5000) # bootstrap the distribution
quantile(fit2_boot_CFZ, probs = 0.05) # Extract 5th percentile and credible intervals

summary(fit2_boot_CFZ) # summarise the distribution parameters
```

 # Build dataframes for graph from distributions

```
x_Conc <- 10^(seq(log10(0.01), log10(1000), length.out = 1000)) # create x vector
pp_CFZ <- apply(fit2_boot_CFZ$estim, 1, function(x) pgamma(x_Conc, x[1], x[2]))
# fit bootstrapped distribution to x vector
bootdat_CFZ <- data.frame(pp_CFZ)  # convert into dataframe

bootdat_CFZ$x_Conc <- x_Conc # add x-values
bootdat_CFZ <- melt(bootdat_CFZ, id.vars = 'x_Conc') # bring to long format
cis_CFZ <- apply(pp_CFZ, 1, quantile, c(0.025, 0.975), na.rm = TRUE) # get CI from
                                                                     bootstraps

rownames(cis_CFZ) <- c('lwr' ,'upr')
pdat_CFZ <- apply(pp_CFZ, 1, median, na.rm = TRUE) # get CI from bootstraps
pdat_CFZ <- data.frame(pdat_CFZ)
pdat_CFZ$x_Conc <- x_Conc # add x-values
pdat_CFZ <- melt(pdat_CFZ, id.vars = 'x_Conc') # bring to long format

pdat_CFZ <- cbind(pdat_CFZ, t(cis_CFZ)) # add CI
df_CFZ$fit <- 10^(log10(df_CFZ$Conc) + 0.2) # add x coordinates for species names from fitted
                                              values
```

 # Solve CDF in respect to concentration to get fraction affected at particular concentrations

```
T<-function(x) {b(x, fit2_boot_CFZ$CI[1,1],fit2_boot_CFZ$CI[2,1])}

Create values for PNECs and MECs
PNECL_CFZ <- 0.15 # PNEC lowest
PNECA_CFZ <- 0.15 # PNEC A. flos-aquae
PNECS_CFZ <- 4.53 # PNEC S. leopoldensis
PNECR_CFZ <- 1 # PNEC resistance
MECm_CFZ <- 0.15 # MEC median
MECh_CFZ <- 42.93 # MEC highest

T(PNECL_CFZ)
T(PNECA _CFZ)
T(PNECS _CFZ)
T(PNECR _CFZ)
T(MECm _CFZ)
T(MECh _CFZ)
```

 # Plot of SSD and relevant data (PNECs and MECs)

```
CFZ_Plot <-
  ggplot()+
  geom_line(data = bootdat_CFZ, aes(x = x_Conc, y = value, group = variable), col = '#e0f3f8', a
lpha = 0.05) + # bootstraps
  geom_point(data = df_CFZ, aes(x = Conc, y = frac)) + # data points
  geom_line(data = pdat_CFZ, aes(x = x_Conc, y = value), col = 'red') + # SSD/CDF
  geom_line(data = pdat_CFZ, aes(x = x_Conc, y = lwr), linetype = 'dashed') + # Lower CI
  geom_line(data = pdat_CFZ, aes(x = x_Conc, y = upr), linetype = 'dashed') + # Higher CI
  geom_text(data = df_CFZ, aes(x = fit, y = frac, label = Species), hjust = 0, size = 3) + # data la
bels
  theme_bw() +
  scale_x_log10(breaks = c(0.01, 0.01, 0.1, 1, 10, 100, 1000), limits = c(0.01, 3000)) +
  scale_y_continuous(limits = c(0,1)) +
  labs(x = expression(paste('Concentration of cefazolin (', mu, 'g ', L^-1,')')),
       y = 'Fraction of species affected') +
  theme_bw() +
  theme(panel.grid.major = element_blank(), panel.grid.minor = element_blank()) +
  theme(text=element_text(family="Arial")) +
  #geom_vline(xintercept = PNECL_CFZ, linetype = 4, colour = "#fc8d59") + # Lowest PNEC fro
m my results
  geom_vline(xintercept = PNECA_CFZ, linetype = 2, colour = "#fc8d59") + # ANA1 PNEC from
my results
  geom_vline(xintercept = PNECS_CFZ, linetype = 3, colour = "#fc8d59") + # SYN1 PNEC from
my results
  geom_vline(xintercept = PNECR_CFZ, linetype = 2, colour = "#2c7bb6")  # Resistance PNEC f
rom AMR industry alliance
CFZ_Plot
```

 # Integrate the distributions to get ETR

```
F <- function(x) {dlnorm(x, meanlog = MEC_Fit_CFZ_lnorm$estimate[1], sdlog = MEC_Fit_CFZ
_lnorm$estimate[2]) * pgamma(x, fit2_boot_CFZ$CI[1,1], fit2_boot_CFZ$CI[2,1])}
c <- integrate( F, lower = 0, upper = 1000000)
c
```

This code was partially adapted from both the MOSAIC SSD platform (http://pbil.univ-lyon1.fr/software/mosaic/ssd) and from Eduard Szoecs (https://edild.github.io/ssd/).

Kon Kam King, G., *et al*. (2014). "MOSAIC_SSD: A new web tool for species sensitivity distribution to include censored data by maximum likelihood." Environmental Toxicology and Chemistry 33(9): 2133-2139.

Species sensitivity distributions based upon NOEC data

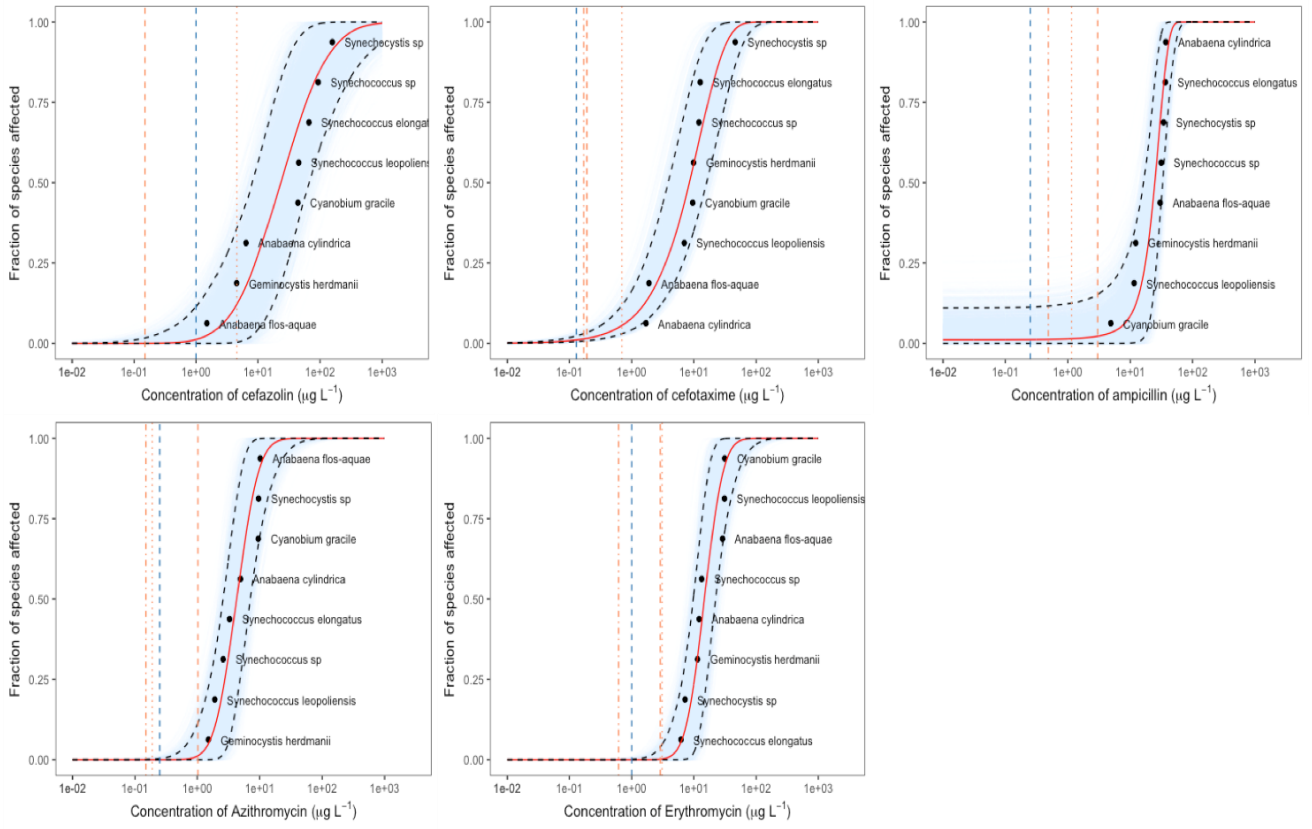

Figure S1. Species sensitivity distributions of cyanobacteria based upon no observed effect concentrations exposed to five antibiotics. Cefazolin and cefotaxime (cephalosporin), ampicillin (penicillin), azithromycin and erythromycin (macrolide). Red line indicates the modelled species sensitivity distribution. Dashed black lines represent upper and lower 95% confidence limits. Blue shaded area indicates results of bootstrapped distributions. Orange vertical lines indicate predicted no effect concentrations (PNEC): Dot-dash orange line= PNEClowest; Dashed orange line = PNECA. flos-aquae; Dotted orange line = PNECS. leopoldensis; Solid blue line = PNECR as described by the Antimicrobial Resistance Industry Alliance.

Antibiotic	Protection limit	Concentration (µg/L)	Lower 95% CI	Higher 95% CI	Fraction of cyanobacteria affected (%)
Cefazolin	HC5	2.33	0.41	10.62	5.00
	$PNEC_{Lowest}$	0.15	-	-	0.01
	$PNEC_{A.\ flos\text{-}aquae}$	0.15	-	-	0.01
	$PNEC_{S.\ leopoldensis}$	4.53	-	-	11.94
	$PNEC_R$	1.00	-	-	1.17
Cefotaxime	HC5	0.63	0.28	1.17	5.00
	$PNEC_{Lowest}$	0.17	-	-	1.38
	$PNEC_{A.\ flos\text{-}aquae}$	0.19	-	-	1.55
	$PNEC_{S.\ leopoldensis}$	0.70	-	-	5.58
	$PNEC_R$	0.40	-	-	1.06
Ampicillin	HC5	7.05	-6.62	19.08	5.00
	$PNEC_{Lowest}$	0.49	-	-	1.18
	$PNEC_{A.\ flos\text{-}aquae}$	3.00	-	-	2.11
	$PNEC_{S.\ leopoldensis}$	1.15	-	-	1.38
	$PNEC_R$	0.25	-	-	1.11
Erythromycin	HC5	6.02	3.15	11.13	5.00
	$PNEC_{Lowest}$	0.62 *	-	-	0.00
	$PNEC_{A.\ flos\text{-}aquae}$	2.90	-	-	0.13
	$PNEC_{S.\ leopoldensis}$	3.10	-	-	0.19
	$PNEC_R$	1.00	-	-	0.00
Azithromycin	HC5	1.50	0.66	3.04	5.00
	$PNEC_{Lowest}$	0.15 *	-	-	0.00
	$PNEC_{A.\ flos\text{-}aquae}$	1.02	-	-	1.26
	$PNEC_{S.\ leopoldensis}$	0.19	-	-	0.00
	$PNEC_R$	0.25	-	-	0.00

Table S1. Protection limits; 5% hazardous concentration (HC5) based upon a species sensitivity distribution (SSD) using no observed effect concentrations (NOEC), predicted no effect concentrations (PNECs) and the fraction of cyanobacteria affected based upon the cyanobacteria SSD. PNECs determined as specified in current environmental risk assessment. $PNEC_{Lowest}$ represents the PNEC based on the most sensitive cyanobacteria in the microplate assays. $PNEC_{A.\ flos\text{-}aquae}$ and $PNEC_{S.\ leopoldensis}$ are based on the data of species recommended in the OECD 201 test guideline (OECD 2011). $PNEC_R$ is the PNEC for resistance as described by the Antimicrobial Resistance Industry Alliance. * $PNEC_{Lowest}$ for erythromycin is < 0.62 and < 0.15 for azithromycin. ** CI was determined to be <0.

Antibiotic	Expected total risk (%)	Measured concentration	Concentration (µg/L)	Fraction of cyanobacteria affected (%)
Cefazolin	6.75	Median	0.15	0.01
		Highest	42.93	67.11
Cefotaxime	2.21	Median	0.04	0.31
		Highest	41.90	96.78
Ampicillin	6.33	Median	0.10	1.07
		Highest	263.30	100.00
Erythromycin	0.13	Median	0.06	0.00
		Highest	7.84	11.89
Azithromycin	1.53	Median	0.09	0.00
		Highest	9.70	90.00

Table S2. Expected total risk, measured environmental concentrations (MEC) and the fraction of cyanobacteria affected based upon the cyanobacteria species sensitivity distributions using no observed effect concentrations and MECs obtained from Umweltbundesamt's 'Pharmacuiticals in the environment' database (Umwelt bundesamt 2018).

Table of distributions used for species sensitivity and measured environmental concentrations distributions

Antibiotic	SSD distribution NOEC	SSD distribution EC_{10}	MEC distribution
Cefazolin	Log normal	Gamma	Log normal
Cefotaxime	Exponential	Exponential	Log normal
Ampicillin	Normal	Normal	Log normal
Erythromycin	Log normal	Log normal	Log normal
Azithromycin	Log normal	Log normal	Log normal

Table S3. Distributions used in the probabilistic modelling of the measured environmental concentrations (MEC) and species sensitivity distributions (SSD) based upon the no observed effect concentrations (NOEC) and 10% effective concentrations (EC_{10}).

Chapter 7

Discussion

As a result of antibiotic use in human and veterinary medicine antibiotics are regularly found in aquatic environments, including surface waters, ground waters, rivers, estuaries, marine waters and sediments (Carvalho and Santos 2016; Gaw *et al*. 2014; Kümmerer 2009; Pei *et al*. 2006). They are also found across terrestrial matrices, especially soils (Kinney *et al*. 2006; Tolls 2001). Antibiotics are designed/selected to target pathogenic bacteria but they may also adversely affect non-pathogenic bacteria that perform a wide range of roles in the normal functioning of healthy ecosystems. Despite this, our knowledge of how antibiotics may affect microbial communities is limited. Furthermore, microbial representation in environmental risk assessment (ERA) is severely lacking, bringing into question the ability of ERA to establish adequate protection limits for these hugely important organisms (Agerstrand *et al*. 2015; Brandt *et al*. 2015; Le Page *et al*. 2017).

The work presented in this thesis demonstrates that the current ERA of antibiotics may not always be protective of bacterial populations in the environment and highlights the knowledge gaps and uncertainties in the current regulatory practice. In chapters 2, 5 and 6 it is shown that reliance upon a single cyanobacteria species in the ERA to represent all prokaryotic diversity means that protection limits can fall short of protecting sensitive bacterial taxa, even when an assessment factor of 10 is applied to account for interspecies sensitivity. We demonstrate that including a wider range of cyanobacteria assays would go some way to improving ERA, but that additional testing on different bacterial clades may be necessary to ensure truly effective protective limits. In this work, I suggest that this could include the use of pre-clinical minimum inhibitory concentrations (MIC) data.

In chapters 2 and 3 it is demonstrated that antimicrobial (AMR) protection limits need to be considered equally to that of ecotoxicological limits because neither are always been protective of the other using currently available methods. The findings from this research have already been used whereby the ‘AMR Industry Alliance’ have now proposed to use both ecotoxicological and AMR data for

establishing antibiotic manufacturing discharge limits (AMR Industry alliance 2018).

In the last empirical data chapter (chapter 6) we provide further evidence that a protection limit (in this case a predicted no effect concentration (PNEC)) based upon the no observed effect concentration (NOEC) and an assessment factor of 10 may be over or under protective depending on the species selected for testing, the class of antibiotic and the magnitude of interspecies variability in sensitivity. Finally, the expected total risk from antibiotics on environmental cyanobacteria populations was explored. In this work it was found that for the five antibiotics analysed, there is a limited risk based on the species sensitivity distributions of the eight species of cyanobacteria tested in the microplate assays conducted in chapter 5. However, for those environments with high antibiotic concentrations, such as hospital and waste water treatment effluents, there is likely to be adverse effects on cyanobacteria populations. It is important to highlight however, that the risk calculated in chapter 6 reflects a limited dataset of MECs that represent a wide variety of matrices and geographic locations. Thus, although the expected total risk and fraction of affected species at higher MECs are useful for comparing between antibiotics and getting a snapshot of antibiotics/potential locations or discharge sources for further investigation, they can not be interpreted in the context of ERA or environmental management.

Screening assays and their application

In order to experimentally examine the findings of our meta-analysis that testing on a single species of cyanobacteria may not allow for adequate estimation of a protection limit, we needed to develop an assay that allowed for the direct comparison of growth rate inhibition across a range of cyanobacteria (chapter 4). Growth inhibition studies on cyanobacteria are generally lacking in the literature (chapter 2) and there are even less examining interspecies differences in sensitivity. The notable exceptions to this were the studies by (Ando *et al*. 2007; Dias *et al*. 2015; Guo *et al*. 2016). The former tested eight species of cyanobacteria and, as we found in chapter 5, also recorded large variability between species of over 2 orders of magnitude. A limit in the work of Ando *et al* however, was the fact that no chemical analysis was conducted to confirm the exposure concentrations. The study by (Guo *et al*. 2016) on the other hand had

supporting chemistry but only tested two cyanobacteria species alongside other primary producers. (Dias *et al*. 2015) also showed high interspecies variability but based their results upon minimum inhibitory concentrations that aren't directly comparable to the endpoints used for ERA due to being at different ends of the dose-response curve.

The microplate assay was designed (and validated against the reference toxicant potassium dichromate) with the aim for it to be i) comparable to traditional shake flask methods and ii) to enable a faster throughput compared with the more cumbersome shake flask system. We also adjusted the exposure period for each individual species so that the assay was conducted, as far as possible, during the balanced growth phase and under the same environmental conditions. This allowed for a direct interspecies comparison that was both environmentally relevant (all species are exposed to the same environmental conditions rather than a range optimised for laboratory growth) and in line with internationally recognised test guidelines (OECD 2011). Adopting this assay approach however required performing the assays under a constant light regime and at a temperature of 28°C, thus lacking direct environmental realism. This however was seen as an acceptable trade-off to achieve balanced growth in a timeframe comparable to the more traditional 72 hour shake flask test.

The exposure studies conducted in this thesis were limited in duration and to only one part of the growth cycle (balanced growth). It cannot be excluded therefore that longer-term effects might arise from exposure to the antibiotics that were not detected here or whether other phases of the growth cycle are affected. Imamura *et al*. (2005) found that the pathogen *Pseudomonas aeruginosa* was not as sensitive to azithromycin in the exponential growth phase when compared with in the stationary growth phase; in the stationary growth phase the minimum bactericidal concentration was over two orders of magnitude lower compared with the growth phase; 1 µg/mL versus 128 µg/mL, respectively. This finding may have serious consequences for ERA where, as discussed in chapters 1 and 4, although there is a clear advantage in terms of reliability and reproducibility for running assays with cultures in balanced growth, the results may not always reflect well the most sensitive parts of the growth cycle. This requires further research that may identify more suitable test

methodologies or the need to incorporate additional assessment factors in establishing protection limits in order to compensate for this uncertainty.

Although consideration of the limitations of the assay systems is important, they are likely to be much less significant compared with the limitations any laboratory based, single species study has when used to extrapolate the assay results to microbial communities in the field. In this case there are the additional complexities to be considered including resource limiting conditions, biological and ecological processes (predation, competition etc) and multiple environmental stressors (temperature changes, toxic mixtures, disturbance etc). The assessment factor of 10 used in establishing a protection limit is intended to acknowledge this uncertainty but, as demonstrated in chapters 2 and 5, it may be insufficient to protect against interspecies sensitivity alone, before considering these additional differences in sensitivity that might arise due to the complexities of what may be occurring within real life microbial communities.

The implications of the thesis findings for ERA

The work in this thesis, coming from both the meta-analysis and the experimental testing of the selected antibiotics (chapters 2 and 5), clearly demonstrates shortcomings of current ERA for the protection of cyanobacteria populations (and most likely other bacterial taxa).

The experimental work in chapter 5 found that it was the case that the large difference observed in species sensitivity through the meta-analysis (chapter 2) was not seen of the same level (up to and over 5 orders of magnitude) with the species assayed. This may be a reflection of the variety of methodologies adopted in the meta-analysis compared to our assays used in chapter 5 that may have been superior in terms of consistency (and species run under the same conditions, by a single experimenter, in the same laboratory). Nevertheless, this does not mean that the greater variation in responses in cyanobacteria than found in our laboratory based analysis doesn't actually occur because the meta-analysis included a wider range of species. To illustrate this, in the meta-analysis the most sensitive species tended to be *Microcystis aeruginosa* but we were unable to get this species into balanced growth in the microplate assay within 72 hours and thus it was not tested. Furthermore, some of the least sensitive cyanobacteria in the meta-analysis

were from the *Nostoc* genus and this genus was not represented at all in our assays (Ando *et al*. 2007; Le Page *et al*. 2017). This difference across chapters 2 and 5 prompted the investigation in to species sensitivity distributions (SSD) in chapter 6. From this work, it was concluded that using an SSD based on mode of action (MoA) relevant species offers a valuable approach in ERA for seeking to obtain more reliable protection limits that don't under or over estimate the effect level. An SSD approach incorporating more bacterial species therefore has a better chance of setting adequate protection limits compared with the current approach of calculating a NOEC (with the limitations associated with it (Green *et al*. 2013)) and adding what is essentially an arbitrary assessment factor.

My thesis work compared the NOEC, 10% and 50% effective concentrations (EC_x) with the MIC of clinically relevant bacteria as found in the EUCAST database (EUCAST). The rationale for this work was that ERA would be better served via the addition of more bacterial diversity, but very little has been published regarding the effect of antibiotics on environmental bacteria that is useful in the context of ERA (chapter 2). The clinical data from the EUCAST database provides an extensive source of effect data upon a wide range of bacteria, but does have the limitation that a direct comparison is not possible given that the endpoints (NOEC and MIC) represent different ends of the dose-response curve and this must be taken into consideration (chapter 3). It was hypothesised that for some antibiotics the clinically relevant Gram-positive and negative bacteria would have been more sensitive than cyanobacteria species (chapters 2, 5 and 6). Assuming these pathogenic bacteria may be representative of other Gram-positive and negative bacteria we concluded that cyanobacteria may not adequately represent the bacterial taxa as a whole and an increase in bacterial diversity to incorporate other bacterial taxonomic clades is required. Further work in identifying suitable bacteria with a wide diversity would be hugely beneficial. Additionally, it would be of great value if the data for clinically relevant bacteria (CRB) in the EUCAST database included the whole dose-response curve rather than just the MIC, allowing for more sophisticated analysis for ecotoxicological testing and AMR.

In pesticide regulations, if the active ingredient is a herbicide multiple MoA relevant algal species must be tested from across broader sensitive taxonomic

clades, including green algae, a diatom and a macrophyte (EFSA 2013; US EPA 2017), although they do not necessarily require an SSD to be modelled based on the data. An approach such as this, where testing is performed using a variety of MoA relevant species, ideally from a variety of bacterial taxonomic clades, for the ERA of antibiotics would be a significant step forwards to gaining more confidence around the protection limits established currently.

The need for an increase in bacterial diversity in the ERA of antibiotics can be exemplified using the differences in cell wall structure between bacterial clades. The permeability, or lack of permeability, of the cell wall is likely to be a key determinate in the sensitivity of a bacterial species to an antibiotic (chapter 5). For example, sensitivity may be driven by the species-specific constitutional makeup of the outer membrane of Gram-negative bacteria and cyanobacteria that restricts hydrophobic molecules such as most macrolides (Delcour 2009; Stock 2005); or due to the presence of porins that allow the passage of smaller molecules such as β-lactums across the membranes. Indeed, it has been demonstrated that the cyanobacteria, *Synechocystis sp*., had an outer membrane that was 20 times less permeable than that of E.coli (Kowata *et al*. 2017). The difference in the outer membrane of Gram-negative and cyanobacteria is of particular importance when considering the effectiveness of the approach of ERA to protect prokaryotic populations, communities and their functions. If cyanobacteria have intrinsically less permeable membranes than Gram-negative bacteria, a protection limit based upon a single cyanobacteria growth inhibition test may be unlikely protect Gram-negative bacteria, at least for antibiotics that would normally cross Gram-negative membranes through porins. This could potentially lead to the disproportionate loss of more sensitive Gram-negative bacteria in a community and the selection of more resistant Gram-negative species and cyanobacteria. This will be further complicated since bacterial communities are comprised of other bacteria clades with variable cell envelopes that have different structures and properties. For example, Gram-positive bacteria, lack an outer membrane in their cell envelope structure and it is therefore reasonable to assume that there may be even greater differences in uptake and thus antibiotic sensitivity between Gram-positive bacteria and those that have an outer membrane such as cyanobacteria and Gram-negative bacteria. It is of critical importance to understand the implications of these different bacterial features to identify if particular

species/clades are likely to be particularly sensitive and thus confidently protect prokaryotic species in the environment. Further research is urgently required to examine if it is reasonable to base a protection limit that aims to be protective of all bacteria on a single cyanobacteria growth inhibition test where the bacteria it should represent may have considerable morphological and physiological differences.

Further differences in sensitivity may occur due to the various morphological forms of bacteria, such as filamentous compared to single celled bacteria. Some investigators have shown that the outer membrane of filamentous cyanobacteria, specifically those with heterocysts, is both continuous (surrounding all cells and producing a shared periplasm) and has decreased permeability in order to prevent the loss of metabolites, such as sucrose, that are to be shared between cells (Flores *et al*. 2006; Nicolaisen *et al*. 2009a; Nicolaisen *et al*. 2009b). As such, it is plausible that uptake of antibiotics into these species may be more restrictive than for single cells for which the outer membrane maybe less restrictive. I don't however see any strong evidence to support this hypothesis within our data in chapter 5, where of the eight cyanobacteria the two *Anabaena* species were filamentous but also some of the more sensitive species to some antibiotics.

In chapter 5 we also found that the MoA might explain the interspecies differences in sensitivity observed and, with further investigation, may also allow the identification of particular bacterial clade(s) that will be particularly sensitive. Further research such as genome analysis on the presence and similarity of drug targets of this kind may potentially allow for a more intelligent testing approach in ERA in the future that targets the most vulnerable species.

Finally, this thesis also shows that the requirement to test antibiotics on MoA irrelevant fish and invertebrate species is unnecessary and that, in accordance with the 3Rs (Hutchinson *et al*. 2016), the environment would be better served by directing resources to test on a greater diversity of bacterial species for improving ERA.

Protection from antibiotics in the environment–the wider picture on what is still lacking?

The many assumptions required in testing a single species of bacteria is hugely limiting in the current ERA for antibiotics. The suggestions in this thesis for increasing bacterial diversity in the toxicity testing and establishing a SSD will go some way to increasing confidence for establishing protection limits, but I now attempt to consider how this extrapolates to real world microbial communities. For example, whether the ecological functions served by these bacteria are protected in the current ERA process.

At this point it is perhaps worth highlighting that protection goals in chemical regulations tend to be vague and it is difficult to ascertain whether we should aim to for the protection of ecosystem function or biodiversity more generally (Brown *et al*. 2017). This is an important distinction because if the aim is for the protection of function it is theoretically possible to lose species providing there is sufficient functional redundancy in the community to continue normal functioning of the ecosystem. If however, the aim is to protect biodiversity or community structure, or a given percentage of them (e.g. 95%), the protection of function may be inherently included (Backhaus *et al*. 2012), although this hypothesis will be examined further below. In chapter 3 it was argued that in the absence of knowledge regarding functional redundancy in communities, together with huge uncertainty regarding the consequences of chronic exposure to multiple stressors, that a conservative approach that aims to protect biodiversity is preferable to best ensure the protection of function. The protection of biodiversity rather than function is also in line with recent initiatives such as the European commissions biodiversity strategy (European Commission 2011). This would mean however, that in some circumstances the protection limit will be overprotective where sensitive species are not present due to the location being an unsuitable habitat (Brown *et al*. 2017).

Due to recent technological advances in genomics and phylogenetics, the effects of chemicals on microbial community diversity and structure can be assessed more easily than ever before. It is clear that antibiotics generally have a negative effect on microbial diversity and can affect community structure in most matrices: for example soil (Cui *et al*. 2014), aquatic biofilms (Proia *et al*. 2013) and sediment (Laverman *et al*. 2015). But due to the inherent complexity

involved, few studies have been able to unpick the effects that antibiotics have on ecosystem functions and biogeochemical processes. There is however, an increasing body of evidence suggesting links between antibiotic exposure, community structure and ecosystem function with recent reviews of the literature showing that antibiotic effects on ecological functions have been often observed, albeit much of this research has been directed towards soil communities and parts of the nitrogen cycle, in laboratory controlled conditions (anaerobic ammonium oxidation, nitrification and denitrification) (Ding and He 2010; Grenni *et al*. 2018; Roose-Amsaleg and Laverman 2016). To illustrate some of these findings, exposure to ciprofloxacin has been shown to affect soil microbial communities by decreasing the bacteria to fungal ratio and increasing the ratio of Gram-positive versus Gram-negative bacteria, in turn leading to a decrease in denitrification rates at high antibiotic concentrations and an increase at lower concentrations (Cui *et al*. 2014). In a statistical analysis of 82 datasets Graham *et al*. (2016) found that incorporating community structure into models explaining environmental carbon and nitrogen processes increased the explanatory power of the model. Studies on the human gut have shown that two antibiotic courses of ciprofloxacin over a ten month period caused a loss of diversity and changed the microbial community structure in the human gut after the initiation of each course (Dethlefsen and Relman 2011). This was subsequently followed by a partial, but incomplete recovery to the original community following completion of the course. Thus, repeated antibiotic exposure here led to an alternative stable community, the functional consequences of which, however, are not known (Dethlefsen and Relman 2011). Given the vast number of clinical studies on the effects of antibiotics on microbial communities perhaps more of this knowledge can be used to supplement the limited environmental evidence on how antibiotic exposure might affect microbial communities and their functions. It should be emphasised that most studies investigating the effects of antibiotics on ecosystem functions have adopted concentrations that are environmentally unrealistic and closer to known therapeutic doses and there is a need for future investigations on chronic exposures at environmentally realistic concentrations (Roose-Amsaleg and Laverman 2016). There are a few cases however where investigators have demonstrated functional affects for antibiotics exposure at environmentally relevant concentrations. Examples of this include the negative affects on

microbial denitrification rates (47% inhibition at 1.2µg/L of sulfamethoxazole (Underwood *et al*. 2011) and 17% inhibition at 10µg/L of sulfamethazine (Ahmad *et al*. 2014)). These affects on denitrification, however, do not appear to occur for all antibiotics, or at least not in all microbial communities or environmental scenarios (Laverman *et al*. 2015).

The magnitude of an antibiotic effect upon ecosystem function will depend on the level of functional redundancy in the community, but this is not well understood in microbial communities and may vary over time and space (Antwis *et al*. 2017). Some investigators have indicated high functional redundancy (Dopheide *et al*. 2015; Frossard *et al*. 2011) whilst others report lower levels of functional redundancy (Galand *et al*. 2018; Strickland *et al*. 2009; Wang *et al*. 2011). Indeed, it is likely that the levels of functional redundancy will depend, in part, on which functions are measured, and including the type of ecosystem and the local environmental conditions (biotic and abiotic). Broad functional endpoints, such as oxygen respiration, that are performed by many taxa will have greater redundancy than for more specialised functions, such as the transformation of specific contaminants (Louca *et al*. 2018). If the protection goal of ERA is to protect ecosystem function, the challenge is to identify and protect those functional groups that may have more limited redundancy, with the additional complexity of the heterogeneous nature of ecosystems.

Above, I state that that the protection of diversity would inherently protect function and would thus be a more conservative approach given the uncertainty involved. Although this true, it is more complicated than simply protecting an arbitrary proportion of the overall diversity (e.g. 95%) after which functional redundancy will compensate and ensure normal ecological functioning. Louca *et al*. (2018) concluded that the taxonomic and functional composition of microbial communities are de-coupled and that the factors that shape taxonomic diversity are different compared with those that may shape functional diversity. This means that there will not always be a link, and certainly not a consistent link across all microbial communities between taxonomic diversity and ecological function, possibly explaining the broad range of results observed in the literature trying to examine this. Protection of diversity in ERA may therefore be insufficient by itself and should be complemented, or perhaps replaced, by functional and community structure endpoints. However, it is

impractical for ERA to consider the vast number of bacterial metabolic functions that contribute to overall ecosystem functioning. Future research is required to establish which ecological functions are representative and applicable in most ecosystems, which functional endpoints are particularly sensitive due to more limited redundancy and, if possible, what proportion of microbial diversity should be protected to ensure no (or minimal) degradation of these ecological functions. This is likely to lead to the requirement of additional functional and community based assays to be incorporated in ERA that are able to address these concerns (Brandt *et al*. 2015; Le Page *et al*. 2017). Furthermore, many bacterial communities in the environment will already be impacted by anthropogenic activities and may already have lost some proportion of biodiversity. It may be that these partially degraded communities will have less functional redundancy than a healthy community in pristine conditions. For example, bacterial communities in agricultural soil, where nutrients have been eroded and replaced with fertilisers may have lower functional redundancy than a forest soil that has less anthropogenic disturbance and much higher organic nutrient content. An open question that needs to be addressed is whether ERA should consider the impact of antibiotics on healthy or compromised communities.

A further consideration that is neglected in the ERA of chemicals (although not always within pesticide ERA) is how a community or ecosystem may recover (or not), which may be driven by redundancy or immigration from outside the affected ecosystem. Using wetland mesocosms Weber *et al*. (2011) showed that although ciprofloxacin reduced bacterial diversity and catabolic activity over the exposure period the microbial communities recovered after a period of 2 to 5 weeks. Interestingly, there was a negative effect on plant health and hydrological parameters in the mesocoms that did not recover within the timescale of the study. Although this study was carried out at a relatively high (but not entirely environmentally unrealistic) concentration of 2mg/L at which it is bactericidal (Silva *et al*. 2011), it demonstrates that both the recovery of the microbial community and wider ecological indirect effects might be important to consider in establishing risk. Furthermore, it is not possible to identify these potential affects within the current approach to ERA that only assesses the growth rate of bacteria in short term single species tests as in our microplate assays (chapter 5). Further research is required to identify the wider

environmental consequences of antibiotic exposure and to assess whether the current protection limits established in ERA are suitably protective of environmental health.

Laboratory based growth inhibition tests on a single bacteria species (as in chapter 5) or even probabilistic modelling of multiple species (chapter 6) fail to address the heterogeneous nature of environmental microbial communities whom experience a multitude of stressors over space and time (chemical mixtures, biological pressures, changes in physical conditions; temperature, hydrological parameters etc). More research is urgently required to identify the long-term risks of persistent, pseudo-persistent and repeated-dose exposure to microbial communities and their ecological functions, especially in the presence of multiple stressors. The bacteriostatic or bactericidal nature of an antibiotic may be of importance in the outcome of such exposures and both should be carefully considered. Additionally, if functional redundancy, in some cases, can mitigate the loss of some of the more sensitive species, we need to identify if this loss of diversity reduces the community and functional resilience to additional stressors that may occur alongside of following antibiotic exposure. The complexity of unravelling these relationships makes such research very challenging, but recent scientific advances in genomics, metagenomics and statistical modelling provide the tools in which to begin addressing these questions (Faust *et al*. 2015; Li *et al*. 2016; Muller *et al*. 2018).

A final consideration when assessing the impacts of antibiotics on microbial communities is that these communities are also comprised of non-bacterial species and that there will also be indirect effects upon these non-bacterial species and thus the structure, diversity and potential ecological functioning of the community as a whole. This again is not considered in current ERA except for the justification of the arbitrary assessment factor of 10. For example, exposure to tetracycline, sulfamonomethoxine and an antibiotic mixture were shown to increase the proportion of fungi in soil communities relative to bacteria (Lin *et al*. 2016). Changes in this ratio can have significant functional effects on microbial communities such as carbon sequestration, decomposition and available nitrogen (in the form of ammonium or nitrates) (Strickland and Rousk 2010). Archaea are another significant part of microbial communities and although little is known regarding their functional roles beyond key processes

such as methanogenesis (Moissl-Eichinger *et al*. 2018), any evaluation of the effects of antibiotics upon microbial communities would be incomplete without their consideration. Archaea tend not to be sensitive to antibiotics and those that do show some sensitivity do so at environmentally unrealistic concentrations (Khelaifia and Drancourt 2012). As such, there seems to be limited evidence that antibiotics may directly affect archaeal populations, but disruption to bacteria within these communities by antibiotics may have indirect effects upon them (Khelaifia and Drancourt 2012). Archaea are the only microbes that perform methanogenesis and recent evidence shows that there is little functional redundancy in methanogens and thus disruption to these communities directly or indirectly, could potentially have environmental consequences (Sierocinski *et al*.). This further exemplifies the complex nature of microbial communities and strengthens the need to consider antibiotic effects on microbial communities more broadly, including the associated changes in diversity and structure that may lead to effects on ecosystem functions. The chapters within thesis go some way to examine the role of single species testing for setting protection limits, but it is clear that there are considerable unknowns regarding the extrapolation from these tests to communities in the field.

The incorporation of antimicrobial resistance in ERA

How antimicrobial resistance (AMR) is incorporated into ERA is problematic, with one of the key issues being the lack of empirical methodology from which to be able to assess the concentration at which resistance determinates are selected for. In chapters 2 and 3, PNECs for resistance, as determined by (Bengtsson-Palme and Larsson 2016) and other available experimental data, were compared with PNECs in surface water and we found that neither protection limit was fully protective of the other. From this we concluded that whilst using the current available methodologies both protection limits for ecotoxicology and AMR should be determined within ERA. This approach has since been taken up by the ‘AMR Industry Alliance’ (AMR Industry alliance 2018).

Since publishing chapters 2 and 3, some investigators have published methodologies that may help to fill the lack of empirical data on AMR and further inform the discussion of how to integrate AMR into ERA. For example (Murray *et al*. 2018) measured resistance alleles in a complex bacterial

community and determined a minimum selective concentration (MSC; minimum concentration that selects for AMR) of 0.4 µg/L for cefotaxime. This is comparable to the HC5 obtained from our SSD in chapter 6 of 0.67 µg/L with 95% confidence limits of 0.3 to 1.1 µg/L, although our data in the SSD was based on measured concentrations whilst the MSC was based on nominal concentrations and may be lower in reality. A PNEC for cefotaxime, derived from the most sensitive cyanobacteria in our microplate assays (chapter 5) using the NOEC and assessment factor of 10 would have been 0.17 µg/L and the PNEC for AMR predicted by (Bengtsson-Palme and Larsson 2016) was 0.13 µg/L. These results are thus comparable in terms of effect levels (0.13 – 0.67 µg/L).

The link between AMR and ecological function is unclear, although some investigators have suggested that the promotion of resistance genes have less of an effect on function than changes in community structure (Wu *et al*. 2017). If this is the case, from an ecological perspective, the ecotoxicological consequences of antibiotic exposure maybe a greater concern, although any increase in AMR genes could be a significant concern for human health.

Next steps

There are several key steps that can be taken In order to further develop and expand upon the conclusions drawn throughout this thesis and these are briefly considered below:

- Additional bacterial testing – This thesis focused upon cyanobacteria as it is this bacterial clade that is used in current regulatory procedures. We showed however, using the MICs of Gram-positive and Gram-negative bacteria in the EUCAST database (EUCAST) and findings from other investigators in the literature that there is likely to be vast differences in bacteria sensitivity across bacterial clades. Future testing is required to experimentally confirm this, using methodologies that allow for the direct comparison (comparable endpoints and test conditions) of a much more diverse selection of bacteria. Such testing should also consider how sensitivity differences between bacterial clades may be affected by the antibiotic mode of action. For example, delmanid inhibits mycobacterial cell membranes by preventing the synthesis of methoxy mycolic acid and

ketomycolic acid, two components that are not found in the cyanobacteria or Gram-negative bacteria cell membrane. For this antibiotic, it is likely mycobacterial species will be most suitable for establishing protection limits rather than other bacteria such as cyanobacteria.

- Mixtures – Bacterial communities in the environment are not exposed to just one antibiotic, but rather they are more typically exposed to a cocktail of chemical contaminants. As such, future work needs to consider how mixtures of antibiotics may interact to cause toxicity and whether synergism or antagonism can be predicted. Furthermore, how the toxicity of antibiotics may influence, or be influenced, by other classes of chemical compounds or other additional stressors (both biotic and abiotic) needs to be established.

- Recovery – Current ERA employs tests that tend to have relatively short term, and where possible, consistent dosing of a chemical (i.e. the test concentrations are kept stable over the test period). This is not realistic of the exposure profile organisms have in the environment where chemical concentrations are likely to fluctuate over short time scales (hours/days/weeks) and longer timescales (months/seasons/years), possibly allowing for the recovery of populations. Future work should consider how different dosing regimes might affect the risk posed by antibiotics when their concentrations are in flux.

- Functional redundancy – In this thesis I discuss how protection limits might be impacted by functional redundancy, but as previously mentioned the relationship between redundancy and biodiversity is not well understood. A key future step is to consider this further and to establish which ecological functions may have more limited redundancy in the presence of antibiotics. Following this, assays that reflect these findings are required that can be incorporated into ERA to establish adequate protection limits.

Final thoughts and future perspectives

This thesis has critically analysed the current ERA of antibiotics and found it to be lacking in its assessment for toxic effects on bacteria, their communities and the functions they perform. The work in this thesis includes the development of a simple, medium to high throughput and cost efficient screen that allows for a more effective assessment of cyanobacteria sensitivity to antibiotics with benefits for support in prioritising legacy compounds for further research and enabling environmental assessment earlier within the drug discovery process, with potentially major cost benefits. This thesis work highlights that the impact of antibiotics on environmental communities remains are poorly understood and greater efforts are required to establish how protective ERA is of diversity and ecosystem function. It is also the case that ERA based on the effects of individual species and populations will not necessarily provide a high level confidence for setting protection limits relevant to real life microbial communities.

This thesis work highlights the need for a greater diversity of bacteria taxa in ERA and that this is clearly necessary even before the cumulative effects of antibiotic mixtures and multiple stressors can be truly accounted. Links between effects on bacterial populations to effects on ecosystem functions that they perform is poorly understood and in such extrapolations and assessments careful consideration of functional redundancy within bacterial communities is needed. But here too, the relevant information to do so is generally lacking. It is becoming increasingly obvious that future ERA needs to develop to consider both taxonomic diversity and ecological functions. This will likely involve studies that experimentally test on communities rather than individual populations.

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Thesis appendix

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Review article

Integrating human and environmental health in antibiotic risk assessment: A critical analysis of protection goals, species sensitivity and antimicrobial resistance

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ABSTRACT

Antibiotics are vital in the treatment of bacterial infectious diseases but when released into the environment they may impact non-target organisms that perform vital ecosystem services and enhance antimicrobial resistance development with significant consequences for human health. We evaluate whether the current environmental risk assessment regulatory guidance is protective of antibiotic impacts on the environment, protective of antimicrobial resistance, and propose science-based protection goals for antibiotic manufacturing discharges. A review and meta-analysis was conducted of aquatic ecotoxicity data for antibiotics and for minimum selective concentration data derived from clinically relevant bacteria. Relative species sensitivity was investigated applying general linear models, and predicted no effect concentrations were generated for toxicity to aquatic organisms and compared with predicted no effect concentrations for resistance development. Prokaryotes were most sensitive to antibiotics but the range of sensitivities spanned up to several orders of magnitude. We show reliance on one species of (cyano)bacteria and the 'activated sludge respiration inhibition test' is not sufficient to set protection levels for the environment. Individually, neither traditional aquatic predicted no effect concentrations nor predicted no effect concentrations suggested to safeguard for antimicrobial resistance, protect against environmental or human health effects (via antimicrobial resistance development). Including data from clinically relevant bacteria and also more species of environmentally relevant bacteria in the regulatory framework would help in defining safe discharge concentrations for antibiotics for patient use and manufacturing that would protect environmental and human health. It would also support ending unnecessary testing on metazoan species.

1. Introduction

Antibiotics are crucial in human healthcare. They are used in the treatment of bacterial infectious diseases, supporting surgical interventions, and in cancer and prophylactic treatment. Antibiotics are also used widely in livestock and domestic animal veterinary treatments and as growth promoters in aquaculture. Global production of antibiotics for human use is valued at $40 billion a year (O'Neill, 2015) illustrating their societal and economic importance. Antibiotic consumption is on the rise and between the years 2000 and 2010 there was an estimated 36% increase in use globally for human healthcare (Van Boeckel et al., 2014).

Antibiotics, as other pharmaceuticals, enter the environment via patient and animal use, through manufacturing plants and/or improper disposal. Common points of entry into the environment from human therapeutic use are via effluents from hospitals, domestic sewerage treatment plants, as well as via leachates from landfill sites. Antibiotics can enter into surface waters from sewerage treatment plants directly or they can be transferred via surface run off. Ground waters can be exposed from agricultural land treated with sewage sludge biosolids as a source of fertiliser (Kümmerer, 2009). Veterinary antibiotics enter the aquatic environment either directly, if treated animals are poorly managed and have access to surface water, or via groundwater from the manure of treated livestock (Davies, 2012; Kümmerer, 2009). Antibiotics in surface waters and sewerage treatment plant effluents/wastewaters are generally measured at concentrations ranging between 0.01 and 1.0 μg/L (Batt et al., 2007; Miao et al., 2004; Monteiro and Boxall, 2010; Watkinson et al., 2009). The highest levels of antibiotic

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residues in effluents - in the milligram per litre range, with records in excess of 1000 mg/L - are reported from manufacturing plants in China and India (Larsson, 2014; Larsson et al., 2007; Li et al., 2008; O'Neill, 2015). Hospital effluents too can contain antibiotic residues in the milligram per litre concentration range (Brown et al., 2006; Watkinson et al., 2009).

Antibiotics affect prokaryotic cells via a number of distinct mechanisms of action, including the inhibition of cell envelope synthesis, inhibition of protein synthesis or inhibition of nucleic acid (DNA/RNA) synthesis. Antibiotics are designed for use in the treatment of bacterial infection in humans and livestock and are thus developed to avoid, or limit, effects on mammalian cells. It is, therefore, reasonable to assume that environmental bacteria are more likely to be adversely affected as a result of non-therapeutic exposure compared with aquatic vertebrates, such as fish.

Within Europe, an environmental risk assessment (ERA) is required for a medicine if the predicted environmental concentration exceeds 10 ng/L (EMA, 2006). In the USA effect studies are triggered if the expected environmental concentration exceeds 100 ng/L (US Food and Drug Administration, 1998). The ERA aims to establish the safe concentrations for the protection of wildlife populations, ecosystem structure and function and includes the calculation of three predicted no effect concentrations (PNEC) for aquatic organisms, namely $PNEC_{surfacewater}$ ($PNEC_{SW}$), $PNEC_{microorganism,}$ and $PNEC_{groundwater}$ (EMA, 2006). These are determined by establishing a no observed effect concentration (NOEC, the test concentration at which there is no statistically significant effect in the response being tested, such as on growth rate or reproduction) for a range of aquatic taxa and applying an assessment factor of ten to account for variability in species sensitivity and extrapolation from laboratory data to the field. $PNEC_{microorganism}$ is based on the 'activated sludge respiration inhibition test' (ASRIT, OECD, 2010) and is primarily used to establish risk to microorganisms in (and the function of) sewerage treatment plants. The $PNEC_{groundwater}$ is based on a chronic test with *Daphnia magna* (e.g. OECD 211 test guideline, (OECD, 2012)) and $PNEC_{SW}$ is calculated from the toxicity to three eukaryotic species – a green algae, invertebrate and fish. For antibiotics, in Europe the ERA guidance encourages ecotoxicity testing with prokaryotes rather than a green algae "*as they are [a] more sensitive indicator organisms than green algae*" (EMA, 2006), and this is conducted in one species of cyanobacteria only.

There is concern that the ERA for antibiotics is biased towards testing on metazoan species (invertebrates and fish in this instance), and does not consider fully the possible impacts of antibiotics on microbial community structure, function and resilience (Agerstrand et al., 2015; Brandt et al., 2015). This is a major shortfall considering the fundamental ecosystem services microbial communities provide (e.g. primary production, nutrient cycling, metabolism and degradation of organic, inorganic and synthetic compounds). A major aim of this meta-analysis therefore was to test if current ERA is protective of vulnerable populations in the environment.

Microorganisms exposed to antibiotics at low, sub-lethal or sub-inhibitory exposure concentrations can develop, or acquire, antimicrobial resistance (AMR) and this has been identified as a major threat to public health (Smith and Coast, 2002; World Health Organization, 2014). AMR is likely to persist and disseminate in diverse environments, including in aquatic ecosystems (Laxminarayan et al., 2013; Taylor et al., 2011). Where the benefit of possessing and expressing the resistance gene outweighs the fitness costs of carriage, antibiotics in the environment may select for and enrich resistance genes in bacterial populations/communities which can then harbour these resistance determinants and transfer them to human pathogens (Ashbolt et al., 2013).

To ensure clinical efficacy and protection of human health, minimum inhibitory (growth) concentrations (MICs, the lowest concentration at which there is no observable growth) are monitored in clinically relevant bacteria (CRB) and recorded in the European Committee on Antimicrobial Susceptibility Testing database (http://www.eucast.org). In addition to monitoring MICs in clinically relevant species, studies with clinical isolates have also identified the lowest concentration that will select for AMR, called minimum selective concentrations (MSCs). MSCs are the minimum concentration at which the presence and expression of resistance gene(s) give bacteria a fitness advantage over non-resistant cells of the same species/strain. This can occur at concentrations considerably below the MIC of the non-resistant cells (Gullberg et al., 2011). Indeed, selection may occur at exposures up to two orders of magnitude lower than the MIC for growth (Gullberg et al., 2011; Hughes and Andersson, 2012; Lundström et al., 2016).

From both human and environmental health perspectives, it is important that risk assessment frameworks incorporate the risk of AMR selection. An approach to establish a surrogate PNEC for AMR ($PNEC_R$) has been suggested adopting MICs from CRB, which are available through the European Committee on Antimicrobial Susceptibility Testing database (Bengtsson-Palme and Larsson, 2016). This is the most comprehensive dataset available where theoretical PNECs ($PNEC_{R(T)}$) have been calculated for 111 antibiotics. This approach uses growth (via the MIC) to predict upper boundaries for resistance, although there has been no verification of an increase in resistance determinants. The approach also assumes that the CRB are representative of the diversity of bacteria in nature. Furthermore, whilst AMR maybe enriched at concentrations well below the MIC of clinical bacteria, the AMR enrichment could potentially occur at concentrations below the effects determined in traditional ERA ecotoxicity growth tests on cyanobacteria. This meta-analysis therefore also sought to determine the relationship between protection goals proposed to protect against resistance development and the traditional aquatic protection goals; i.e. establish if the proposed methods used to derive a PNEC for AMR development ($PNEC_R$) are protective of those currently used for aquatic ecosystem function ($PNEC_{sw}$) and vice versa.

Recognising that antibiotic releases from drug production and formulation facilities represent 'hot spots' for the development of AMR it is critical that these discharges are minimised and managed effectively across the whole supply chain. To address this concern, the pharmaceutical industry recently established an AMR Road map which included a commitment to "establish science-driven, risk-based targets for discharge concentrations for antibiotics and good practice methods to reduce environmental impact of manufacturing discharges, by 2020" {IFPMA, 2016 #415}.

To improve the testing paradigm for antibiotics for use in prospective regulatory frameworks and to establish safe discharge concentrations for antibiotic production, we conducted a meta-analysis based on a systematic review of the publically available aquatic ecotoxicity data and clinically relevant MICs for antibiotics. Specifically we; 1) assess the relative sensitivity of commonly used taxa in aquatic ecotoxicity, with a MOA perspective, to evaluate the reliability of the current ERA of antibiotics to identify risk to vulnerable populations; 2) assess the value of extending the toxicity testing for bacteria through an assessment on the relative sensitivity of several cyanobacterial species, the marine bacteria *Vibrio fischeri* and the CRB MICs; 3) critically evaluate the current proposed approaches for determining the risk of AMR and its incorporation into risk assessment for the protection of human health; i.e. whether a $PNEC_R$ is more or less protective than $PNEC_{SW}$ calculated using traditional ecotoxicity testing; 4) test the assumption that CRB adequately represent environmental bacteria and evaluate the use of pre-clinical MIC data for the protection of other bacterial species through a comparison of the NOECs for cyanobacteria with the adjusted MIC, calculated by Bengtsson-Palme and Larsson (2016) from CRB and; 5) use the empirical data collected in these analysis to help establish science-driven, risk-based targets for manufacturing discharge concentrations for antibiotics.

2. Methods

2.1. Data search strategy

A comprehensive literature search was carried out to identify studies reporting toxicological effects of antibiotics on aquatic taxa commonly used in ERA. These taxa included cyanobacteria, green algae, macrophytes (the latter currently used in ERA for agrochemicals, but not pharmaceuticals), invertebrates and fish. Data were also collected for the effects of antibiotics on *Vibro fischeri*, for the ASRIT test and *Pseudomonas putida* (where available). Data were used in our analyses only if they met the following criteria: 1) the endpoint calculated was a NOEC, 50% effective concentration (EC50) or 50% inhibition concentration (IC50), the concentration at which 50% of the population are effected or inhibited respectively; 2) the methodology adopted was according to (or with minor deviations from) currently accepted regulatory protocols (e.g. Organisation for Economic Co-operation and Development (OECD) or International Organisation for Standardisation (ISO) test guidelines); 3) the aquatic species belong to the taxa described above; 4) exposures were for single species not multiple species/community exposures (with exception of the ASRIT which is a community based exposure) and; 5) organisms were exposed to a single antibiotic (not a chemical mixture).

The aim of this paper was to conduct a meta-analysis of available data in the context of current regulatory guidance that uses population-relevant endpoints to establish PNECs. Therefore NOECs and EC/IC50s for growth, reproduction or mortality only (or accepted surrogates e.g. luminescence in *V. fischeri* or respiration in the ASRIT) were collected and analysed. Moreover, interpretation of biomarker endpoints in relation to population-based NOECs and EC/IC50s are not well established.

Searches and data collections were conducted for the following public databases and literature:

- Environmental data on antibiotics from the trade organisation for the research-based pharmaceutical industry in Sweden (LIF), obtained from the Swedish fass.se database (www.fass.se accessed Jan 2016).
- Environmental data for antibiotics from the 'European public assessment report' database (www.ema.europa.eu, accessed Jan 2016).
- All published data in the Wikipharma database (http://www.wikipharma.org, accessed Jan 2016).
- All relevant data in the study by Vestel et al. (2015) which included the antibiotics azithromycin, bedaquiline, ceftobiprole, doripenem, linezolid, meropenem, sulfamethoxazole and trimethoprim.
- Data for sulfadiazine, neomycin and gentamycin, kindly provided by Merck Sharp & Dohme (MSD) through the 'Innovative Medicines Initiative' iPIE project (https://www.imi.europa.eu/content/ipie).
- A GoogleScholar search focused on cyanobacteria with the following search criteria for the 111 antibiotics listed in the paper by Bengtsson-Palme and Larsson (2016): *Antibiotic* cyanobacteria "OECD 201" OR "ISO8962" OR "ISO 8962" OR "850.4500" OR "E1440-91"
- The theoretical $PNEC_R$ ($PNEC_{R(T)}$) and the size-adjusted MIC (MIC_{aj}) for antibiotics were collected from Bengtsson-Palme and Larsson (2016). For antibiotics where < 40 species have been tested in the European Committee on Antimicrobial Susceptibility Testing database, Bengtsson-Palme and Larsson (2016) calculated a size-adjusted MIC. This is a theoretical adjustment to the MIC to include 99% of CRB. The number derived from that calculation was rounded down to the nearest concentration in the range operated in the European Committee on Antimicrobial Susceptibility Testing protocol. $PNEC_{R(T)s}$ were calculated by applying an assessment factor of 10 to account for differences between inhibitory concentrations and selective concentrations of the antibiotics. Experimentally derived MSCs were identified from literature following a GoogleScholar search with search criteria: "Minimum selective concentration" MSC AND "antibiotic resistance". We highlight here that currently there is no internationally standardized test method for MSC and that extrapolation to the environment is poorly understood due to the complex nature of resistance enrichment, the complex nature of communities and a range of environmental factors that may influence the MSC (Khan et al., 2017; Quinlan et al., 2011).
- Antifungal and antiviral drugs obtained through our search criteria were excluded from this assessment.

All data derived from these searches are provided in the supplemental material, Table S1 and a flowchart to illustrate the data collection and statistical processes for these analyses is provided in Fig. S1.

2.2. Assessment of data reliability

Assessments on data reliability were undertaken using the 'Criteria for reporting and evaluating ecotoxicity data' (CRED) system that is specifically designed for the evaluation of ecotoxicity data for regulatory use (Moermond et al., 2016). In this system reliability is defined as "the inherent quality of a test report or publication relating to (preferably) standardized methodology and the way the experimental procedure and results are described to give evidence of the clarity and plausibility of the findings". The CRED system categorises the reliability of studies into one of four scores; R1 (reliable without constraints), R2 (reliable with constraints), R3 (unreliable) or R4 (not assignable). Studies identified as R3 are considered unsuitable for use in regulatory decision-making; whereas caution needs to be applied on a study-by-study basis for studies categorised as R2 or R4. The CRED evaluation method also provides guidance on the evaluation of the relevance of data (Moermond et al., 2016). This, however, was not applied as the data were considered relevant for this meta-analysis having fulfilled the selection criteria outlined in Section 2.1. The CRED reliability score for each study is given in Table S1.

2.3. Relative taxa sensitivity data

The lowest 'reliable' NOEC and EC50 for each taxa were identified for each antibiotic. Data from studies that had CRED reliability scores of R1 and R2 were prioritised, without bias between R1 and R2, over those in the categories of R3 or R4. R4 data were selected over R3 data as the majority of R4 studies were assigned R4 due to unpublished/missing information in an otherwise (apparently) reliable study compared with R3, which were assigned unreliable for defined reason. The lowest 'reliable' NOEC and EC50 were applied in the analysis of relative taxa sensitivity and are presented in the Table S2. This conservative approach was deemed more appropriate rather than taking an average of all available data that has imbalanced taxa representation and varying data reliability.

An analysis of the relative sensitivity of cyanobacterial species adopted the same CRED criteria as described above to establish the lowest 'reliable' EC50. EC50s were used rather than NOECs as there was a larger dataset for cyanobacterial EC50s. These data are presented in Table S3.

2.4. Censored data

For some antibiotics the data was either left or right censored, meaning that the value was not a precise number and was given as greater than (>) or less than (<) the value reported (i.e. no effect at the highest test concentration or an observed effect at the lowest tested concentration, respectively). Censored data values were used when no other data were available (> than numbers would represent conservative values and < numbers were included only when they represented the lowest 'reliable' data value). Where data were censored,

this is indicated in Table S1.

2.5. Establishing relative taxa sensitivity to antibiotics

A sensitivity ratio (SR) was calculated between the different taxa and cyanobacteria for each antibiotic, where data were available. The SR was calculated using the lowest NOEC (or NOEC and MIC_{aj} in the case of CRB) or EC50 using the following equation:

$$Log_{10}SR = logE_{cyanobacteria} - logE_{taxa}$$

where E is the endpoint (NOEC, EC50 or MIC_{aj}).

A SR $>$ 0 indicates that the cyanobacteria are more sensitive than the other taxa and less sensitive when SR $<$ 0. Each unit of SR is equivalent to an order of magnitude difference in sensitivity.

The difference between a SR calculated from NOECs compared with those calculated from EC50s was examined to identify how the endpoint used might impact the sensitivity ratio. Briefly, a generalised linear model (GLM) (Gaussian error family with identity link function) was constructed using the 'lmer' package with the restricted maximum likelihood method (Bates et al., 2015) in R (version 3.3.0; R Project for Statistical Computing, Vienna, Austria). The model residuals were normally distributed and significant differences identified using the "lmerTest" package in R (Kuznetsova et al., 2013). SRs were used only where a NOEC and EC50 were from the same species and publication in order to exclude effects of different methodologies. The SRs calculated from EC50s were significantly higher by 0.5 ($p = 0.05$) than those calculated from NOECs i.e. cyanobacteria were less sensitive as measured by EC50s. As such, SRs calculated from EC50s were only included in subsequent analyses comparing taxa sensitivities where NOEC SRs were not available. We acknowledge that this will have a small effect on the output of the models. However, because of the sparse dataset and the relatively small difference in SR between EC50s and NOECs compared with the differences between taxa, the inclusion of the EC50 SRs where NOEC SRs are not available increases the number of SRs for comparison and robustness of the models.

We established a GLM in R (version 3.3.0; R Project for Statistical Computing, Vienna, Austria) to determine the effects of exposure duration on the EC50 for *V. fischeri,* as EC50 are often reported for 5, 15 and 30 min and for 24 h. Censored data were removed and the remaining EC50s were log_{10} transformed before use in the GLM (Gaussian error family with inverse link function) that was constructed as described for comparing NOEC and EC50 SRs above. Significant differences were identified by applying a TukeyHSD post hoc test. Twenty four hour EC50s were significantly lower ($p \leq 0.001$) than those following shorter exposure periods and data for this time point only were therefore used in subsequent analyses on relative taxa sensitivities.

Differences in SR across all taxa for all antibiotics were analysed using a GLM. The aim of the analysis was to compare the sensitivity of all taxa to cyanobacteria. Cyanobacteria were chosen as the comparator because they are assumed to be the most mode-of-action relevant taxa (therefore, most sensitive species) in current ERA, and thus expected to drive the $PNEC_{SW}$. Briefly, to assess for statistical differences in SR the GLM was constructed forcing the intercept through 0 (the SR value of cyanobacteria). Therefore, the statistical differences identified by "lmerTest" (Bates et al., 2015) represent the statistical difference from 0 and thus the statistical difference between the taxa and cyanobacteria. This allowed for the exclusion of cyanobacterial SRs in the GLM as the sensitivity of cyanobacteria were already accounted for in the calculation of the SRs. TukeyHSD post hoc tests were applied to identify any further differences between the taxa groups. Details on model construction and validation are provided in the Supplemental Material. Adopting the same process and validation steps, further GLMs were established for analyses of antibiotics with different mechanisms of actions and, where sufficient data were available, for antibiotic classes (a more detailed methodology for this is presented in Supplementary Material).

Antibiotics were classified into three groups based on their broad mode of action, specifically, cell envelope inhibitors (Anatomical Therapeutic Chemical (ATC) classification system codes J01C and J01D), Nucleic acid synthesis inhibitors (ATC codes J01E and J01 M) and protein synthesis inhibitors (ATC codes J01A, J01B, J01F, J01G, J01XC, J01XX08, J01XX11 and QJ01XQ).

It is important to note that in addition to comparing different endpoints and methodologies, representation of antibiotics - in both potency and number of antibiotics with data - varied between and within taxa and antibiotic classes. We acknowledge this may introduce some uncertainty and potential bias in our analysis and have thus avoided the use of more complex model designs that might otherwise have introduced random factors and interactions. However, the biases mentioned above are unlikely to have an impact on the overall conclusions drawn from these analyses.

2.6. Calculation of PNECs

Where a full set of ecotoxicity data for an European Medicines Agency Phase 2 ERA was available (cyanobacteria, invertebrate and fish tests) a $PNEC_{SW}$ was calculated by taking the lowest NOEC of the three studies and applying an assessment factor of 10, as described in the regulatory guidance (EMA, 2006). A theoretical $PNEC_R$ ($PNEC_{R(T)}$) was taken directly from (Bengtsson-Palme and Larsson, 2016). An experimental $PNEC_R$ ($PNEC_{R(Exp)}$) was calculated from the lowest experimental selective concentration and applying an assessment factor of 10.

There was not enough data to conduct species sensitivity distribution analysis and calculate 95% percentile protective limits, as this requires a minimum of 10 species and preferably $>$ 15 (Echa, 2008).

2.7. 5th Percentile determination

The calculated 5th percentiles for the NOEC and MIC data subsets were not normally distributed or fitting to other known distributions (e.g. gamma and weibull) before or following transformations (log, log_{10} or boxcox). The 5th percentile therefore was established using the non-parametric Harrell-Davis quantile estimator method. Analysis was conducted in R (version 3.3.0; R Project for Statistical Computing, Vienna, Austria) using the hdquantile function in the 'Hmisc' package (Harrell, 2016).

3. Results

Ecotoxicity data were collected for 79 antibiotics (Table S1) representing 48% of the 164 approved antibiotics identified in www.drugbank.ca and (Santos et al., 2017). Information on the ecotoxicity in cyanobacteria was available for 41 of these 79 antibiotics, but with NOECs for only 27 (16%). Antibiotics with NOECs for cyanobacteria were well distributed across all ATC sub-classes under J01, with exception of J01XX ('other antibacterials'; Fig. S2).

A complete Phase 2, ERA dataset that included the full range of taxa for calculating a $PNEC_{SW}$ (EMA, 2006) was available for only seven of these antibiotics. This may reflect the lack of pharmaceutical ERA datasets placed in the public domain and/or that few antibiotics have been approved since the existing European Medicines Agency guideline came into force in 2006 requiring full chronic toxicity testing on cyanobacteria/microalgae, invertebrates and fish and consequently lack a full ecotoxicity data set.

3.1. Relative species sensitivities

Overall, cyanobacteria were the most sensitive taxa of those currently recommended in the ERA of human pharmaceuticals (EMA, 2006; US Food and Drug Administration, 1998) ($p \leq 0.001$, Fig. 1A)

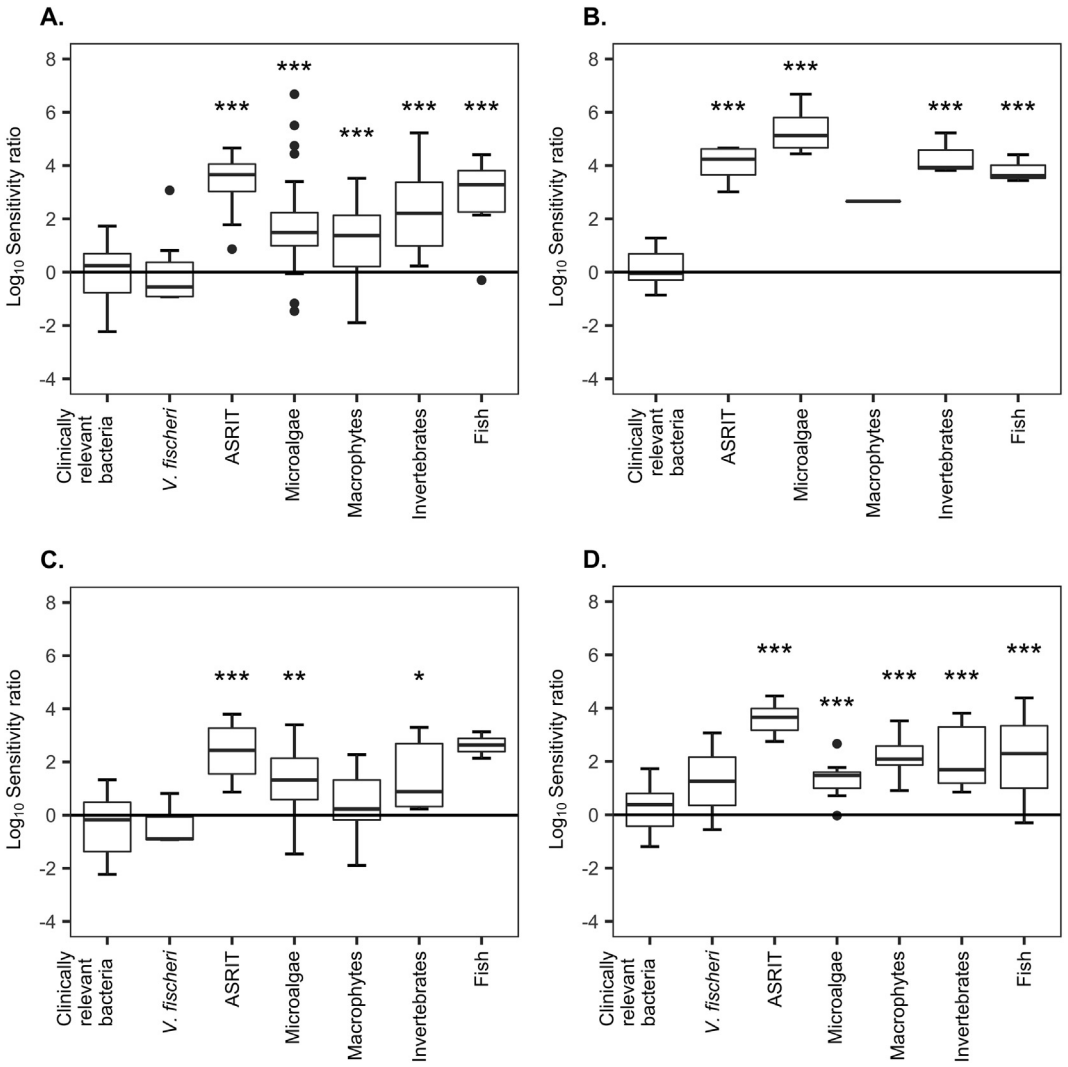

Fig. 1. Boxplots of Log_{10} sensitivity ratio (SR) between cyanobacteria and other species/phyla for A) all antibiotics ($n = 37$), B) cell envelope inhibitors ($n = 8$), C) Nucleic acid synthesis inhibitors ($n = 12$) and D) protein synthesis inhibitors ($n = 16$). SR calculated based on log_{10}cyanobacteria NOEC or EC50 – log_{10}taxa NOEC or EC50. Where SR = 0 the sensitivity of the taxa is equal to cyanobacteria, represented by horizontal line, where SR > 0 taxa had a lower sensitivity and < 0 indicates higher comparative taxa sensitivity. Significant differences of SR from cyanobacteria in the generalised linear mixed models are indicated by: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Statistical tests were not performed on macrophytes in cell envelope inhibitors as there was only one antibiotic tested in macrophytes.

and they were equally sensitive as other bacteria (CRB and *V. fischeri*) and more sensitive than macrophytes (that are not currently required in ERA of pharmaceuticals; $p \leq 0.001$).

The sensitivity of cyanobacteria and CRB were not significantly different for any of the three broad antibiotic mechanisms of actions (Figs. 1B-D); NOECs in cyanobacteria were lower than CRB MIC_{aj} for half (12 out of 24 antibiotics; Fig. 2A). If we were to adopt the lowest MIC, instead of the modelled $MIC_{aj,}$ in this meta-analysis there would be more cases (18, rather than 12, out of 24) where the cyanobacteria were the most sensitive. Although there was no clear relationship between the CRB MIC_{aj} and cyanobacterial NOECs the difference in sensitivity was up to two orders of magnitude for specific individual antibiotics (Fig. 2A and 6C).

There were no significant differences in sensitivity to DNA or protein synthesis inhibiting antibiotics between *V. fischeri* and cyanobacteria (Fig. 1; there were no data for cell-envelope inhibiting antibiotics). Of the seven antibiotics where SRs could be determined five were for quinolones giving an antibiotic class bias for the *V. fischeri* data. EC50s for *V. fischeri* were lower than those for the cyanobacteria on six occasions (Fig. 2B), three of these were almost an order of magnitude lower (flumequine, lomefloxacin and oxolinic acid). *V. fischeri* was also the most sensitive organism to olfoxacin, with a NOEC one order of magnitude lower than the CRB MIC_{aj} (Fig. 2A) and an EC50 half that for the cyanobacteria (Fig. S3).

Pseudomonas putida, a model (soil) gram-negative bacteria used in standard growth inhibition test guideline (ISO, 1995) was more sensitive than cyanobacteria for one out of five antibiotics (meropenem; Fig. 2A and B).

The ASRIT (OECD, 2010) was consistently between two and four orders of magnitude less sensitive than cyanobacteria, with the exception of trimethoprim (Figs. 1 and 2 $p \leq 0.001$).

There were large differences in sensitivity between cyanobacterial genera and species, with between two and three orders of magnitude difference in EC50s for 10 out of the 16 antibiotics, and approximately five orders of magnitude difference in response to the β-lactams amoxicillin and ampicillin (Fig. 3). Overall, *Microcystis aeruginosa* was the most sensitive species (in half of the 16 antibiotics). *Anabaena cylindrical*, *Synechococcus leopoliensis* and *Microcystis wesenbergii* were each the most sensitive cyanobacterium for 2 of 16 antibiotics for which there were data on multiple species. *A. flos-aquae,* one of the cyanobacterial species recommended for testing in the OECD 201 test guideline, was the most sensitive species for only 1 of the 13 antibiotics

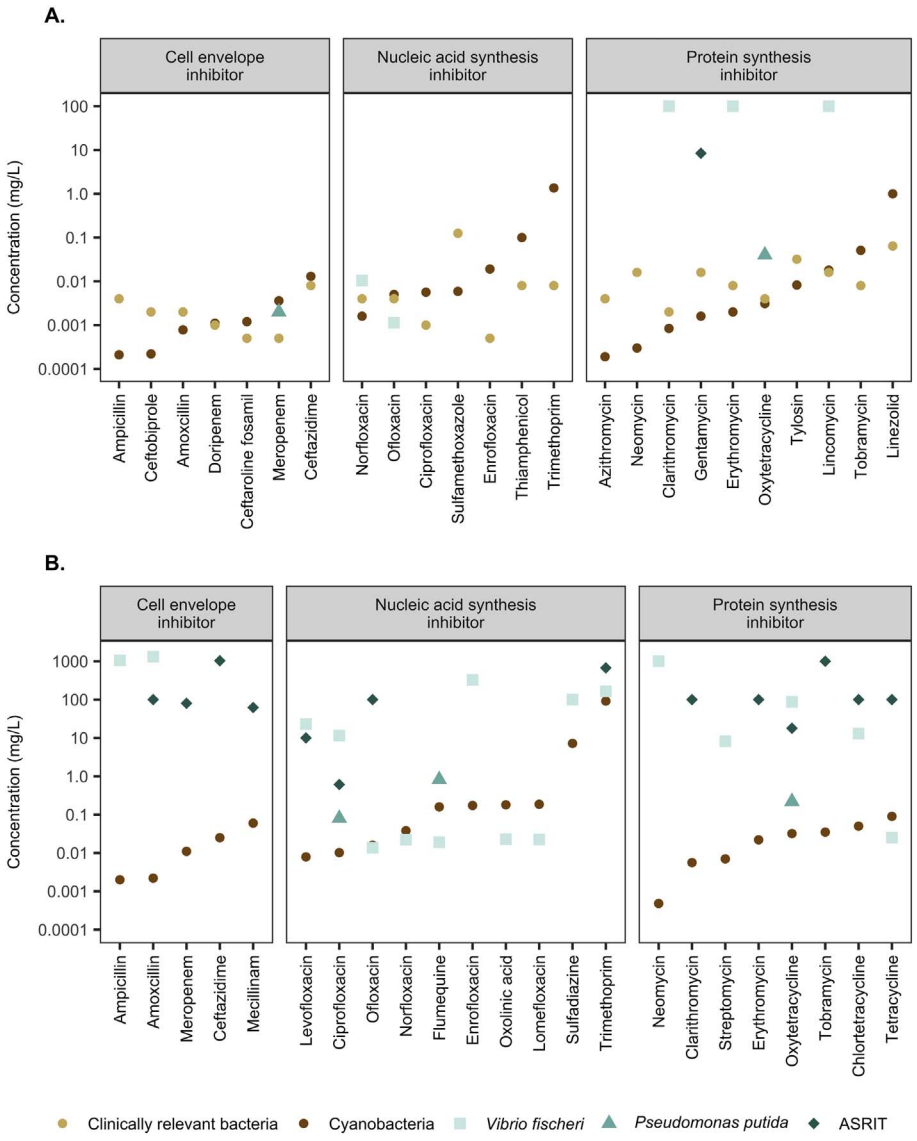

Fig. 2. Chronic exposure effects of antibiotics on A) environmental bacteria and clinically relevant bacteria (no observed effect concentrations (NOEC) and adjusted minimum inhibitory concentrations respectively) and B) environmental bacteria 50% effective concentrations.

in which it was tested. When considering antibiotic sensitivity based on their mechanisms of action, *Microcystis* species appeared to be more sensitive to nucleic acid synthesis inhibitors (7 out of 9 antibiotics). *Microcystis* and *Synechococcus* species were the most sensitive to cell envelope inhibiting antibiotics. *Anabaena* genera were the most sensitive to the protein synthesis inhibitors (3 out of 6) and in two cases by more than an order of magnitude.

Overall, macrophytes were generally less sensitive to antibiotics compared with cyanobacteria with a wide range of SRs (Fig. 1, $p \leq 0.001$). However, they showed equal sensitivity with cyanobacteria to nucleic acid synthesis inhibitors (average SR = 0.42; $p = 0.3$). The NOECs for trimethoprim and sulfadimethoxine were lower for macrophytes than for cyanobacteria (Fig. 4A). A comparison of macrophyte and environmental bacteria EC50s is provided in Fig. S3.

Microalgae were also generally less sensitive to antibiotics than cyanobacteria (Fig. 1, $p \leq 0.001$). However, for sulfadiazine and sulfadimethoxine the NOECs in microalgae (0.135 and 0.529 mg/L, respectively) were over an order of magnitude lower than for the lowest in the cyanobacteria (Fig. 4A). We interpret these data with caution, however, as the results for the cyanobacteria were derived from a study based on nominal (i.e. not measured) test exposure concentrations (Ando et al., 2007). A comparison of the EC50s for microalgae with environmental bacteria is shown in Fig. S3.

Metazoans (fish and invertebrates) were significantly less sensitive across all antibiotics compared with cyanobacteria and often by between two and four orders of magnitude (with exception of tedlizolid phosphate, Figs. 1 and 4, $p \leq 0.001$, for both fish and invertebrates). There was substantial variation in SR between cyanobacteria and the metazoan taxa (as illustrated by the standard errors in the data; Fig. 1). In the case of tedlizoid phosphate, a pro-drug, fish appeared more sensitive than cyanobacteria (NOECs of 0.032 versus 0.063 mg/L, respectively; Fig. 4B). A MIC_{aj} for tedozolid (the active pharmaceutical ingredient) was not available from the Bengtsson-Palme and Larsson (2016) study, but a MIC of 0.016 mg/L (based on 12 species), corresponding to a $MIC_{aj} < 0.008$ mg/L was recently (January 2017) reported the European Committee on Antimicrobial Susceptibility Testing database. This suggests that CRB are substantially more sensitive to tedozolid compared with fish and cyanobacteria. The fact that tedizolid phosphate (pro-drug) requires activation by phosphatases in the blood to convert it into the active ingredient (tedizolid), and the ecotoxicity assessments in cyanobacteria appear to be based on the pro-drug only, may explain why cyanobacteria were relatively insensitive. In no cases were the chronic NOECs for invertebrates lower than the NOECs for cyanobacteria (Fig. 4). The daphnid EC50 for the antifolate trimethoprim, however, was lower than the EC50 for cyanobacteria (8.21 and 91.68 mg/L, respectively. Fig. S3). This was not the case for the NOECs

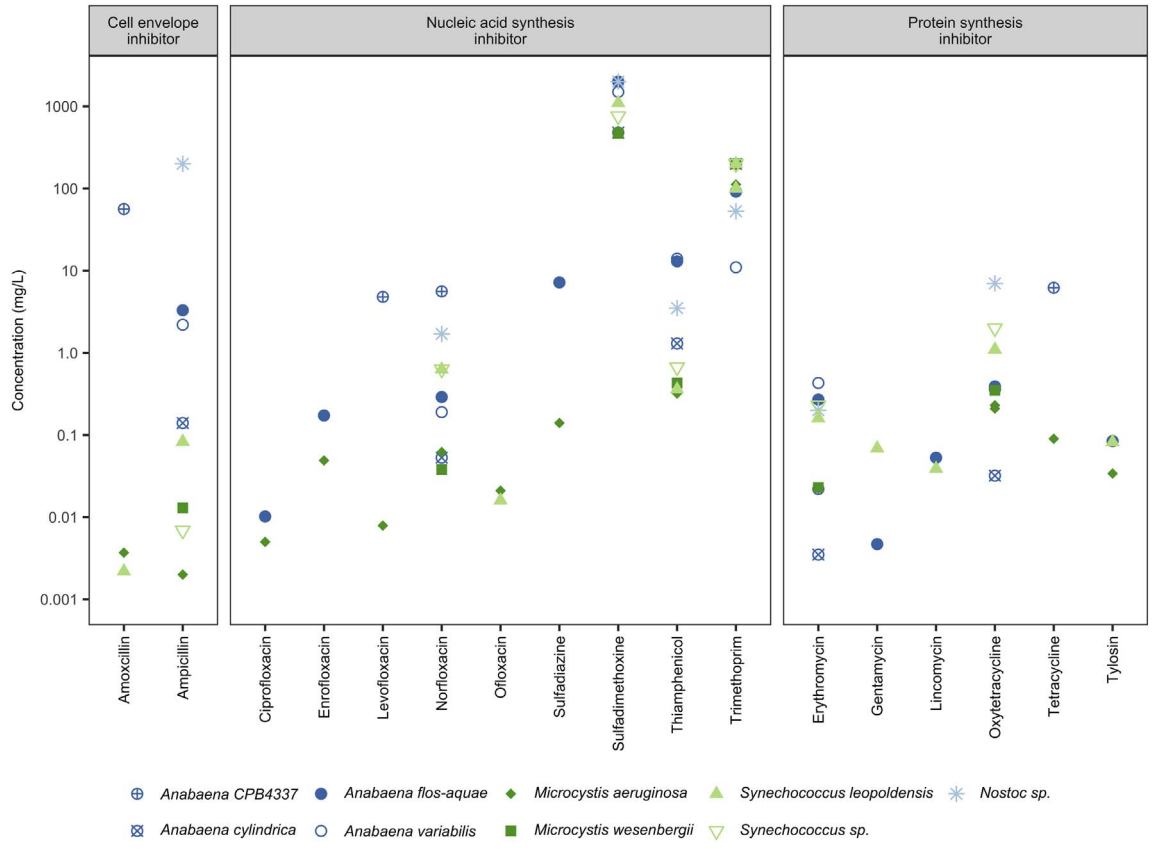

Fig. 3. Chronic exposure effects (EC50s) of antibiotics on different cyanobacteria species.

for the same compound, indicating differences in the shape of the dose-response curve. Importantly, in this case cyanobacteria would still drive the $PNEC_{SW}$.

3.2. PNEC comparisons

For the limited number of antibiotics where a definitive $PNEC_{SW}$ could be calculated ($n = 7$) an analysis of the relationship between traditional ERA PNECs and those for AMR was conducted. Within this meta-analysis the theoretically determined PNEC for resistance development $PNEC_{R(T)}$) obtained from Bengtsson-Palme and Larsson (2016) for the different antibiotics was not always protective of (lower than) the $PNEC_{SW}$ (Fig. 5A). The $PNEC_{R(T)}$ was lower than $PNEC_{SW}$ for ceftaroline, ciprofloxacin and tobramycin. However, the $PNEC_{SW}$ was approximately ten-fold lower than $PNEC_{R(T)}$ for ceftobiprole, sulfamethoxazole and azithromycin.

Where experimentally derived MSCs existed, the $PNEC_{R(Exp)}$ was lower than $PNEC_{R(T)}$ for three out of five antibiotics with available data (Fig. 5B). However, $PNEC_{R(T)}$ overestimated the risk of resistance development for streptomycin by an order of magnitude. $PNEC_{R(T)}$ and $PNEC_{R(Exp)}$ were similar for trimethoprim (Fig. 5B; trimethoprim $PNEC_{R(Exp)}$ was < 0.2 µg/L). The $PNEC_{SW}$ for erythromycin and streptomycin were lower than their $PNEC_{R(T)}$ and $PNEC_{R(Exp)}$ (Fig. 5B). The $PNEC_{R(Exp)}$ for erythromycin however, did not have a definitive value, (i.e. < 0.2 mg/L) and as such we assign caution to this comparison.

3.3. Establishing 5th percentiles

We determined the 5th percentile for growth inhibition data for cyanobacteria and environmental bacteria and MICs for CRB (See table S4). The rationale for this was to establish an environmental protection goal for antibiotic production discharges that would be protective of bacterial NOECs with 95% confidence. The 5th percentiles ranged from 225 to 2028 ng/L, depending on the bacteria and endpoints used. The lowest NOECs for environmentally relevant bacteria (cyanobacteria, *P. putida* and *V. fischeri*) gave the lowest value (225 ± 71 ng/L, Fig. 6A).

4. Discussion

In our evaluation of the current regulatory ERA guidance we show that of the taxa tested, as expected based on the mechanisms of action, prokaryotes were most sensitive to antibiotics. However, we also show that reliance on one species of (cyano)bacteria to set protection levels (e.g. PNECs), as operates currently, is unlikely to be protective of environmental and human health (through AMR). Individually, neither traditional aquatic PNECs nor the AMR based PNECs protect fully against the effects of antibiotics. We thus recommend the inclusion of both clinically important bacteria and a wider range of species of environmentally relevant bacteria to improve the prospective regulatory framework for human and ERA. This approach will help also in defining more appropriate safe discharge concentrations for antibiotic production, and help to exclude unnecessary ERA testing on metazoan species.

4.1. Species relative sensitivity: The need for more bacteria

During their development, the efficacy and safety of new antibiotics are assessed in preclinical and clinical studies before market approval. It is therefore unlikely that toxic effects will occur in an aquatic vertebrate (such as fish) at water concentrations lower than those affecting prokaryotic species (target or non-target). As expected, in our analyses, those species evolutionarily more distant to pathogenic bacteria were generally less sensitive to antibiotics compared with clinically relevant and environmental bacteria. Our results also indicate that neither cyanobacteria, CRB nor other environmental bacteria (*V. fischeri* and *P. putida*) provide a single organism/test that is fully protective of the diversity of bacteria in the environment. Thus, a $PNEC_{SW}$ determined

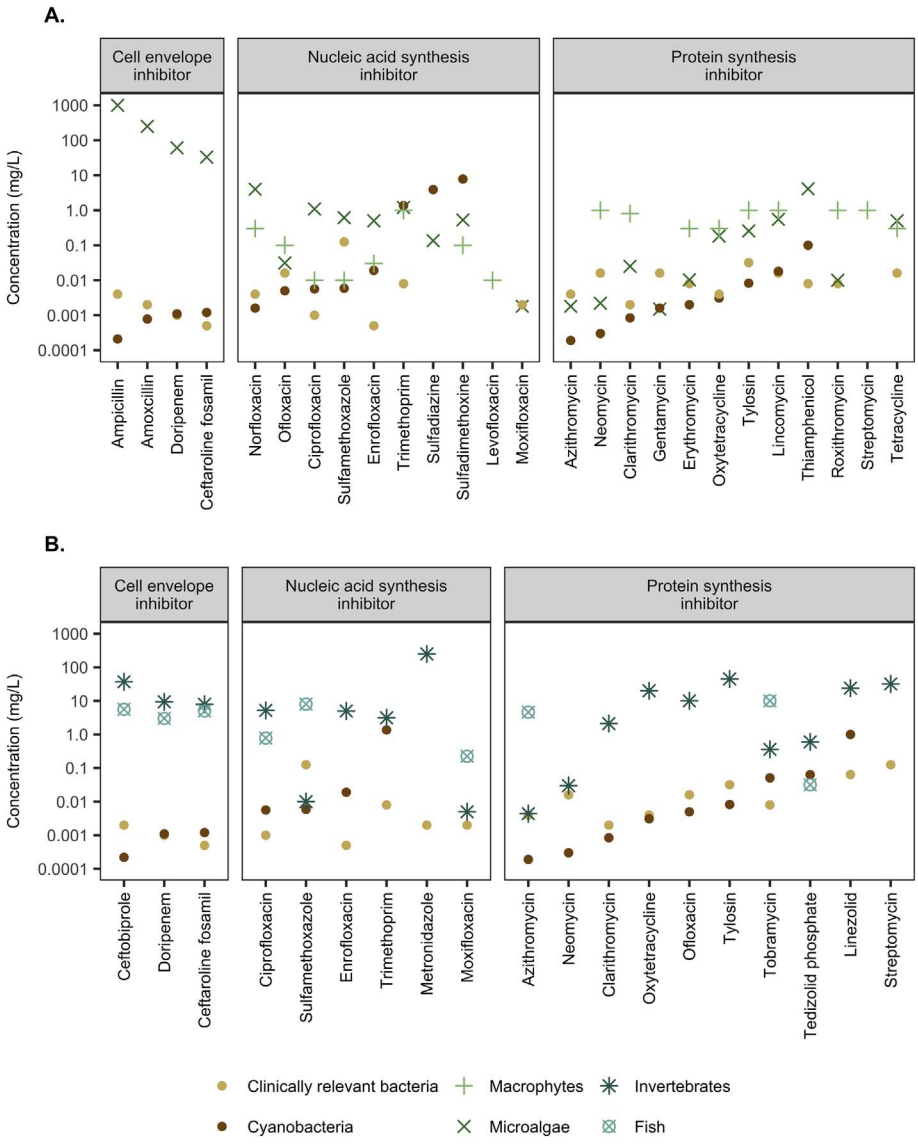

Fig. 4. Chronic exposure effects of antibiotics on cyanobacteria and clinically relevant bacteria (no observed effect concentrations (NOEC) and adjusted minimum inhibitory concentrations respectively) compared with A) NOECs for microalgae and macrophytes and B) NOECs in invertebrates and fish.

according to the current ERA guidance (EMA, 2006; US Food and Drug Administration, 1998) will not always be protective of the environment.

Sensitivity to any one antibiotic differed by up to five orders of magnitude across different species of cyanobacteria. Patterns of sensitivity for the different genera were observed across the different antibiotic mechanisms of actions, but no one species was consistently the most sensitive. Cyanobacteria are one of the most diverse phyla on the planet (Shih et al., 2013; Whitton, 2012) and this large range in sensitivity to antibiotics might therefore be expected. In ERA *A. flos-aquae* is the most regularly used of the two OECD test guideline recommended cyanobacterial species (the other being *S. leopoliensis;* (OECD, 2011)) but *A. flos-aquae* was the most sensitive cyanobacteria for only one of the 13 antibiotics for which data were available for multiple genera and species. In the cases of ampicillin, erythromycin, norfloxacin, oxytetracycline, sulfdiazine and trimethoprim (35% of antibiotics with multiple cyanobacterial EC50s) the difference in sensitivity between *A. flos-aquae* and the most sensitive taxon was greater than the assessment factor (× 10) used to generate a PNEC for the risk assessment. For ampicillin, reliance on *A. flos-aquae* could underestimate the $PNEC_{SW}$ by more than three orders of magnitude. This questions the current over reliance on a single cyanobacteria test species within ERA frameworks and we propose at least three cyanobacteria genera should be included within these risk assessment frameworks. The case above for ampicillin highlights a further important issue relating to the relevance of high sensitivity for some cyanobacteria. Ampicillin is not persistent in the environment and undergoes partial degradation by bacteria; indeed, primary degradation is the resistance mechanism. If degradation were factored in, from an ecotoxicological point of view, exposure and environmental effects would be low, although community structure changes could impact resilience. Furthermore, since the resistance mechanism partially degrades the antibiotic resulting in a lower concentration of ampicillin in the environment care needs to be taken not to assume a low measured concentration of ampicillin necessarily equates with an absence of selection for AMR development and human health risk.

The cyanobacteria adopted for toxicity testing has been based largely on experimental convenience (e.g. the ability to grow them and measure cell density in the laboratory) with little knowledge on how representative they are of other cyanobacteria. No consideration has been given to how they grow and function in non-pelagic habitats, e.g. biofilms. From our analyses, *M. aeruginosa* would potentially provide a relatively high sensitivity to most antibiotics. This species however, has a slower growth rate and the current test with this species may therefore have to be extended to make the test comparable in terms of the growth and replication dynamics with that for *A. flos-aquae* and *S.*

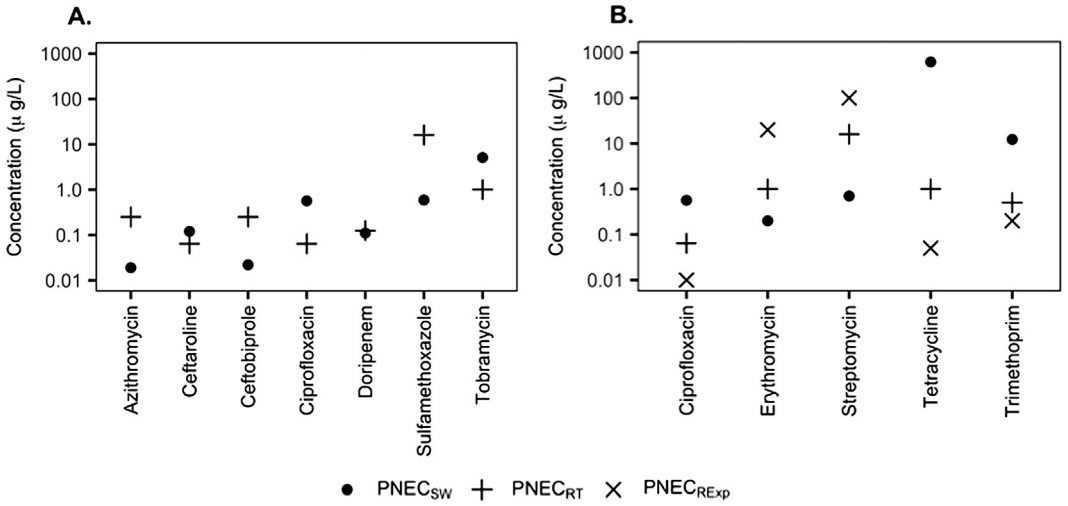

Fig. 5. Comparisons of predicted no effect concentrations (PNEC) for antimicrobial resistance and ecotoxicity for aquatic taxa in surface water. A) Comparison of theoretically derived PNEC for resistance development ($PNEC_{R(T)}$) based on clinically relevant bacteria (Bengtsson-Palme and Larsson, 2016) and PNEC for ecotoxicity in surface water ($PNEC_{SW}$). (B) Comparison of $PNEC_{R(T)}$, $PNEC_R$ based on experimentally derived minimum selective concentrations ($PNEC_{R(EXP)}$) and $PNEC_{SW}$. In A) data are presented for antibiotics only where a full data set including cyanobacteria, invertebrate and fish tests were available and calculated from no observed effect concentrations as described in (EMA, 2006). $PNEC_{SW}$ in B) are calculated from cyanobacteria NOECs regardless of a complete ecotoxicity data set where a $PNEC_{R(EXP)}$ was available. $PNEC_{R(EXP)}$ is a less than (<) value in erythromycin and trimethoprim. $PNEC_{R(EXP)}$ based on strain specific MSC in ciprofloxacin, erythromycin, streptomycin and trimethoprim. $PNEC_{R(EXP)}$ based on community based MSC in tetracycline. EC50 for cyanobacteria was used because NOEC were not available for $PNEC_{SW}$ in streptomycin and tetracycline therefore NOEC may be up to an order of magnitude lower.

leopoliensis. We highlight that the requirement for optimized conditions for culturing a species and variation in life history components across species (e.g. growth rates and lag time) create further challenges for interspecies substance effects analyses. For example, exposure time can have a direct impact on the perceived sensitivity. In this meta-analysis we have used data that are based on regulatory approved guidelines in which exposure time and exposure conditions have been optimized for the different organisms to ensure that growth in the controls do not reach the plateau phase, thus maximizing the ability to detect for any effects against treatment groups. Longer exposure periods could potentially result in lower effective exposure concentrations, as we demonstrate for the EC50 in *V. fischeri* (for a 24 h exposure compared with shorter test periods) and as has been shown for the ASRIT (Kümmerer et al., 2004). Extending exposure periods in growth tests however needs to ensure that this does not compromise the ability to distinguish for effects i.e. additional time does not result in the controls being limited in their growth dynamics by the available resources and thus affect the comparison with the treated groups. It needs to be recognized, however, that differences between test conditions optimized for different species (e.g. chemical constituents of the culture media, pH, temperature, light intensity and test length, to name just a few) could all impact the fate and behavior of the antibiotic and its bioavailability, distribution, metabolism and excretion in test organisms, which in turn may influence the perceived relative sensitivity. Distinction needs to be made on whether the exposure adopted is optimized for assessment of effects relative to controls (as is the case in the OECD 201 test guideline for green algae and cyanobacteria) or focused more on environmental relevance (for example in the ASRIT analyzing for impacts within hydraulic residence time in sewerage treatment works). Species sensitivity analyses and /or functional impacts are arguably better addressed under context specific conditions that consider the microbial community structure(s) and physicochemical conditions that occur in those natural systems.

Available study information was not sufficiently comprehensive to allow for consideration of these variables within our meta-analysis and we were thus restricted to endpoint data (EC_{50} and NOEC) that we derived from reliable studies. Further investigation is warranted into the physiological basis for the differences in sensitivity to antibiotics to help identify species, or groups of species, that best represent the phylum for their protection and the critical ecosystem services (e.g. primary productivity and food source) they provide.

V. fischeri and *Pseudomonads* were more sensitive than cyanobacteria to some antibiotics and may potentially provide valuable additional species for inclusion within the ERA. Furthermore, they already have internationally recognized test guidelines (ISO, 1995, 2007). *V. fischeri*, is a marine bacterium that would not normally be considered in ERA for freshwaters, but is sometimes used in whole effluent assessments (ECETOC, 2004). It is, nevertheless, a prokaryotic species and antibiotics and antibiotic resistant bacteria have been detected in estuaries and marine environments emanating from sewerage treatment plant discharges and manufacturing effluents (Schaefer et al., 2009; Webster et al., 2004; Zheng et al., 2011; Zou et al., 2011). The compiled data show that *V. fischeri* was more sensitive than cyanobacteria for six antibiotics, and for half of these by nearly an order of magnitude (flumequine, lomefloxacin and oxolinic acid). The inclusion of this test could therefore be of value to ERA if performed with an exposure time of 24 h (results based on exposure lengths of < 24 h showed significantly less sensitivity). *Pseudomonads* have been shown to be less sensitive than the other soil bacteria to tetracycline, chlortetracycline, and oxytetracycline and in some instances by over an order of magnitude (Halling-Sørensen et al., 2002). The low sensitivity observed in *Pseudomonas* species has been attributed to their apparent high natural resistance to some antibiotics (Halling-Sørensen et al., 2002; Kittinger et al., 2016). Thus, our findings suggest that additional testing with *P. putida* could be of value to the ERA, but it may still not be protective of other soil bacteria. Any consideration to incorporate the test with *P. putida* in antibiotic ERA would need to first characterise the strain in terms of its chromosomal and plasmid resistance to help prevent biasing any function or growth based assessment (Brandt et al., 2015).

The ASRIT (OECD, 2010) was several orders of magnitude less sensitive to antibiotics than cyanobacteria and other bacterial species, confirming reports that this test is largely insensitive to antibiotics (Kümmerer et al., 2004). As such, the ASRIT would not influence the outcome of the ERA. This lack of sensitivity may be due to several factors, including the short exposure time (3 h) of the test (Kümmerer et al., 2004), the lack of antibiotic bioavailability due to adsorption to the sludge solids (e.g. Golet et al., 2002) or that the microbial community in the activated sludge has an innate resistance having been exposed previously to the antibiotic (Davies, 2012). It was not possible to assess the effect of extending the ASRIT test duration due to a lack of

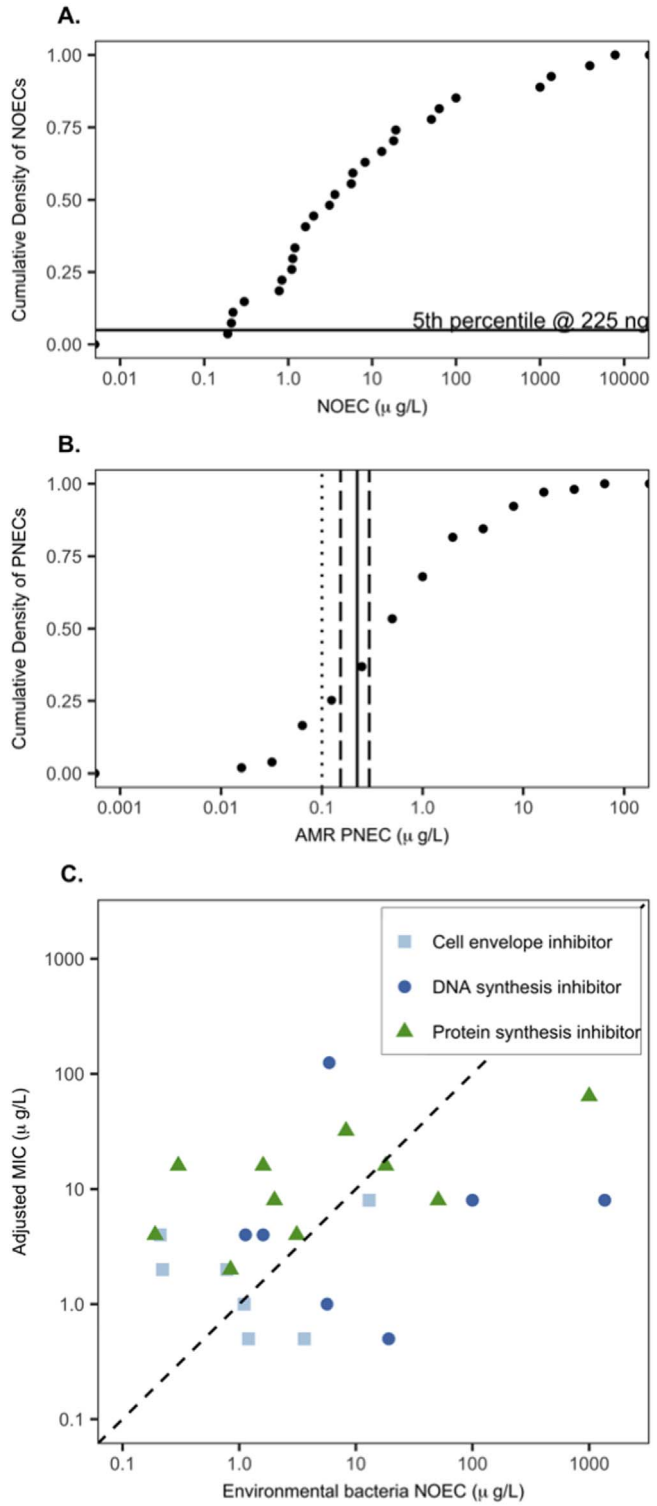

Fig. 6. A) Cumulative density plot of the NOECs for environmental bacteria for 27 antibiotics, showing the 5th percentile. B) Cumulative density plot of PNECs for AMR for 103 antibiotics, as calculated by Bengtsson-Palme and Larsson (2016). The vertical solid line represents the 5th percentile of the bacteria NOECs, dashed lines represent the standard error and dotted line indicates the proposed discharge limit. Note each point can represent up to 17 antibiotics. C) Comparison of NOECs for environmental bacteria and clinically relevant bacteria minimum inhibitory concentrations.

available data and because most ASRIT results are reported as censored data of > 100 mg/L. Furthermore, the endpoint of respiration, may not be suitable for all mechanisms of actions (Brandt et al., 2015) and it does not equate with changes in bacterial diversity or community structure. We thus support the need to replace and/or complement the ASRIT with other assays (Brandt et al., 2015), which are relevant for all pharmaceuticals.

In order to build greater confidence in the ERA for antibiotics we sought to gain a better understanding on the differences observed in sensitivity between the species and to establish both how often and for which antibiotic classes these differences exceed the assessment factor of 10. Overall, across all the antibiotics assessed, cyanobacteria and CRB were equally sensitive to antibiotics (fig. 1). Thus, neither CRB nor cyanobacteria were consistently more sensitive than the other. In this meta-analysis, the inclusion of CRB in ERA would drive the PNEC in 40% of cases further supporting a more holistic 'one health' approach that uses clinical and environmental data. There were, however, substantial differences in sensitivity to antifolates observed between the cyanobacterial species and CRB. The folate synthesis pathway that antifolates inhibit is present in cyanobacteria and so the reason for the apparent lack of sensitivity in some cyanobacteria is unknown. However, de Crécy-Lagard et al. (2007) reported that cyanobacteria possess a protein that may act as a folate transporter allowing the bypassing of some of the folate synthesis pathway. Our analysis suggests therefore that cyanobacteria may not always be a suitable representative for bacteria for full protection against antifolate antibiotics.

Macrophytes appear especially sensitive to antifolates and quinolones. The folate synthesis pathway in bacteria, algae and plants is fundamentally the same (Basset et al., 2005) and they are, therefore, all potentially susceptible to antifolates. Indeed, sulfamethoxazole has been reported to act as a competitive agonist to *p*-aminobenzoic acid in both *Lemna gibba* (Brain et al., 2008b) and *Arabidopsis thaliana* (Zhang et al., 2012). Macrophytes were also more sensitive than cyanobacteria to five quinolones. Quinolones cause toxicity by forming complexes with DNA gyrase or topoisomerase IV resulting in the inhibition of DNA replication and transcription (Aldred et al., 2014). Chloroplasts are descended from cyanobacteria (Falcon et al., 2010) and some plants and red algae have been shown to contain DNA gyrases in their plastids (including chloroplasts) and mitochondria (Moriyama and Sato, 2014; Wall et al., 2004). Quinolone antibiotics are reported to have anti-chloroplastic activity (Brain et al., 2008a; Brain et al., 2004; Ebert et al., 2011) which can affect photosynthesis in plants (Brain et al., 2008a). Indeed, organellar DNA gyrase has been shown to be the primary target of ciprofloxacin in *Arabidopsis thaliana* (Evans-Roberts et al., 2016). Thus, our findings indicate that for some antibiotics in these classes, macrophytes could potentially drive the protection goal. Consequently, these species should be considered for inclusion within risk assessment frameworks for antibiotics.

The metazoan taxa were never found to be the most sensitive compared with all bacterial taxa. This questions the necessity of resource intensive metazoan testing of antibiotics, as required by European Medicines Agency and Food and Drugs Administration guidance (EMA, 2006). Inclusion of appropriate (and additional) bacterial testing in the ERA for antibiotics would potentially allow for the exclusion of some unnecessary testing on metazoan species, acknowledging the principles of the 3R's to replace, reduce and refine studies that use 'protected' animals, such as fish (Hutchinson et al., 2016; Scholz et al., 2013).

We performed this meta-analysis based on data that was deemed most reliable according to the CRED system (Moermond et al., 2016). The conclusions however, are still drawn upon data that were conducted in different labs, with different procedures and of varying quantity (in terms of test performance and meta-data) and quality of reporting. We strongly emphasise the need to collect and report suitable control data, chemical analysis and meta-data in order to assist in reliable comparisons of studies.

An analysis of appropriate additional bacterial species for inclusion in the ERA needs to consider potential differences in sensitivity due to pharmacokinetic considerations including bioavailability, charge, uptake, elimination, metabolism, degradation rates or binding affinities,

or a combination of them. Differences in bacterial morphologies and innate resistance may also account for some of the differences in sensitivity between species. Some bacteria have several different growth forms depending on the environmental conditions. As an example, increased temperature and light intensity causes aggregation of *Synechococcus elongates* cells (Koblížek et al., 2000) and this aggregation may have an impact on the sensitivity of the cells to antibiotic exposure. Several studies have demonstrated that cells in biofilms are less sensitive/more protected from chemical exposure (Balcázar et al., 2015). A better understanding of how physiological and morphological differences in cells and community structure affect the toxicity of chemicals to bacteria is required to fully understand the risk posed by antibiotics in the environment.

Bacteria are fundamental to many vital ecosystem services, but little is understood regarding species loss and functional redundancy and thus, the resilience of ecosystem function. Some investigators, however, have begun to address this. For example, Lundström et al. (2016) found no change in the overall taxonomic diversity when biofilms were exposed to tetracycline, however, the community composition was altered and the functional diversity, as measured by utilization of carbon sources, decreased with increasing tetracycline concentrations. Ciprofloxacin exposure altered the bacterial community structure in marine sediments at 0.2 mg/L, resulting in a decrease in the community ability to degrade pyrene (Näslund et al., 2008). It was also found to increase overall biomass in salt marsh microbial communities, favouring gram negative and sulfate-reducing bacteria (Cordova-Kreylos and Scow, 2007). Several studies have shown that bacterial diversity has a positive relationship with ecosystem function (Bell et al., 2005; Langenheder et al., 2010). Delgado-Baquerizo et al. (2016) demonstrated that loss of diversity in aquatic bacterial communities caused a decrease in both broad (microbial respiration) and specialized (toxin degradation; of mycrocystin-LR and triclosan degradation) endpoints and the communities showed little or no functional redundancy. These studies indicate that a small drop in bacterial diversity may potentially impact negatively on the ecosystem services they provide.

From this, we conclude that the ERA framework for antibiotics needs to be based upon a suitable range of bacteria. This should include CRB and capture a wider range of ecologically important functional groups. Previous investigators have identified standard studies that may fulfill some of these data gaps e.g. nitrifying bacteria, methanogens and sulfate-reducing bacteria (Brandt et al., 2015) although more research is required to identify if these tests will be protective of all functional bacterial groups or if further standard tests will need to be developed. The effect of antibiotics on these functional groups is currently outside risk assessment frameworks and environmental and non-therapeutic human impacts are considered in isolation. Furthermore, a measure of the change in community structure would add value, especially looking at diversity in terms of clinical and environmental relevance, and understanding to changes in functional endpoints in bacterial multi-species/community tests to determine whether ecological resilience is being compromised.

4.2. PNECs for AMR verses traditional ecotoxicological effects

AMR is a serious risk to human health globally and currently sits outside the ERA regulations. Both theoretical methodologies and empirical data available for assessing AMR selection and transfer in the environment are limited. Consequentially, evidence is lacking to assess the best approach for the risk of AMR development, how resistance in the environment may lead to enrichment of resistance in human pathogens and how the risk posed by antibiotics by AMR development compares to their effects upon ecosystem function and services. Previous investigators have explored resistance selection using a variety of approaches, for example, comparing predicted environmental concentrations with MICs (Kümmerer and Henninger, 2003), using MICs to calculate potentially affected fractions of communities (Singer et al., 2011) and using growth and competition experiments to demonstrate resistance selection (Negri et al., 2000) and calculate MSCs (Gullberg et al., 2011). The theoretical approach proposed by Bengtsson-Palme and Larsson (2016) is a recent contribution and provides a good basis for this discussion, using MIC data to assess reduction in antibiotic efficacy due to erosion by resistance. However, it is important to note that this approach assumes growth can be used to predict resistance and is not verified through direct testing of resistance markers and as such any conclusions drawn from this analysis must therefore be considered with this in mind.

Our findings suggest that the $PNEC_{R(T)}$ defined by Bengtsson-Palme and Larsson (2016) is not always lower than the $PNEC_{SW}$; for 7 antibiotics $PNEC_{SW}$ was lower in four cases (fig. 5). This may be due to either the $PNEC_{R(T)}$ underestimating the risk or cyanobacteria being more sensitive to some antibiotics compared with the CRB. Experimentally determined MSCs were derived largely from laboratory strain competition experiments (four of the five cases; Fig. 5B), where strains that differ in only the presence/absence of the resistance genes under investigation are compared (Gullberg et al., 2014; Gullberg et al., 2011). These strain competition experiments have limitations in scaling up to more complex microbial communities (Bengtsson-Palme et al., 2014). There are very few cases where analyses have been conducted for more complex communities but it is hypothesised that the combined effects of changes in community structure (due to loss of the most sensitive species), protective morphological forms (e.g. bacteria maybe less susceptible in biofilms compared to those within the water column (Balcázar et al., 2015)), difficulty in defining the 'true' antibiotic exposure concentration, and alternative selection pressures (e.g. nutrient limitation, predation and other chemical/physical stressors) may negate the fitness benefit of the resistance (Bengtsson-Palme and Larsson, 2016; Brosche and Backhaus, 2010; Day et al., 2015; Gullberg et al., 2014; Lundström et al., 2016; Quinlan et al., 2011). Most studies that have considered effects of antibiotics on complex communities have been taxon independent, assessing AMR gene copy number relative to 16SrRNA, rather than providing species specific information. Investigations into AMR following tetracycline exposure, however, have found that resistance was increased in periphyton at the lowest test concentration of 0.5 μg/L (Quinlan et al., 2011), horizontal gene transfer (HGT) was promoted at 10 μg/L (Jutkina et al., 2016) and resistant bacteria and resistance genes was increased in biofilms at concentrations below 1 μg/L (Lundström et al., 2016). Assuming an assessment factor of 10, from this data a $PNEC_{R(Exp)}$ would be 0.05 μg/L, which is 20 times lower than $PNEC_{R(T)}$ of 1 μg/L (Bengtsson-Palme and Larsson, 2016). There is no NOEC data for tetracycline in cyanobacteria, but in *Microcystis aeruginosa* a EC50 is reported at 90 μg/L (Halling-Sørensen, 2000) and in *Anabaena* sp. an EC10 of 2.5 mg/L (González-Pleiter et al., 2013), suggesting that resistance for tetracycline may occur at concentrations nearly 100-fold lower than effects on growth inhibition in cyanobacteria. This again emphasizes the need for a more holistic approach to the setting of protection goals for antibiotics and the development of validated assays to assess MSCs in complex and simple systems, as well as generating toxicity data for cyanobacteria and other environmental and/or clinical bacteria.

It should be recognized that although studies that are used to guide regulatory decision-making require standardized test methodologies to help ensure reliable and repeatable results, the link between these single species studies and those operating in the complex systems in the field is largely unknown and, as mentioned previously, the link to ecosystem services is not made. The application of mesocosm studies that enable community response and effects upon ecosystem functions to be assessed have good utility here to help provide insights into the development of AMR in environmentally realistic scenarios (Knapp et al., 2008; Knapp et al., 2010; Quinlan et al., 2011). In addition to living in complex communities in the environment, it is important to note that organisms are also likely to be exposed to antibiotic mixtures and the relationship between single exposure laboratory testing and

mixtures toxicity is unknown and requires further research (Backhaus et al., 2000; Brosche and Backhaus, 2010; González-Pleiter et al., 2013; Liu et al., 2014).

In the context of current regulatory guidance, MSCs derived from experimental data, albeit they are limited, in some cases supported the theoretically derived $PNEC_{R(T)}$. There were cases also where $PNEC_{R(T)}$ was not necessarily appropriate (optimal) for risk assessment for AMR. Nevertheless, until there is an internationally accepted method for the experimental determination of $PNEC_R$ - which may require further knowledge on resistance mechanisms, model variability and the application to mixed communities that vary over time and space - the theoretical approach advocated by Bengtsson-Palme and Larsson (2016), based on MIC data in the European Committee on Antimicrobial Susceptibility Testing database, provides a valuable alternative as part of a broader evidence-based approach to ERA. Moreover, it provides an efficient and cost effective method to address concerns and prioritise legacy antibiotics that have already been registered and are present in the environment. It should be noted, however, that there are clear limitations to this approach (as identified by the paper's authors). These include the test conditions for determining the MIC in CRB, that are largely environmentally irrelevant, the assumptions that growth inhibition can be used to predict selection for resistance. There is also an assumption that an assessment factor of 10 will provide a suitable safety margin to account for selection below the MIC and conversely that adjusting the MIC down to account for species numbers and then applying a further assessment factor of 10 isn't overprotective. Finally, MIC-derived protection goals will change over time, as MICs are determined for more species with variable sensitivity and as a consequence periodic updates will be required.

Our analysis suggests that the susceptibility of species in European Committee on Antimicrobial Susceptibility Testing is not always protective of environmental bacteria, such as cyanobacteria and therefore a $PNEC_{R(T)}$ using CRB MIC data as a surrogate for resistance may not be protective of the risk of AMR development in environmental bacteria. Furthermore, we show that a $PNEC_{R(T)}$ may not be protective of ecosystem function traditionally determined using the growth inhibition test with cyanobacteria. From this we conclude that despite evidence that resistance will occur at lower concentrations than the effects on population density (Gullberg et al., 2011; Hughes and Andersson, 2012), both a $PNEC_R$ and a $PNEC_{SW}$ are needed to establish safe concentrations for the protection of ecosystem function and against the development of resistance.

It is noteworthy that from an environmental health perspective (rather than human health), AMR can provide an ecosystem service or benefit. For example, bacteria expressing beta-lactamase enzyme activity degrade and reduce the environmental burden of beta-lactam antibiotics and this in turn could contribute positively in sewerage treatment plants where high antibiotic concentration might otherwise compromise functional efficiency.

4.3. Production discharge limits

In addressing the impact of antibiotic pollution on ecosystem function, AMR development and human health, safe discharge limits for antibiotic production facilities need to be established (Agerstrand et al., 2015; Larsson, 2014; Pruden et al., 2013). However, there are few data available in the public domain to support the development of such limits and this is especially so for experimental data on AMR development. Most data that are available are based on growth inhibition tests and we have therefore identified the lowest NOEC values for 27 antibiotics representing sensitive phyla (cyanobacteria, *V. fischeri* and *P. putida*) and using these data we estimate the 5th percentile to be 225 ± 71 ng/L. Thus, a conservative limit of 154 ng/L would account for uncertainty. Provided that these 27 antibiotics are representative of all antibiotics, the cyanobacterial NOECs are, with 95% confidence, likely to be higher than 154 ng/L.

The lowest MSC reported in the literature is 100 ng/L with many others between 10 and 1000 times higher (Brosche and Backhaus, 2010; Gullberg et al., 2014; Gullberg et al., 2011; Lundström et al., 2016). Setting a threshold limit of 100 ng/L for antibiotic discharges would, therefore, appear to be protective of environmental bacterial populations (with 95% confidence) and match the lowest empirical evidence of AMR development. However, it would not be protective for 16% of the theoretical $PNEC_{R(T)}$s, described by Bengtsson-Palme and Larsson (2016) (Fig. 6B) highlighting that safe discharge limits may need to be lower than this for some antibiotics in order to consider the potential to select for resistance in clinical and environmental isolates. It should be noted, however, that the $PNEC_{R(T)}$ incorporates a correction factor that adjusts the MIC according to the number of species it is based upon and a further assessment factor of 10 to account for AMR. In turn, the corrections could cause the $PNEC_{R(T)}$ to be over protective (as shown for some antibiotics in Fig. 5B).

A single, protective threshold limit that could be applied as an interim measure in the absence of other reliable empirical clinical and or environmental data (and standardized methodologies for AMR), which is based on empirical data would be of great value. Based on the antibiotic compounds for which we were able to obtain NOECs from environmentally relevant bacteria and from the available MSCs in the literature, we suggest a production discharge limit of 100 ng/L for each antibiotic, applied in the mixing zone downstream of the point source discharge for protection of ecosystem function and the risk of AMR development. The use of a single protection goal rather than a range, for production facilities offers pragmatic benefits to industry and suppliers. Compliance with a single protection value provides simplicity and ease of implementation compared with the 111 values advocated for the different antibiotics suggested by Bengtsson-Palme and Larsson (2016), of which some would not be protective of the environment or the MSC. Consideration is required for how this limit would apply in the case of antibiotic mixtures, although this falls out of scope of this meta-analysis.

This approach could also help prevent the use of conflicting values for a single antibiotic. However, it is important to ensure that this value proves to be protective. So where other data are available (e.g. empirical or $PNEC_{R(T)}$) that suggest a lower limit is required to be protective, the 100 ng/L should be adjusted accordingly to provide the required protection. Equally, a higher limit may be applicable where there are substantive data to support its increase. We advocate this as an interim measure only until more data are obtained to support the risk analysis for antibiotics. Furthermore, as methodologies for the assessment of AMR are developed these values should also be incorporated and protection goals updated.

5. Concluding remarks and considerations for ERA

Our analysis shows that frameworks for ERA and human health protection (through protection for the risk of AMR) for antibiotics need to consider the impact of antibiotics on relevant vulnerable species and the essential ecosystem services they provide. The current framework for ERA based on just one cyanobacterial species is, in many cases, inadequate and it does not address risk to critical ecosystem services. There is also an urgent need to better establish the effects of antibiotics on bacterial diversity, community structure, ecosystem function and resilience in order to better understand the effects of antibiotics in the environment.

We emphasise that the presence of antibiotics in the environment does not necessarily lead to the development of AMR in bacterial communities and studies are required that better establish the toxic effects of antibiotics, AMR and the relationship between them in environmentally relevant contexts. In the environment other selection pressures (e.g. nutrient availability and predation) may be more significant than that posed by exposure to low levels of antibiotics. As a consequence AMR may not be observed at the same concentrations as in

the laboratory studies. However, it is also the case that the fitness cost of carrying some resistance genes may be very low or even neutral and therefore the genes coding for resistance could remain in the bacterial communities after only a short exposure. Understanding these complexities in AMR development in the environment is crucial for establishing interrelationships with human pathogens and in turn managing and mitigating the risk of antibiotics in the environment for the protection of human health.

From our analyses on relative species sensitivity we highlight the following as key considerations for the use, and development of human and ERA frameworks for antibiotics.

1. The need for inclusion of a larger selection of bacterial species for testing to account for the variability in sensitivity between species and for greater confidence in the protection of bacterial communities and the ecosystem services they provide.
   a. Brandt et al. (2015) have identified a number of suitable established standard tests for other bacteria (including *P. putida*) and for ecosystem services (e.g. nitrification and carbon transformation) and these should be considered as additional tests in the ERA of antibiotics.
   b. We show that pre-clinical MIC data of CRB could be used to increase the diversity of bacterial species represented in ERA at little cost. The use of pre-clinical and clinical data is often advocated to identify environmental risk (Boxall et al., 2012) but the realisation of this is limited with 'bridging' studies and methods still being developed.
   c. We reaffirm that the only required community test, the ASRIT, is not sensitive to antibiotics and thus its suitability for determining the effect of antibiotics to environmental bacteria and sewerage treatment plant microorganism communities is questionable. Consideration for its replacement by tests to assess the effects on bacterial community function or impacts on population growth are warranted.
2. Testing of antibiotics on metazoans may not be required.
   a. Metazoans were generally 2 to 4 orders of magnitude less sensitive to antibiotics than cyanobacteria. Further investigation is required to assess and confirm these results on a wider series of empirical in vivo exposures, however this meta-analysis provides a starting point for this discussion and the possible reduction in the use of metazoans in antibiotic testing.
3. Our meta-analysis highlights that the relative high sensitivity of microalgae and macrophytes to some antifolate and quinolone antibiotics (compared with cyanobacteria) supporting their inclusion in risk assessment frameworks for these compound classes. Further research into the relative sensitivity of macrophytes and microalgae to these classes of antibiotics is warranted.
4. Test systems to determine PNEC or MSC for AMR development are urgently required for clinical and environmental species. Our analysis, suggests that the CRB in the European Committee on Antimicrobial Susceptibility Testing database are not always representative of the diversity of sensitive bacteria in nature. This illustrates that ERA needs to incorporate both $PNEC_{SW}$ and $PNEC_{R}$. There is a need to develop a standardized method to experimentally determine an MSC in environmental and clinical bacteria, exemplified by three out of five experimental values being lower than the theoretical value.
5. A discharge limit of 100 ng/L maybe a protective and pragmatic approach to address environmental concerns around antibiotic production in the absence of sufficient reliable clinical and environmental data, whilst urgently needed methodologies and empirical data are obtained to draw firmer conclusions. Where data exists that suggest a higher or lower concentration is required to be protective that value should be used instead.

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GLP is a former employee and current shareholder of AstraZeneca PLC. JRS is an employee and shareholder of AstraZeneca PLC.

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Antibiotic risk assessment needs to protect both environmental and human health

In our recent meta-analysis on antibiotic ecotoxicity data published in *Environment International* (Le Page et al., 2017) we suggest that because of the great diversity in species sensitivity, environmental risk assessment (ERA) would be improved by testing a more diverse range of bacteria (including both environmental bacteria and clinically relevant bacteria (CRB)). We also conclude that tests on antibiotics should consider endpoints of relevance to ecosystem function. Comparing the protection goals for environmental heath with those for human health (protection against antimicrobial resistance (AMR) development) we, furthermore, identify that neither protection goal is always protective of the other whilst using current methodologies (with surrogate endpoints for each goal and very limited bacterial biodiversity tested); supporting the need for both in any comprehensive health protection system for antibiotics.

In a correspondence to our paper Bengtsson-Palme and Larsson (2018) point out a bias in our sensitivity analysis favouring environmental bacteria (including cyanobacteria). We acknowledge this, but equally in this correspondence we challenge some of their points made on how this impacts on the significance of our data. We also address points relating to the lack of clarity on protection goals for antibiotics in the discussion of our paper and discuss what data are most suitable for establishing those protection goals. We emphasise that the main conclusion drawn from our original paper has not changed and we maintain that a holistic approach including both environmental health and resistance selection is required to drive an effective overall protection limit for antibiotics.

1. Sensitivity analyses skews

Bengtsson-Palme and Larsson (2018) rightfully point out that our analysis skews the apparent sensitivity in favour of the environmental bacteria because the endpoints compared for CRB (minimum inhibitory concentrations, MIC) and environmental bacteria (no observed effect concentrations, NOEC) for growth inhibition are derived from different ends of the dose response curve; MICs are derived from the top of the dose-response curve (full inhibitory effect on growth) and the NOECs for environmental bacteria from the bottom of the response curve (concentration with no inhibition). In some cases therefore CRB may be more sensitive than environmental bacteria than our analysis suggests. However, it should be highlighted that this doesn't necessarily mean that environmental bacteria will not represent the most sensitive taxa for individual antibiotics. This is because, in the first instance, in the cases where environmental bacteria were more sensitive by an order of magnitude or more compared with CRB in our analysis, environmental bacteria are likely to be comparable, if not more sensitive to those antibiotics. In our meta-analysis this would be the case for 6 out of 24 antibiotics (including azithromycin and ampicillin). Secondly, very large differences in sensitivity can occur between different species of bacteria (our meta-analysis showed sensitivity spanned five orders of magnitude in 8 species cyanobacteria exposed to ampicillin) and because of the far greater species number and diversity tested in CRB compared with environmental bacteria there is likely to be a sensitivity bias in favour of CRB. The size-adjusted MIC value used as our comparative endpoint for CRB was calculated from the MICs of up to 70 species in up to 5 families (Bengtsson-Palme and Larsson, 2016). In stark contrast to CRB, cyanobacteria antibiotic test data were generally derived from only one or two species giving far greater uncertainty in the sensitivity calculation for this group.

2. Uncertainty in protection targets

ERA for antibiotics in the European Union is legislated by the Medicinal Products for Human Use directive (EC, 2001) where the protection goal is to prevent “any risk of undesirable effects on the environment”. Current practice is to calculate a PNEC using chronic growth and/or reproduction data on single species, which for antibiotics is normally based on the $PNEC_{SW}$ driven by a cyanobacterium. The relationship however, between individual species sensitivity, ecosystem function and functional redundancy is not well understood (Antwis et al., 2017) and what constitutes an “undesirable effect” is unclear. As Bengtsson-Palme and Larsson (2018) point out, clarity is, therefore, required in the definition and objectives of these protection goals. The issue of functional redundancy, and to what extent it is possible to eradicate or lose a microbial species without compromising that ecosystem function is a hugely important consideration for environmental protection. There is some evidence that microbial communities may be less functionally redundant than macroorganism communities (Delgado-Baquerizo et al., 2016). Thus, although we reiterate our support of the inclusion of ecosystem function based tests, given the uncertainties relating to functional redundancy, at this time ecosystem level protection may be best served by a conservative protection goal based upon bacterial biodiversity (and therefore inherently ecosystem function).

Bengtsson-Palme and Larsson (2018), highlight that the risk of AMR and human health concerns are generally the main driving force for antibiotic protection goals but they also agree with our conclusions that a holistic approach that considers both environmental health and AMR should be taken. The meta-analysis shows that for some antibiotics the environmental protection limits may be lower than the protection limits predicted for AMR (using current methodologies and surrogate endpoints for biodiversity and AMR). To illustrate this, here (Fig. 1) we compare the $PNEC_r$ determined using the size-adjusted MIC data (Bengtsson-Palme and Larsson, 2016) and $PNEC_{sw}$ calculated from the lowest NOEC in our meta-analysis with the $PNEC_{fw}$ (PNEC in freshwater) determined for the 5 antibiotics in the European commission environmental quality standards watch list (Carvalho et al., 2015). In each case the $PNEC_r$ represents the highest PNEC for each antibiotic (i.e. is least protective as a whole).

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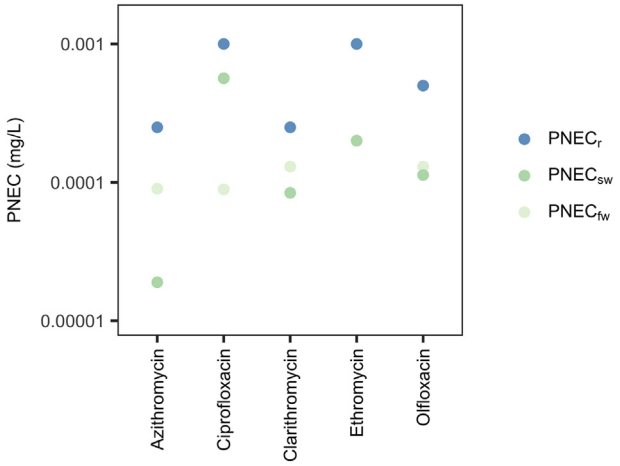

Fig. 1. Predicted no effect concentrations (PNEC) for the antibiotics in the European commission watch list under the environmental quality standards directive (Carvalho et al., 2015). $PNEC_{fw}$ is the PNEC that is determined for freshwater in the European commission directive (Note that the assessment factor for $PNEC_{fw}$ may be up to 50 rather than 10 in these examples due to the lack of a full phase II base set of data – algae/cyanobacteria, invertebrates and fish (EMA, 2006). The $PNEC_{fw}$ for ciprofloxacin is thus most likely overprotective); $PNEC_r$ is the PNEC calculated from minimum inhibitory concentrations (Bengtsson-Palme and Larsson, 2016); $PNEC_{sw}$ is the PNEC determined from the lowest, publically available, environmental bacteria no observed effect concentration (Le Page et al., 2017). $PNEC_{sw}$ uses an assessment factor of 10 for each antibiotic.

As Bengtsson-Palme and Larsson (2018) point out, protection against antibiotic pollution for environmental health is more of a localised impact, whereas AMR has a wider and more pervasive global significance, directing stakeholders towards the need for two different protection targets determined from appropriate data and methodologies. We still maintain however, that an overall protection limit should protect both environmental and human health. Environmental protection and associated legislation differs across countries, but equally there is a social responsibility to ensure that product provenance is conducted to the highest possible levels.

3. Discharge limit

In response to stakeholder calls to address the risk of antibiotics released from manufacturing operations, which currently sits outside of the regulatory ERA framework, in our original paper we proposed an interim production discharge limit of 100 ng/L for each antibiotic, to be applied in the mixing zone to both protect environmental bacteria populations and reduce the risk of AMR development. This interim limit recognised that (i) because most antibiotics were authorised before the current guidelines came into force, many either lack or have very limited ecotoxicology data, and (ii) the need to establish science-based limits in the absence of such data. We were explicit in our paper to point out, however, that as sufficient data become available for mode of action relevant species we support the use of higher or lower protection limits based on these empirical data. Bengtsson-Palme and Larsson (2018) questioned this conservative limit for antibiotics because it may incur higher manufacturing costs through the need for infrastructure investment to reduce discharges and based on the fact that some antibiotics have relatively low toxicity and do not exert a strong selection pressure for antibiotic resistance. These are important points to debate. A single interim value helps the pharmaceutical industry, many of whom are currently reviewing their antibiotic manufacturing operations, to prioritise interventions and actions. These interventions may include generating relevant environmental toxicology data where empirical data does not exist or when a possible risk is identified at a site. A single value will also enable the pharmaceutical industry to benchmark existing suppliers more effectively to identify best practice in waste management. The requirement for infrastructure investments, as highlighted by Bengtsson-Palme and Larsson (2018), represents a last resort and these would only be required where risks could not be refined and managed through other interventions. Where infrastructure upgrades are required to meet scientifically robust limits, then the costs of these upgrades will need to be evaluated and justified as part of a wider socio-economic assessment into the stewardship of antimicrobial chemotherapy. In most cases, however, these interventions are not likely to incur excessive costs; the manual wipe down of equipment prior to cleaning washes, separation and incineration of the wastewater from the first wash of equipment, or the installation of inline filters to remove undissolved material can all significantly reduce environmental concentrations of APIs, in most cases by > 90% (Hargreaves et al., 2017). The logistics for antibiotic supply can be extremely complex with many suppliers manufacturing a whole range of antibiotics for numerous contractors and there can be language barriers and many suppliers lack the expertise to determine safe concentrations for themselves. In this case the use of a single interim limit has practical as well as scientific value. It may help remove conflicting limits (e.g. where two contractors provide different safe values or no level of protection), and minimise confusion amongst the pharmaceutical industry and their suppliers in the absence of data.

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GLP is a former employee and current shareholder of AstraZeneca PLC. JRS is an employee and shareholder of AstraZeneca PLC.

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