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

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Abstract

Once released into the environment antibiotics can kill or inhibit the growth of bacteria, and in turn potentially have effects on bacterial community structure and ecosystem function. Environmental risk assessment (ERA) seeks to establish protection limits to minimise chemical impacts on the environment, but recent evidence suggests that the current regulatory approaches for ERA for antibiotics may not be adequate for protecting bacteria that have fundamental roles in ecosystem function. In this study we assess the differences in interspecies sensitivity of eight species of cyanobacteria to seven antibiotics (cefazolin, cefotaxime, ampicillin, sulfamethazine, sulfadiazine, azithromycin and erythromycin) with three different modes of action. We found that variability in the sensitivity to these antibiotics between species was dependent on the mode of action and varied by up to 70 times for β-lactams. Probabilistic analysis using species sensitivity distributions suggest that the current predicted no effect concentration PNEC for the antibiotics may be either over or under protective of cyanobacteria dependent on the species on which it is based and the mode of action of the antibiotic; the PNECs derived for the macrolide antibiotics were over protective but PNECs for β-lactams were generally under protective. For some geographical locations we identify a significant risk to cyanobacteria populations based upon measured environmental concentrations of selected antibiotics. We conclude that protection limits, as determined according to current regulatory guidance, may not always be protective and might be better derived using SSDs and that including toxicity data for a wider range of (cyano-) bacteria would improve confidence for the ERA of antibiotics.
41 **Keywords:**

42 Antibiotics; Antimicrobial resistance; Environmental risk assessment;

43 Pharmaceuticals; Species sensitivity distribution; Predicted No Effect

44 Concentration
1. 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 their release into the environment however, non-target bacteria may be affected and the vital ecosystem services they facilitate may be disrupted as a consequence, which include primary productivity, nutrient cycling and contaminant degradation (Dopheide et al., 2015; Grenni et al., 2018; Kümmerer, 2009). Aquatic ecosystems are especially at risk due to the concentrations of antibiotic inputs received from manufacturing plants and hospital effluents, wastewater treatment plants (WWTP), aquaculture, and run-off from agriculture (Batt et al., 2007; Brown et al., 2006; Cabello, 2006; Jaimes-Correa et al., 2015; Larsson, 2014; Larsson et al., 2007; Li et al., 2008; Liu et al., 2017; Watkinson et al., 2009). The European regulatory environmental risk assessment (ERA) for antibiotics aims to establish protection limits that prevent “risk of undesirable effects on the environment” (EC, 2001), but the effectiveness of the current approach to do so has been questioned (Agerstrand et al., 2015; Brandt et al., 2015; Le Page et al., 2017). In addition, many antibiotics lack data for environmental bacteria due to the regulatory requirement for ERA testing only coming into force in 2006; before which most antibiotics had already been approved (Le Page et al. 2017). Consequently, there is an urgent need to assess whether the protection limits currently derived according to the current ERA guideline for antibiotics are able to protect against undesirable effects on the environment.
In the European ERA for human medicinal products and the VICH guidelines for veterinary antibiotics, protection limits for pharmaceuticals, including antibiotics, are represented by a predicted no effect concentration (PNEC). This is calculated by applying an assessment factor (AF) of 10 to the lowest no observed effect concentration (NOEC) following testing upon a cyanobacteria (green algae when not an antibiotic), invertebrate, fish and an activated sludge respiration inhibition test (ASRIT); i.e the PNEC is calculated by dividing the lowest NOEC by 10. The ASRIT however, is not sensitive to antibiotics (Kümmerer, 2009; Le Page et al., 2017) and consequently only a single species of cyanobacteria represents all bacterial diversity in an antibiotic ERA that also measures a single functional endpoint: primary productivity. Additionally, most tests use either *Anabaena flos-aquae* (particularly in the case of regulatory studies) or *Microcystis aeruginosa*, providing a limited understanding of cyanobacteria interspecies sensitivity. In a revised version of the EMA guidance for ERA that is currently under consultation, however, it is advocated that two cyanobacteria species should be tested and fish are only tested when the pharmaceutical targets are present. The AF is applied to account for uncertainty due to interspecies variability and the extrapolation from controlled laboratory studies to the field. But the application of an AF of 10 for antibiotics is unsupported by experimental data and evidence shows that in some cases interspecies bacterial sensitivity may exceed this by several orders of magnitude (Chapman *et al.*, 1998; Le Page *et al.*, 2017). Consequently, there may be cases where the PNEC is not protective of all species in the environment. Moreover, a PNEC calculated this way has two potential drawbacks: firstly, the NOEC has been heavily criticised due to its dependence on the design of the experiment.
conducted to derive it (Green et al., 2013) and secondly, because it uses only a single effect value (the NOEC); quantification of the uncertainty around the PNEC is not determined (Chapman et al., 1998).

A second approach for establishing protection limits that overcomes some of the problems associated with the current PNEC approach is through the construction of a species sensitivity distribution (SSD). A SSD is a probability model of interspecies variability across a toxicity endpoint following chemical exposure (e.g. NOEC or ECₙ) and it allows 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 in plant protection product regulations (EFSA, 2013) or 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 no more than 5% of species (HC5), although it has been suggested that the lower 95% confidence limit of the HC5 (HC5₂.₅%) should be used to ensure a truly protective limit (Verdonck et al., 2001; Wheeler et al., 2002). This lower, more protective limit, however, will have more statistical uncertainty. An AF 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).

In the past SSDs have been criticised for being ecologically unrealistic and for a lack of statistical robustness but recent advances 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, as well as the choice of taxa, their sensitivity to the mode of action and the even representation of the taxonomic groups of interest. 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 computation of reliable statistics from a limited dataset (<10 data points) (Kon Kam King et al., 2014; Wheeler et al., 2002). Fewer species therefore are arguably required to reliably model the lower tail of the SSD (comprising the most sensitive species) from which a protection limit could be derived, providing they are all known to be sensitive to the mode of action (MoA) of the chemical. This is because the SSD focuses upon the species most at risk and the distribution will not be impacted by non-sensitive species or taxonomic clades (Schmitt-Jansen et al., 2008; Segner, 2011).

It is not uncommon for the measured environmental concentrations (MECs) of antibiotics to exceed the PNEC in the environment, especially in WWTP, 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 risk to bacterial communities and the ecosystem functions that they provide. By considering these MECs in relation to a SSD it is possible to obtain an indication of the proportion of species that may be at risk.

In a recent meta-analysis of all publicly available literature we identified that cyanobacteria sensitivity to antibiotics may vary by up to 100,000 times (Le Page et al., 2017). For some antibiotics a sensitivity difference exceeding the AF of 10
occurred between the most sensitive species, most commonly, *Microcystis aeruginosa*, and the two species recommended in the OECD 201 test guideline for establishing protection limits, namely *Anabaena flos-aquae* and *Synechococcus leopoliensis*. Although in some cases when *A. flos-aquae* was the most sensitive species the assessment factor of 10 did appear to be protective. The aforementioned meta-analysis was based on an assessment of published data and collated studies performed using different methodologies and test conditions in different laboratories by different researchers. 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 phylogenetically diverse cyanobacteria (as assessed by their genome sequences (Shih *et al.*, 2013)) culturable under laboratory conditions that are of environmental relevance (Le Page *et al.* under review). We focused on cyanobacteria due to their current key role within ERA and because they are a 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).

Seven antibiotics were selected that spanned both a range of antibiotic classes and modes of action (MoA) in order to assess the impact MoA may have on the
degree of interspecies sensitivity observed. These included: i) three cell envelope
synthesis inhibiting antibiotics, β-lactams, which target penicillin binding
proteins (which catalyse the building of the peptidoglycan cell membrane of
bacteria) namely, cefazolin and cefotaxime (1st and 3rd generation
cephalosporins, respectively) and ampicillin (a penicillin); ii) two DNA synthesis
inhibitors, sulfadiazine and sulfamethazine (sulfonamides) that prevent the
production of folic acid, a key precursor in the DNA synthesis pathway; and iii)
two protein synthesis inhibitors, erythromycin and azithromycin (macrolides),
which inhibit the normal functioning of the bacterial ribosome. The macrolides,
azithromycin and erythromycin are both candidates to be priority substances in
the EU Water Framework Directive watch list (Carvalho et al., 2015) and US EPA
contaminate list 3 (US EPA, 2009). Cefazolin and sulfamethazine 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).

We addressed the hypothesis that current protection limits for antibiotics in
surface water (PNEC_{SW}) are not fully protective of all cyanobacteria populations.
We first determined the interspecies sensitivity differences of eight species of
cyanobacteria by performing growth inhibition assays. We then established SSDs
and compared the PNEC calculated according to current guidance with the HC5
and HC5_{2.5%} to determine the proportion of species that would be affected
following exposure to the PNEC determined from these results. Finally, based on
our SSDs, we calculated the proportion of cyanobacteria likely to be affected
using published MECs.
2. Materials and methods

2.1. 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). The basis for the selection of each species is given in Supplementary material A.

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

2.2. 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). These antibiotics span three MoAs that are detailed above in the introduction. Additional rationale for their choice was based upon
one or a combination of the following; i) being a compound of regulatory concern (Carvalho et al., 2015; US EPA, 2009), ii) having suitable solubility in the test media, and iii) having limited or no cyanobacteria data available in the literature. A summary of the chemical properties is given in table 1.

2.3. 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 which was developed as a medium throughput test and aims to identify which species are susceptible than others and of which the development and validation is documented in (Le Page et al., under review). Biomass was measured using phycocyanin fluorescence as a surrogate (excitation = 590 nm, emission = 650 nm, cut-off = 635 nm; 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, Le Page et al., under review).

A pre-culture for each species was prepared between three and four days prior to the start of the test in 50 mL of BG-11 under the experimental exposure conditions (but 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 double the nominal test concentrations.
100µL of test solution was added to 100 µL of cyanobacteria inoculum to achieve a final cyanobacteria density 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™ 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 Supplementary material A.

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 ensure toxicity testing was carried out, as best as possible, during exponential growth for each species, and these were: 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. The exception here was for the exposure of the *Synechocystis* sp. to sulfadiazine where due to a slower growth rate than expected, an exposure period of 72 hours was adopted; iii) 72 hours for the slower growing species, *A. cylindrical*, *C. gracile* and *G. herdmanii*. (Le Page *et al.*, under review) provides further discussion around the selection of exposure times and the potential consequences for their extension or reduction, although we highlight that with the current set up we cannot discern the magnitude of effect of the technical uncertainty caused by comparing several species and how this may impact on the biological differences observed.

Daily cell density determinations were made for each well via measurement of phycocyanin fluorescence. 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 ($p < 0.01$) were observed in the solvent control (two tailed 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.
### Table 1 – Chemical properties of antibiotics.

- **Antibiotic**
- **Primary pharmacological Target**
- **Log Kow**
- **pKa**
- **Log Dow (pH 8)**
- **Solubility at pH 8.0 (g/L)**

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Primary pharmacological Target</th>
<th>Log Kow</th>
<th>pKa</th>
<th>Log Dow (pH 8)</th>
<th>Solubility at pH 8.0 (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cefazolin</td>
<td>Penicillin binding protein</td>
<td>-1.52</td>
<td>2.84 (acid)</td>
<td>-5.04</td>
<td>454.5</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>Penicillin binding protein</td>
<td>-1.49</td>
<td>2.73 (acid)</td>
<td>-4.24</td>
<td>455.5</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>Penicillin binding protein</td>
<td>-2</td>
<td>3.24 (acid)</td>
<td>-2.72</td>
<td>0.04</td>
</tr>
<tr>
<td>Sulfadiazine</td>
<td>Dihydropteroate synthetase</td>
<td>-0.39</td>
<td>6.99 (acid)</td>
<td>-0.33</td>
<td>8.91</td>
</tr>
<tr>
<td>Sulfamethazine</td>
<td>Dihydropteroate synthetase</td>
<td>0.65</td>
<td>6.99 (acid)</td>
<td>-0.06</td>
<td>4.72</td>
</tr>
<tr>
<td>Azithromycin</td>
<td>Bacterial ribosome</td>
<td>2.44</td>
<td>12.43 (acid)</td>
<td>-0.08</td>
<td>1810</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>Bacterial ribosome</td>
<td>2.6</td>
<td>12.45 (acid)</td>
<td>1.55</td>
<td>43.3</td>
</tr>
</tbody>
</table>

\(^{a}\) according to drugbank (www.drugbank.ca).

\(^{b}\) predicted by ChemAxon (www.chemicalize.org)

#### 2.4. Chemical analysis

The concentrations of antibiotics in the stocks and in three exposure replicates for all concentrations and in each species at the end of the tests were measured using liquid chromatography-mass spectrometry (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.

2.5. Measured environmental concentrations

The MECs for each antibiotic were obtained from Umweltbundesamt’s (UBA) ‘Pharmaceuticals 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. Measurements of 0 μg/L were removed as they represent either the absence of the antibiotic or presence below the limit of detection, which make this analysis assume a worst-case scenario by moving the median to higher concentrations. MECs from matrixes such as inflows to WWTP, sewage sludge or untreated hospital and industrial effluents were also removed from the analysis to leave only environmentally relevant MECs.

2.6. Statistical analysis

2.6.1. Growth rate calculations

Growth rate of cyanobacteria was calculated according to equation below based on the phycocyanin fluorescence at the start and the end of the assay.

\[
\text{Growth Rate} = \frac{\ln X_j - \ln X_i}{t_j - t_i}
\]

where

\(X_i = \text{cell density at time } t_i\)

\(t_i = i^{th} \text{ time point}\)
### 2.6.2. Dose-response modelling and ECx 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). For all pairs of bacterial species and antibiotics, growth rate data were fitted to log-logistic (3, 4 and 5 parameters) and Weibull distributions (4 and 5 parameters). Of these, the optimal distribution was selected based of the log-likelihood score to represent the data. From this fitted distribution estimates of the 10 and 50% effective concentrations (ECx) and associated confidence limits were determined. Data handing for the growth rate determinants for each species are provided in Supplementary material A.

### 2.6.3. Species Sensitivity Distributions

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). The 95% confidence intervals of the EC10 for each species 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 EC10 into the SSD and this increases 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.

The HC5 and associated confidence intervals were determined from bootstrapping of 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 but for this the NOEC values were used as non-censored data.

3. Results

3.1. Antibiotic exposure concentrations

The measured concentrations of the antibiotics in each microplate assay, calculated using a geometric mean of the concentrations at the start (stocks) and end (exposure replicates) of the test, are provided in the Supplementary material B (figures S.B1 – S.B14). Overall concentrations of the antibiotics in the test media varied with losses due, in part, to the presence of the bacteria. These losses differed across the various antibiotics tested and species (graphs S.B15 – S.B21; determined as the difference between concentrations in the wells containing cyanobacteria and blank replicates (without cyanobacteria)).

For cefazolin, mean measured concentrations in the exposure replicates ranged between 14 to 32% of the nominal concentrations (Fig S.B1). The greatest losses of cefazolin occurred in the exposures to *S. elongates* and *Synechococcus sp.*

Mean measured concentrations of cefotaxime in the exposure replicates ranged between 18 and 44% of nominal (Fig S.B3). The greatest reductions in the exposure replicates compared with replicates without cyanobacteria were for the *Synechococcus* genus (Fig S.B16).
Mean measured concentrations of ampicillin in the exposure replicates ranged between 44 and 95% of nominal (Fig S.B5). In the presence of the cyanobacteria there were generally between 10 and 30% additional reductions compared with the replicates without bacteria, but was most pronounced in the A. cylindrica exposure replicates (S.B17).

Mean measured concentrations of sulfadiazine in the exposure replicates were between 101 and 142% of nominal (Fig S.B7). The high measured concentrations of up to 142% of nominal occurred in the nominal 2.36, 145 and 1140 μg/L test concentrations and they would increase the uncertainty around toxicity estimates calculated. However, due to the lack of sensitivity of the cyanobacteria to sulfadiazine (see below) this doesn’t affect any conclusions drawn. Reductions in sulfadiazine concentrations due to the presence of the cyanobacteria varied across tests concentrations (S.B18).

Mean measured concentrations of sulfamethazin in the exposure replicates ranged between 87 to and 134% of nominal (Fig S.B9). Extraction errors for nominal concentrations 907 and 1633 μg/L in the S. elongates exposure meant that these had to be excluded for the analyses.

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 concentrations were lowered by up to 50% over the exposure period 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 replicates without cyanobacteria present (Fig S.B20).

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 by between 10 and 15% (Fig S.B21).

3.2. Growth inhibition, species sensitivity distributions and protection

limit analysis:

The dose-response curves for growth inhibition of the eight cyanobacteria for each antibiotic tested are presented in Figure 1. The EC10s, EC50s and NOECs for the experimental data are given in Table 2 (raw data are provided, and shown graphically in Supplementary material C, figures S.C1 – S.C7). All dose-response analyses are based upon geometric mean measured test concentrations. The pHs at the start and at the end of the tests are provided in Supplementary material D.

The data in Figure 2 presents the SSDs, based upon cyanobacteria EC10s for each antibiotic, together with PNECs based upon the NOEC of the most sensitive species tested (PNEC_{lower}) 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-aquae}) and S. leopoldensis (PNEC_{S. leopoldensis}). SSDs
based upon cyanobacteria NOECs are provided in Supplementary material C (figure S.C8). It was not possible to establish the SSDs for sulfamethazine or for sulfadiazine as neither produced a full dose response curve from which to calculate an EC$_{10}$.

The data in Table 2 provide values for the HC5, PNECs and the proportion of cyanobacteria affected at these concentrations, predicted from the SSD (based on the EC$_{10S}$). Table 3 gives the highest and median MECs and the proportion of cyanobacteria affected at these concentrations predicted from the SSD (based on the EC$_{10S}$). The same information as Tables 2 and 3 are provided in Tables S.C1 and S.C2 but here using the SSD based upon NOEC data. Table S.C.3 provides the best fitting distributions used for establishing the SSD.

EC$_{10S}$ for the different cyanobacteria exposure for cefazolin ranged between 2.4 and 124 μg/L and the EC$_{50S}$ ranged between 4.1 to 283 μg/L (Figure 1 and Table 1). Based upon both EC$_{10}$ and EC$_{50}$ A. flos-aquae and G. herdmanii were the most sensitive species to cefazolin. 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$_{50}$. The HC5 for cefazolin, based on EC$_{10S}$, was 1.13 μg/L, which was 7.5 times higher than the lowest PNEC (for *A. flos-aquae*) but 4 times lower than that based on *S. leopoldensis* (Figure 2 and Table 2). The predicted proportion of cyanobacteria affected at the PNECs ranged between 0.95 and 13.3% depending on which species was used to derive the PNEC (Table 2). The HC5 based on the NOEC data was 5 μg/L, 4 times higher than when based on the EC$_{10}$ (Table SC.1). The median MEC was predicted to affect a small fraction
of cyanobacteria (<1%) based on the SSD. The highest recorded MEC (42.9 μg/L; the maximum concentration observed in a range of effluents in Taiwan, including manufacturing and hospital effluents (Lin et al., 2008)) was predicted to affect 60.2% of cyanobacteria (Table 3) with the second highest MEC of 6.2 μg/L affecting 16.2% of cyanobacteria. The median MEC of 6 hospital effluents in the same study, of 6.2 μg/L (Lin et al., 2008), would affect 17.3% of cyanobacteria based on the SSD.

EC$_{10}$s for the different cyanobacteria exposure to cefotaxime ranged between 1.2 and 39.8 μg/L and EC$_{50}$s ranged between 2.2 and 98 μg/L (Figure 1 and Table 1). The maximum difference in sensitivity (45 times) occurred between A. flos-aquae (the most sensitive) and Synechococcus sp (the least sensitive). 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. The HC5 for cefotaxime, based upon EC$_{10}$s, was 0.67 μg/L, which was 4 times higher than the lowest PNEC (for A. cylindrica) and approximately the same value as for the PNEC based upon S. leopoldensis (Figure 2 and Table 2). The predicted proportion of cyanobacteria affected at the PNECs ranged between 1.3 and 5.2% depending on which species was used to derive the PNEC (Table 2). The HC5 based upon the NOEC data was approximately the same as when based on the EC$_{10}$ (Table S.C.1). The median MEC had little effect upon cyanobacteria based on the SSD. The highest recorded MEC (41.9 μg/L; the maximum concentration observed in a range of effluents in Taiwan, including manufacturing and hospital effluents (Lin et al., 2008)) was predicted to affect 95.9% of cyanobacteria (Table
3). The median MEC of 6 hospital effluents in the same study, of 0.413 μg/L (Lin et al., 2008), would affect 3.1% of cyanobacteria based on the SSD.

EC$_{10s}$ for the different cyanobacteria exposure to ampicillin ranged between 5.9 and 44.6 μg/L and EC$_{50s}$ ranged between 8.4 and 81.4 μg/L (Figure 1 and Table 1). Based on the EC$_{50}$, there was a difference in sensitivity of approximately 10 times (9.7) between the most sensitive (C. gracile) and least sensitive species (A. cylindrical). C. gracile was 3 times more sensitive than the next most sensitive species, S. leopoliensis. The remaining cyanobacteria all had similar sensitivities with EC$_{50s}$ of between 52 and 81.4 μg/L. The HC5 for ampicillin, based on EC$_{10s}$, 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 on A. flos-aquae and S. leopoldensis, respectively (Figure 2 and Table 2). The predicted proportion of cyanobacteria affected at the PNECs ranged between 0.9 and 1.6% depending on which species was used to derive the PNEC (Table 2). The HC5 based upon the NOEC data was approximately the same as those based on the EC$_{10}$ (Table S.C.1).

The median MEC indicated little effect upon cyanobacteria based on the SSD. The highest recorded MEC of 27.1 μg/L (WWTP effluent in India (Mutiyar and Mittal, 2013)) was predicted to affect 44.3% of the cyanobacteria (Table 3).

Exposure to sulfadiazine only caused partial inhibition of growth of the cyanobacteria tested (Figure 1). It was possible to fit log-logistic or Weibull distributions to the growth data but as growth inhibition ceased before the point of 50% growth inhibition EC$_{10}$ or EC$_{50}$ values (and therefore SSDs) could not be
calculated. The highest recorded MEC was 30.5 μg/L; treated WWTP effluent in east China (Chen et al., 2012), whilst the median MEC was 0.019 μg/L. 

As for sulfadiazine, sulfamethazine did not induce full growth inhibition for any of the cyanobacteria tested (Figure 1) preventing the ability to calculate EC\textsubscript{10} or EC\textsubscript{50} values (or SSDs). C. gracile was the most sensitive species to the growth inhibition effects of sulfamethazine (a 50% reduction in growth rate was observed at an exposure concentration of 1465 μg/L). At the highest tested exposure concentration (10,000 μg/L) there was between a 30% and 40% decrease in growth rate in A. flos-aquae, A. cylindrical, S. leopoliensis, S. elongates, Synechococcus sp., and G. herdmani. Synechocystis sp was far less affected with only a 4% inhibition of growth at the highest tested concentration. The highest recorded MEC was 25.4 μg/L; treated WWTP effluent in Korea (Sim et al., 2011), whilst the median MEC was 0.015 μg/L. 

EC\textsubscript{10}s for the different cyanobacteria exposure to erythromycin ranged between 21.1 and 58.8 μg/L and the EC\textsubscript{50}s were between 43.4 and 135.1 μg/L (Figure 1 and Table 1). Based upon the EC\textsubscript{50}, there was only a small interspecies difference in sensitivity; 3.1 times, between the most sensitive (A. cylindrical) and least sensitive species (A. flos-aquae). The HC5 for erythromycin, based upon EC\textsubscript{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 for A. flos-aquae and S. leopoldensis, respectively (Figure 2 and Table 2). The proportion of cyanobacteria affected at the PNECs was <1% for all PNECs irrespective of which species was used to derive it (Table 2). The HC5 based upon the NOEC data was
3.5 times lower than when based on the EC\textsubscript{10} (Table SC.2). No species of cyanobacteria are predicted to be affected by the MECs based on the SSD (highest MEC was 7.8 μg/L; untreated manufacturing discharge in China (Lin and Tsai, 2009)) (Table 3). The HC\textsubscript{5} in this study was 107 times higher (HC\textsubscript{5.25} was 81 times higher) than the PNEC in the European Unions watch list of priority substances for erythromycin is 0.2 μg/L (Loos et al., 2018), but a PNEC based upon the lowest NOEC, based on \textit{S. elongates}, would have been only ≪3 times higher (NOEC ≪0.62 μg/L).

EC\textsubscript{10}s for the different cyanobacteria exposure to azithromycin ranged between 3.2 and 17.7 μg/L and EC\textsubscript{50}s ranged between 5.4 and 33.8 μg/L (Figure 1 and Table 1). Based upon the EC\textsubscript{50}, there was difference in sensitivity of 6.3 times only between the most sensitive (\textit{A. cylindrical}) and least sensitive species (\textit{Synechococcus sp.}). The HC\textsubscript{5} for azithromycin, based on EC\textsubscript{10}s, was 3.2 μg/L, which was 21 times higher than the lowest PNEC (for \textit{G. herdmanii}) and 3.1 and 16.6 times higher than the PNECs for \textit{A. flos-aquae} and \textit{S. leopoldensis}, respectively (Figure 2 and Table 2). The predicted proportion of cyanobacteria affected at the PNECs was ≪1% for all PNECs irrespective of which species was used to derive it (Table 2). The HC\textsubscript{5} based upon the NOEC data was approximately half as much as when based on the EC\textsubscript{10} (Table SC.2). The median MEC had no effect upon cyanobacteria based on the SSD, whilst the highest recorded MEC of 2.8 μg/L (from a WWTP in Las Vegas; (Jones-Lepp et al., 2012)) was predicted to affect 3% of cyanobacteria (Table 3). The HC\textsubscript{5} in this study was 166 times higher (HC\textsubscript{5.25} was 111 times higher) than the PNEC in the European Unions watch list of priority substances for azithromycin, 0.019 μg/L (Loos et al.,
but a PNEC based upon the lowest NOEC, based on *G. herdmenii*, would have been only <8 times higher (NOEC = 0.15 μg/L).
Figure 1 – Fitted concentration - response curves showing the effects of antibiotics on the growth rate of cyanobacteria. Curves are based upon 10 exposure concentrations. Antibiotics are arranged (vertical panels) according to their mode of action. Red dotted line indicates the highest measured environmental concentration (MEC) in UBA database (Umwelt bundesamt, 2018). Raw data plots are presented in Supplementary material C. In some cases the number of concentrations tested falling on the slope of the dose response curve may be low (< 3) and this may influence the confidence (robustness) of the toxicity estimation.
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<td>12.9</td>
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<tr>
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<td>76.1</td>
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<td>68.9                          &lt;</td>
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<td>49.5</td>
<td>61.8</td>
<td>&lt;7.2</td>
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Table 2 – Antibiotic 10% and 50% effective concentrations (EC<sub>x</sub>) and no observed effect concentrations (NOEC) for growth inhibition of eight cyanobacteria species. All concentrations are reported in μg/L. CL = Confidence Limit.<sup>a</sup> Times difference calculated by largest EC<sub>x</sub>/smallest EC<sub>x</sub> – reported value is based on largest range of EC<sub>10</sub> and EC<sub>50</sub>. Mode of Actions: cefazolin, cefotaxime and ampicillin are cell membrane synthesis inhibitors; sulfadiazine and sulfamethazine are DNA synthesis inhibitors (Anti-folates); Azithromycin and Erythromycin are Protein synthesis inhibitors.
Figure 2. Species sensitivity distributions of cyanobacteria exposed to five antibiotics; cefazolin and cefotaxime (cephalosporins), ampicillin (penicillin), azithromycin and erythromycin (macrolides). 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 coloured vertical lines indicate predicted no effect concentrations (PNEC): Dot-dash orange line = PNEClowest; Dashed orange line = PNEC A. flo-aquae; Dotted orange line = PNEC S. leopoldensis.
<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Protection limit</th>
<th>Concentration (μg/L)</th>
<th>Lower 95% CI</th>
<th>Higher 95% CI</th>
<th>Proportion of cyanobacteria affected (%)</th>
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<td>0.95</td>
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<tr>
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<td>PNEC_A. flos-aquae</td>
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<td></td>
<td></td>
<td>0.95</td>
</tr>
<tr>
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<td>Erythromycin</td>
<td>HCS</td>
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<td>16.18</td>
<td>28.76</td>
<td>5</td>
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<td></td>
<td>PNEC_Lowest</td>
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<td></td>
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<td>PNEC_A. flos-aquae</td>
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Table 2. Protection limits; 5% hazardous concentration (HCS) based upon a species sensitivity distribution (SSD) using 10% effective concentrations (EC$_{10}$), predicted no effect concentrations (PNECs) and the proportion of cyanobacteria affected based upon the SSD. PNECs determined as specified in current environmental risk assessment. PNEC$_{Lowest}$ represents the PNEC based on the most sensitive cyanobacteria in the conducted assays. PNEC$_{A. flos-aquae}$ and PNEC$_{S. leopoldensis}$ are based on the data of species recommended in the OECD 201 test guideline (OECD, 2011). * PNEC$_{Lowest}$ for erythromycin is < 0.62 and < 0.15 for azithromycin. ** CI was determined to be <0.
<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Measured concentration</th>
<th>Concentration (μg/L)</th>
<th>Proportion of cyanobacteria affected (%)</th>
</tr>
</thead>
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<tr>
<td>Cefazolin</td>
<td>Median</td>
<td>0.15 (10)</td>
<td>0.95</td>
</tr>
<tr>
<td></td>
<td>Highest</td>
<td>42.9 (Lin et al., 2008)</td>
<td>60</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>Median</td>
<td>0.033 (16)</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>Highest</td>
<td>41.9 (Lin et al., 2008)</td>
<td>96</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>Median</td>
<td>0.021 (15)</td>
<td>0.85</td>
</tr>
<tr>
<td></td>
<td>Highest</td>
<td>27.1 (Mutiyar and Mittal, 2013)</td>
<td>44</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>Median</td>
<td>0.050 (533)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Highest</td>
<td>7.8 (Lin and Tsai, 2009)</td>
<td>0</td>
</tr>
<tr>
<td>Azithromycin</td>
<td>Median</td>
<td>0.054 (255)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Highest</td>
<td>2.8 (Jones-Lepp et al., 2012)</td>
<td>3</td>
</tr>
</tbody>
</table>

Table 3. Proportion (%) of cyanobacteria affected at median and highest measured environmental concentrations (MECs) based on the cyanobacteria species sensitivity distributions using 10% effective concentrations and MECs obtained from Umweltbundesamt’s ‘Pharmaceuticals in the environment’ database (Umwelt bundesamt, 2018). Bracketed numbers indicate number of MECs in median calculation.

4. Discussion

We show that for eight species of cyanobacteria the sensitivity for growth inhibition for antibiotic exposure can vary widely and is influenced by the antibiotic MoA. For the β-lactam antibiotics in particular, the interspecies sensitivity varied by up to 70 times, far exceeding the AF of 10 currently applied to the NOEC to establish the PNEC in ERA (based on a single species of cyanobacteria). The SSD analysis indicated however that the current regulatory approach to ERA in Europe was generally protective of >98% of cyanobacteria populations when the reference test species employed for this was A. flos-aquae.

In contrast, the PNECs derived for cefazolin and cefotaxime when testing was based upon S. leopoldensis (another OECD recommended species) would result in growth inhibition for 13% and 5% of the tested cyanobacteria, respectively. In
the case of macrolides, the SSDs showed that an AF of 10 might be at a level that is suitable for the adequate population protection of cyanobacteria. We show that in some of the more polluted environments with antibiotics, based on the highest published MECs in the literature, up to 60% of cyanobacteria populations may be affected in these specific environments.

4.1. Chemical analysis, fate and behavior 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 the exposure period were high for the β-lactams, likely due to photodegradation (Wang and Lin, 2012 {Arsand, 2018 #177}), and for azithromycin possibly due to adsorption to the culture vessel materials and cellular or extracellular matter given its high partition-coefficient (LogP, 4.02) and adsorption coefficient (Kd, 3100) (National Center for Biotechnology Information, 2018). Erythromycin and the sulfonamides were more stable in the assay system.

Generally, the presence of the cyanobacteria resulted in a reduced amount of antibiotic in the culture medium (measured at the end of the exposure) likely as a consequence of adsorption and/or uptake into the bacterial cells and/or biodegradation by the cyanobacteria. Biodegradation is considered the most likely factor influencing the measured levels between the species studied for any one antibiotic, particularly notable in the β-lactams, as differences in surface binding of the antibiotic alone are very unlikely to account for this variation.
Further discussion on the fate of the antibiotics tested in the assays can be found in Supplementary material B.

4.2. Cyanobacteria sensitivity

**β-lactams:** There was a major difference (up to 70 times) in sensitivity to β-lactams (and in particular the cephalosporins) between the different species of cyanobacteria in our study. The reason for this is unknown but it may reflect differences in uptake rates caused by the quantity and type of porins in the outer membrane (Li et al., 2015; Sugawara et al., 2016). The bilayered outer membrane of cyanobacteria (and 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 to enter the periplasm. Porins tend to let small and non-lipophilic molecules pass through with ease, which includes the β-lactams (as well as fluoroquinolones, tetracycline, chloramphenicol, cycloserine, and aminoglycosides antibiotics) (Delcour, 2009; Li et al., 2015). For the relatively small molecules of cefazolin and ampicillin, we might thus expect that porin channels to be the uptake main route. For the larger antibiotic cefotaxime however, diffusion through the outer membrane may be more important in cellular uptake as it may be too large to easily pass through porins. Indeed, the susceptibility of the Gram-negative bacteria, *K. pneumonia*, was 4–8 times higher to cefotaxime when the strain expressed a larger porin channel (García-Sureda et al., 2011).
Porins 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 allowing more molecules to enter into the cell (Hoiczyk and Hansel, 2000). It is hypothesised that, as autotrophs, cyanobacteria synthesise the large organic molecules they require (Hoiczyk and Hansel, 2000; Kowata et al., 2017), whilst non-autotrophic bacteria need to uptake more (and larger) molecule types 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 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 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 and thus conclusions cannot be drawn, but our data do indicate the possibility of biodegradation for all the β-lactams tested. For cefazolin this (potential) biodegradation was greatest for *S. elongates* and *Synechococcus sp.*, which were also the least sensitive species tested based on growth inhibition. This is in accordance with findings that the Gram-negative *Enterobacteriaceae* family showed interspecies variability in sensitivity to β-lactams, ranging by between one and two orders of magnitude,
which were attributed to differences in chromosomal β-lactamases (Stock, 2005).

**Sulphonamides:** Growth inhibition of cyanobacteria following exposure to sulfonamides was generally limited and in some species the inhibitory effect plateaued with increasing antibiotic concentration, which may suggest the 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 sulfonamides compared to microalgae and macrophytes (Le Page *et al*., 2017). A possible explanation for their 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). 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 EC10s and EC50s for the eight species differed by less than an order of magnitude. Uptake and efflux may also influence the differences in cyanobacteria sensitivity to macrolides. Indeed, (Stock, 2005) hypothesized that Gram-negative bacteria species specific differences are, at least in part, driven by differences in outer membrane hydrophobicity. Due to macrolides large size, uptake is generally thought to be restricted by the outer membrane (Delcour, 2009; Stock, 2005), although there is
some evidence that porin-like uptake may be present (Hahn et al., 2012).

Azithromycin is dicationic and less hydrophobic than erythromycin and may therefore pass through the outer membrane more easily (Farmer et al., 1992; Stock, 2005).

The similar levels of efficacy of the macrolides across the different cyanobacteria species 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. Based on the literature, therefore, the differences 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 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 species. 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 it 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).

### 4.3. Sensitivity comparisons with other bacteria

Due to the limited data available for environmental bacteria 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\textsubscript{10} the concentration that inhibits growth rate by 10%) (Bengtsson-Palme and Larsson, 2018; Le Page et al., 2018). Additionally, the EUCAST data is not based on measured concentrations and since the tests are conducted in the dark, we might thus expect less degradation via photolysis than observed in our assays.

When comparing the effects of β-lactams in this study with the MICs of clinically relevant bacteria in the EUCAST database, the most sensitive cyanobacteria in our study were 3-6 times more sensitive to cefozolin. In accordance with our hypothesis above that cyanobacteria may be less sensitive to the larger antibiotics as they do not have porins that enable their uptake, several of the clinically relevant bacteria appeared to be more sensitive to cefotaxime than cyanobacteria (EUCAST). The effects of ampicillin on the cyanobacteria were similar to those observed on cyanobacteria by (Ando et al., 2007) and within the ranges seen in clinically relevant bacteria in the EUCAST database (EUCAST).

There are limited published data available for sulfadiazine and sulfamethazine. They are both veterinary antibiotics and so neither have EUCAST data and their
ecotoxicological profiles are rather poorly understood. Investigators have found however that the MICs for sulfamethazine tend to be relatively high (>512 mg/L for both Gram-negative and Gram positive bacterial strains) compared with other antibiotics (Salmon and Watts, 2000; Salmon et al., 1995).

Data on azithromycin is limited for ecotoxicologically relevant species but our results are in accordance with those reported in (Vestel et al., 2015) where cyanobacteria (species not provided) had a EC$_{50}$ of 1.8 μg/L. The MICs of clinically relevant bacteria in the EUCAST database suggest that growth in the most sensitive bacteria is inhibited completely at 16 μg/L, which is consistent with that for the more sensitive cyanobacteria in this study. For erythromycin, EC$_{50}$s were generally similar to those obtained for eight species of cyanobacteria by (Ando et al., 2007). They similarly found, A. cylindrical, to be the most sensitive species but calculated the EC$_{50}$s to be over an order of magnitude lower than in this study (3.5 compared to 44 μg/L respectively), albeit their tests exposure period was twice that of in this study (6 days) (Ando et al., 2007)). The most sensitive clinically relevant bacteria to erythromycin in the EUCAST database have MICs from 8 μg/L (EUCAST), suggesting that for this antibiotic that clinically relevant bacteria may be more sensitive than cyanobacteria.

4.4. Implications for ERA

4.4.1. PNECs with an assessment factor of 10 are not always protective

Our data suggest that for the β-lactams, depending on which species the PNEC was derived from, protection of >95% of cyanobacteria species was not be predicted by our SSDs, even with an assessment factor of 10 applied to account
for such interspecies sensitivity differences. For the two macrolides however, the
PNEC was protective of all cyanobacteria regardless of the species from which
the PNEC was derived. 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 and the MoA can be an important factor in this consideration.

The large interspecies variability observed between cyanobacteria exposed to
cell membrane synthesis inhibitors causes the PNEC to have a higher probability
of being under protective because the assessment factor of 10 is likely
inadequate as highlighted in our previous meta-analysis (Le Page et al., 2017).
For other MoAs, such as the macrolides, the smaller interspecies variability
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.

The limited sensitivity of cyanobacteria to sulfonamide antibiotics confirmed the
findings from the previous meta-analysis (Le Page et al., 2017) expressing
care that cyanobacteria may not be suitable for the estimation of
environmental 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, although
the revised ERA currently under consultation for the European Medicine Agency
does require a microalgae in addition to two cyanobacteria species and an
invertebrate (EMA, 2018).

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

Results from this analysis suggest that an approach using a SSD with eight cyanobacteria to derive an HC5 or HC5(2.5) with a small assessment factor (of less than 10) may be more suitable for the determination of protection limits for cyanobacteria populations than the traditional PNEC. But additional testing on other bacterial classes is required to ensure protection of bacteria more generally. We emphasise that a PNEC based on the NOEC and AF of 10 was generally adequately protective providing the species on which it was based was sensitive. If we consider the two species recommended in the OECD 201 test guideline, a PNEC based upon *A. flos-aquae* was protective but a PNEC based upon *S. leopoldensis* was under protective for both cefazolin and cefotaxime. Furthermore, for some MoAs such as sulphonamides, its possible that a PNEC using an AF of 10 will be under protective of bacteria more generally regardless of which cyanobacteria species is used.

For the cephalosporins, the HC5 was generally 4 - 8 times higher than the PNEC_{lowest} but 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. 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 protective of 95% of cyanobacteria (Wheeler *et al.*, 2002) without being over protective in for some MoAs as appears to be the case for the PNECs for
macrolides. The HC5 95% confidence intervals suggest some uncertainty (although this is relatively small) but given that these estimates incorporate the error around the original EC\textsubscript{10} via the use of the 95% confidence limits as censored data, this might be expected. The wider HC5 95% confidence limits for cefazolin 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). Further investigation is required to explore the hypothesis that 5% of species can be affected beyond their EC\textsubscript{10} without adverse effects upon environmental communities and ecosystem function. Such studies are best undertaken using semi-field test designs, as conducted, for example, by (Rico et al., 2014). These authors found disruptions to the nitrogen cycle occurred in mesocosms exposed to enrofloxacin that resulted from reduced numbers of ammonia-oxidising bacteria and archaea leading to higher ammonia and lower nitrate concentrations. In order to better estimate the effects of antibiotics on ecosystem functioning, additional endpoints that better represent functions of interest might usefully be included, for example oxygen evolution (as a proxy for photosynthetic rate) and pigment content (Guo et al., 2016b).

The selection of species for use in an SSD is important (Verdonck et al., 2003). Our analysis reflects only cyanobacteria sensitivity, and even here we studied
only a small selection of classes of cyanobacteria that grew adequately in the assay method adopted. Thus, a more diverse range of bacteria should be included since sensitivity differences between taxonomic clades could be large, even spanning several orders of magnitude. Furthermore, it should be emphasised that non-bacterial taxa including certain macrophytes (Le Page et al., 2017) and diatoms (Guo et al., 2016a) have been shown to be more sensitive to some antibiotics (e.g. sulphonamides and trimethoprim) than cyanobacteria. It is likely that some other bacterial taxa (i.e. not cyanobacteria) could be equally or more sensitive than all eukaryotes and thus it should be possible to select an appropriate diversity of bacteria for ERA testing of antibiotics that provide appropriate limits for the protection of all prokaryotes and eukaryotes.

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, biofilms in the field may provide resilience to chemical toxicity due to the protective nature of complex biofilm communities and extracellular substances (Harrison et al., 2007) and interspecies competition for resources may lower sensitivity to chemical contaminants (Rico et al., 2018). On the other hand, environmental conditions could significantly increase the sensitivity of bacteria to antibiotics due to 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). 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 but more confidence can be applied to an empirically derived HC5/HC5(2.5) with a smaller assessment factor (of less than 10, although further investigation is required as these are still largely arbitrary) and for which error can be quantified. Furthermore, a HC5 has greater certainty compared with the current PNEC and thus a reduced likelihood of underestimating the PNEC where interspecies variability is high and overestimating the PNEC where interspecies variability is low. Finally, a SSD based on an ECx avoids the criticisms of the NOEC that is flawed and dependent of experimental design.

The SSDs highlight that for the majority of MECs there is a limited general effect on cyanobacteria in the natural systems (potentially affected proportions of <1%) from all antibiotics based on the median MEC, which was based on data where the non-detects were excluded and thus a worse case scenario. However, 60, 96 and 44% of cyanobacteria may be affected when exposed to the highest cefazolin, cefotaxime and ampicillin environmentally relevant MECs recorded in the UBA database (Umwelt bundesamt, 2018). Our analysis therefore suggests that there are some cyanobacterial communities that may be severely affected by antibiotic pollution with potential consequences on the ecosystem functions that they provide. Equally, however, our data suggests that these effects are likely to be restricted to a small number of highly contaminated locations. In order to better estimate the risk of antibiotics in the environment, there is an urgent need
for more quantitative data on antibiotics in freshwater systems allowing for a better understanding of the distribution of MECs and more accurate estimations on possible associated risks.

Our results also show that a more comprehensive understanding of the effects of antibiotics upon prokaryotic diversity is needed for appropriate environmental protection. We argue that an ERA 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.

5. 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 general MoAs. Our experimental data verify the findings of a meta-analysis of published literature (Le Page et al., 2017) where large interspecies sensitivity is observed and is influenced by the MoA. To our knowledge, we present the first environmentally relevant bacterial data for cefazolin and sulfamethazine. Although a PNEC established using an assessment factor of 10 on a NOEC appears to generally be protective when a sensitive species for that antibiotic is tested, it may cause protection limits to be either over- or under-protective.
depending on the MoA. This approach may also result in an increased level of uncertainty around the PNEC estimated. We conclude a probabilistic approach using an SSD and several bacterial assays that cover a wider range of bacterial diversity would better protect against the detrimental effects of antibiotics on the environment. 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. The data presented also suggest that cyanobacteria may not be a suitable group of bacteria for determining environmental risk to sulfonamides due to their insensitivity relative to other environmentally important taxa (e.g. other bacterial clades or macrophytes (Le Page et al., 2017)). Finally, we show that the highest recorded MECs in the literature may pose a significant threat to cyanobacteria populations.

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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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