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, sufamethazine, 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.

Keywords:

Antibiotics; Antimicrobial resistance; Environmental risk assessment; Pharmaceuticals; Species sensitivity distribution; Predicted No Effect 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

94 conducted to derive it (Green *et al*., 2013) and secondly, because it uses only a
95 single effect value (the NOEC); quantification of the uncertainty around the PNEC
96 is not determined (Chapman *et al*., 1998).

97 A second approach for establishing protection limits that overcomes some of the
98 problems associated with the current PNEC approach is through the
99 construction of a species sensitivity distribution (SSD). A SSD is a probability
100 model of interspecies variability across a toxicity endpoint following chemical
101 exposure (e.g. NOEC or EC_x) and it allows prediction of the proportion of species
102 affected at any concentration for the species group modelled (Aldenberg *et al*.,
103 2001; Belanger *et al*., 2017; Wheeler *et al*., 2002). SSDs are more commonly used
104 for higher tier ERA in plant protection product regulations (EFSA, 2013) or in the
105 Water Framework Directive (European Commission Joint Research Centre,
106 2003). The protection limit most often derived from a SSD is the hazardous
107 concentration that affects no more than 5% of species (HC5), although it has
108 been suggested that the lower 95% confidence limit of the HC5 ($HC5_{2.5\%}$) should
109 be used to ensure a truly protective limit (Verdonck *et al*., 2001; Wheeler *et al*.,
110 2002). This lower, more protective limit, however, will have more statistical
111 uncertainty. An AF of less than 10 is sometimes applied depending on the
112 specific regulations and quality/quantity of the data on which the SSD is based
113 (EFSA, 2013).

114 In the past SSDs have been criticised for being ecologically unrealistic and for a
115 lack of statistical robustness but recent advances allow for the mitigation of
116 some of these concerns (Forbes and Calow, 2002; Kon Kam King *et al*., 2015; Kon
117 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

167 degree of interspecies sensitivity observed. These included; i) three cell envelope
168 synthesis inhibiting antibiotics, β-lactams, which target penicillin binding
169 proteins (which catalyse the building of the peptidoglycan cell membrane of
170 bacteria) namely, cefazolin and cefotaxime (1st and 3rd generation
171 cephalosporins, respectively) and ampicillin (a penicillin); ii) two DNA synthesis
172 inhibitors, sulfadiazine and sulfamethazine (sulfonamides) that prevent the
173 production of folic acid, a key precursor in the DNA synthesis pathway; and iii)
174 two protein synthesis inhibitors, erythromycin and azithromycin (macrolides),
175 which inhibit the normal functioning of the bacterial ribosome. The macrolides,
176 azithromycin and erythromycin are both candidates to be priority substances in
177 the EU Water Framework Directive watch list (Carvalho *et al*., 2015) and US EPA
178 contaminate list 3 (US EPA, 2009). Cefazolin and sulfamethazine have no
179 ecotoxicological data available for cyanobacteria in the open literature.
180 Sulfadiazine, cefotaxime and azithromycin have very limited ERA relevant
181 ecotoxicological data (Le Page *et al*., 2017).
182
183 We addressed the hypothesis that current protection limits for antibiotics in
184 surface water ($PNEC_{SW}$) are not fully protective of all cyanobacteria populations.
185 We first determined the interspecies sensitivity differences of eight species of
186 cyanobacteria by performing growth inhibition assays. We then established SSDs
187 and compared the PNEC calculated according to current guidance with the HC5
188 and $HC5_{2.5\%}$ to determine the proportion of species that would be affected
189 following exposure to the PNEC determined from these results. Finally, based on
190 our SSDs, we calculated the proportion of cyanobacteria likely to be affected
191 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 Multitron 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.

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)

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 = cell density at time t_i

t_i = i'th 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 (EC_x) 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 EC_{10} 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 EC_{10} 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).

363 Mean measured concentrations of ampicillin in the exposure replicates ranged
364 between 44 and 95% of nominal (Fig S.B5). In the presence of the cyanobacteria
365 there were generally between 10 and 30% additional reductions compared with
366 the replicates without bacteria, but was most pronounced in the *A. cylindrical*
367 exposure replicates (S.B17).
368
369 Mean measured concentrations of sulfadiazine in the exposure replicates were
370 between 101 and 142% of nominal (Fig S.B7). The high measured concentrations
371 of up to 142% of nominal occurred in the nominal 2.36, 145 and 1140 μg/L test
372 concentrations and they would increase the uncertainty around toxicity
373 estimates calculated. However, due to the lack of sensitivity of the cyanobacteria
374 to sulfadiazine (see below) this doesn't affect any conclusions drawn. Reductions
375 in sulfadiazine concentrations due to the presence of the cyanobacteria varied
376 across tests concentrations (S.B18).
377
378 Mean measured concentrations of sulfamethazine in the exposure replicates
379 ranged between 87 to and 134% of nominal (Fig S.B9). Extraction errors for
380 nominal concentrations 907 and 1633 μg/L in the *S. elongates* exposure meant
381 that these had to be excluded for the analyses.
382
383 Mean measured concentrations of erythromycin in the exposure replicates were
384 between 71 and 100% of nominal (Fig S.B11) with exception of the nominal
385 3.77 μg/L test concentration (53% of nominal). Erythromycin concentrations
386 were lowered by up to 50% over the exposure period and the presence of the
387 cyanobacteria in the exposure replicates caused additional erythromycin losses

388 of on average of 10 and 20% (but up to 60%) compared to replicates without
389 cyanobacteria present (Fig S.B20).
390
391 Mean measured concentrations of azithromycin in the exposure replicates
392 ranged between 23 and 79% of nominal (Fig S.B13). Azithromycin
393 concentrations in the exposure and blank replicates at the end of the exposures
394 were considerably lower, by up to 96%. The presence of all species of
395 cyanobacteria had an effect of reducing the test concentrations further by
396 between 10 and 15% (Fig S.B21).

397 3.2. Growth inhibition, species sensitivity distributions and protection
398 limit analysis:

399 The dose-response curves for growth inhibition of the eight cyanobacteria for
400 each antibiotic tested are presented in Figure 1. The EC10s, EC50s and NOECs for
401 the experimental data are given in Table 2 (raw data are provided, and shown
402 graphically in Supplementary material C, figures S.C1 – S.C7). All dose-response
403 analyses are based upon geometric mean measured test concentrations. The pHs
404 at the start and at the end of the tests are provided in Supplementary material D.
405
406 The data in Figure 2 presents the SSDs, based upon cyanobacteria EC_{10}s for each
407 antibiotic, together with PNECs based upon the NOEC of the most sensitive
408 species tested ($PNEC_{lowest}$) and the PNECs, based upon the two Organisation for
409 Economic Co-operation and Development (OECD) test guideline recommended
410 species (from which all PNECs derived for regulatory purposes are likely derived
411 from) *A. flos-aquae* ($PNEC_{A.\ flos\text{-}aquae}$) and *S. leopoldensis* ($PNEC_{S.leopoldensis}$). SSDs

412 based upon cyanobacteria NOECs are provided in Supplementary material C
413 (figure S.C8). It was not possible to establish the SSDs for sulfamethazine or for
414 sulfadiazine as neither produced a full dose response curve from which to
415 calculate an EC_{10}.

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

423 EC_{10}s for the different cyanobacteria exposure for cefazolin ranged between 2.4
424 and 124 µg/L and the EC_{50}s ranged between 4.1 to 283 µg/L (Figure 1 and Table
425 1). Based upon both EC_{10} and EC_{50} *A. flos-aquae* and *G. herdmanii* were the most
426 sensitive species to cefazolin. Species in the *Synechococcus* genera (*S. leopoliensis*,
427 *S. elongates* and *Synechococcus sp.*) along with *Synechocystis sp.* were the least
428 sensitive. *Synechococcus sp.* was up to 70 times less sensitive than the most
429 sensitive species based on the EC_{50}. The HC5 for cefazolin, based on EC_{10}s, was
430 1.13 µg/L, which was 7.5 times higher than the lowest PNEC (for *A. flos-aquae*)
431 but 4 times lower than that based on *S. leopoldensis* (Figure 2 and Table 2). The
432 predicted proportion of cyanobacteria affected at the PNECs ranged between
433 0.95 and 13.3% depending on which species was used to derive the PNEC (Table
434 2). The HC5 based on the NOEC data was 5 µg/L, 4 times higher than when based
435 on the EC_{10} (Table SC.1). The median MEC was predicted to affect a small fraction

436 of cyanobacteria (<1%) based on the SSD. The highest recorded MEC (42.9 µg/L;
437 the maximum concentration observed in a range of effluents in Taiwan, including
438 manufacturing and hospital effluents (Lin *et al*., 2008)) was predicted to affect
439 60.2% of cyanobacteria (Table 3) with the second highest MEC of 6.2 µg/L
440 affecting 16.2% of cyanobacteria. The median MEC of 6 hospital effluents in the
441 same study, of 6.2 µg/L (Lin *et al*., 2008), would affect 17.3% of cyanobacteria
442 based on the SSD.
443
444 EC_{10}s for the different cyanobacteria exposure to cefotaxime ranged between 1.2
445 and 39.8 µg/L and EC_{50}s ranged between 2.2 and 98 µg/L (Figure 1 and Table 1).
446 The maximum difference in sensitivity (45 times) occurred between *A. flos-aquae*
447 (the most sensitive) and *Synechococcus sp* (the least sensitive). The four least
448 sensitive species, *S. leopoliensis*, *Synechocystis sp., S. elongates* and
449 *Synechococcus sp.*, were also the least sensitive species to cefazolin, the other
450 cephalosporin tested, with the same order of relative sensitivity. The HC5 for
451 cefotaxime, based upon EC_{10}s, was 0.67 µg/L, which was 4 times higher than the
452 lowest PNEC (for *A. cylindrica*) and approximately the same value as for the
453 PNEC based upon *S. leopoldensis* (Figure 2 and Table 2). The predicted
454 proportion of cyanobacteria affected at the PNECs ranged between 1.3 and 5.2%
455 depending on which species was used to derive the PNEC (Table 2). The HC5
456 based upon the NOEC data was approximately the same as when based on the
457 EC_{10} (Table SC.1). The median MEC had little effect upon cyanobacteria based on
458 the SSD. The highest recorded MEC (41.9 µg/L; the maximum concentration
459 observed in a range of effluents in Taiwan, including manufacturing and hospital
460 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_{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 (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 EC50s of between 52 and 81.4 µg/L. The HC5 for ampicillin, based on 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 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 SC.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

485 calculated. The highest recorded MEC was 30.5 µg/L; treated WWTP effluent in
486 east China (Chen *et al*., 2012), whilst the median MEC was 0.019 µg/L.
487
488 As for sulfadiazine, sulfamethazine did not induce full growth inhibition for any
489 of the cyanobacteria tested (Figure 1) preventing the ability to calculate EC_{10} or
490 EC_{50} values (or SSDs). *C. gracile* was the most sensitive species to the growth
491 inhibition effects of sulfamethazine (a 50% reduction in growth rate was
492 observed at an exposure concentration of 1465 µg/L). At the highest tested
493 exposure concentration (10,000 µg/L) there was between a 30% and 40%
494 decrease in growth rate in *A. flos-aquae*, *A. cylindrical, S. leopoliensis*, *S. elongates*,
495 *Synechococcus sp.,* and *G. herdmanii*. *Synechocystis sp* was far less affected with
496 only a 4% inhibition of growth at the highest tested concentration. The highest
497 recorded MEC was 25.4 µg/L; treated WWTP effluent in Korea (Sim *et al*., 2011),
498 whilst the median MEC was 0.015 µg/L.
499
500 EC_{10}s for the different cyanobacteria exposure to erythromycin ranged between
501 21.1 and 58.8 µg/L and the EC_{50}s were between 43.4 and 135.1 µg/L (Figure 1
502 and Table 1). Based upon the EC_{50}, there was only a small interspecies difference
503 in sensitivity; 3.1 times, between the most sensitive (*A. cylindrical*) and least
504 sensitive species (*A. flos-aquae*). The HC5 for erythromycin, based upon EC_{10}s,
505 was 21.3 µg/L, which was 34.4 times higher than the lowest PNEC (for
506 S. *elongatus*) and 7.3 and 6.9 times higher than the PNECs for *A. flos-aquae* and
507 *S. leopoldensis,* respectively (Figure 2 and Table 2). The proportion of
508 cyanobacteria affected at the PNECs was <1% for all PNECs irrespective of which
509 species was used to derive it (Table 2). The HC5 based upon the NOEC data was

510 3.5 times lower than when based on the EC_{10} (Table SC.2). No species of
511 cyanobacteria are predicted to be affected by the MECs based on the SSD
512 (highest MEC was 7.8 µg/L; untreated manufacturing discharge in China (Lin and
513 Tsai, 2009)) (Table 3). The HC5 in this study was 107 times higher ($HC5_{2.5}$ was
514 81 times higher) than the PNEC in the European Unions watch list of priority
515 substances for erythromycin is 0.2 µg/L (Loos *et al*., 2018), but a PNEC based
516 upon the lowest NOEC, based on *S. elongates*, would have been only <3 times
517 higher (NOEC <0.62 µg/L).
518
519 EC_{10}s for the different cyanobacteria exposure to azithromycin ranged between
520 3.2 and 17.7 µg/L and EC_{50}s ranged between 5.4 and 33.8 µg/L (Figure 1 and
521 Table 1). Based upon the EC_{50}, there was difference in sensitivity of 6.3 times
522 only between the most sensitive (*A. cylindrical*) and least sensitive species
523 (*Synechococcus sp.*). The HC5 for azithromycin, based on EC_{10}s, was 3.2 µg/L,
524 which was 21 times higher than the lowest PNEC (for *G. herdmanii*) and 3.1 and
525 16.6 times higher than the PNECs for *A. flos-aquae* and *S. leopoldensis,*
526 respectively (Figure 2 and Table 2). The predicted proportion of cyanobacteria
527 affected at the PNECs was <1% for all PNECs irrespective of which species was
528 used to derive it (Table 2). The HC5 based upon the NOEC data was
529 approximately half as much as when based on the EC_{10} (Table SC.2). The median
530 MEC had no effect upon cyanobacteria based on the SSD, whilst the highest
531 recorded MEC of 2.8 µg/L (from a WWTP in Las Vegas; (Jones-Lepp *et al*., 2012))
532 was predicted to affect 3% of cyanobacteria (Table 3). The HC5 in this study was
533 166 times higher ($HC5_{2.5}$ was 111 times higher) than the PNEC in the European
534 Unions watch list of priority substances for azithromycin, 0.019 µg/L (Loos *et al*.,

535 2018), but a PNEC based upon the lowest NOEC, based on *G. herdmenii*, would

536 have been only <8 times higher (NOEC = 0.15 µg/L).

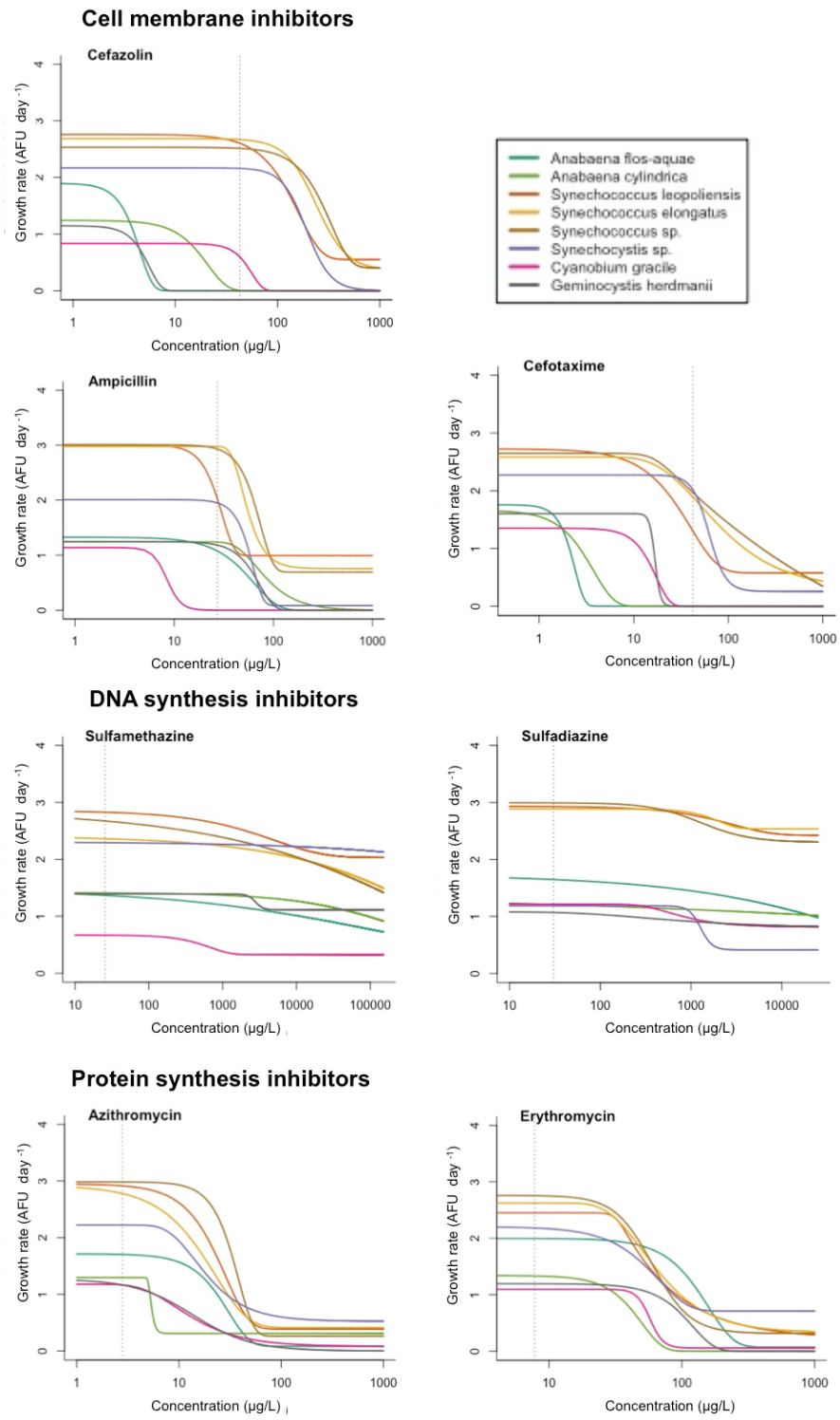

Figure 1 – Fitted concentration - response curves showing the effects of antibiotics on the growth rate of cyanobacteria. Curves are based upon 10 exposure concentrations Antibiotics are arranged (vertical panels) according to their mode of action. Red dotted line indicates the highest measured environmental 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.

545

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]
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	

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]
	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	
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	
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	

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]
	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	
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	

546 Table 2 – Antibiotic 10% and 50% effective concentrations (ECx) and no observed effect concentrations (NOEC) for growth inhibition of eight cyanobacteria
547 species. All concentrations are reported in µg/L. CL = Confidence Limit. [a] Times difference calculated by largest ECx/smallest ECx – reported value is based on
548 largest range of EC_{10} and EC_{50}. Mode of Actions: cefazolin , cefotaxime amd ampicillin are cell membrane synthesis inhibitors; sufadiazine and sulfamethazine are
549 DNA synthesis inhibitors (Anti-folates); Azithromycin and Erythromycin are Protein synthesis inhibitors.

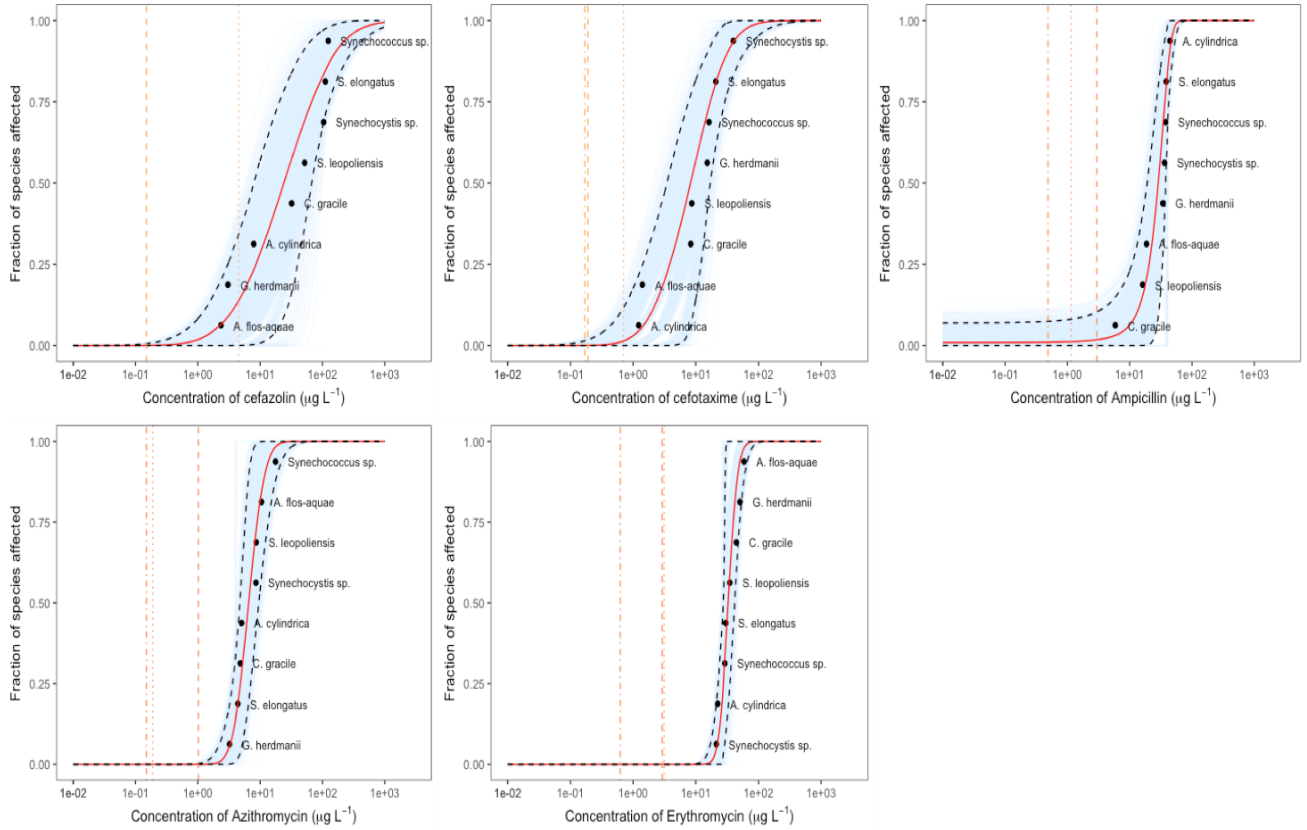

Figure 2. Species sensitivity distributions of cyanobacteria exposed to five antibiotics; cefazolin and cefotaxime (cephalosporins), ampicillin (penicillin), azithromycin and erythromycin (macrolides). Red line indicates the modelled species sensitivity distribution. Dashed black lines represent upper 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= $PNEC_{lowest}$; Dashed orange line = $PNEC_{\textit{A. flos-aquae}}$; Dotted orange line = $PNEC_{\textit{S. leopoldensis}}$.

Antibiotic	Protection limit	Concentration (µg/L)	Lower 95% CI	Higher 95% CI	Proportion of cyanobacteria affected (%)
Cefazolin	HC5	1.13	0.13	19.88	5
	$PNEC_{Lowest}$	0.15	-	-	0.95
	$PNEC_{A.\ flos\text{-}aquae}$	0.15	-	-	0.95
	$PNEC_{S.\ leopoldensis}$	4.53	-	-	13.26
Cefotaxime	HC5	0.67	0.32	1.13	5
	$PNEC_{Lowest}$	0.17	-	-	1.29
	$PNEC_{A.\ flos\text{-}aquae}$	0.19	-	-	1.44
	$PNEC_{S.\ leopoldensis}$	0.7	-	-	5.2
Ampicillin	HC5	8.56	0**	26.47	5
	$PNEC_{Lowest}$	0.49	-	-	0.91
	$PNEC_{A.\ flos\text{-}aquae}$	3	-	-	1.56
	$PNEC_{S.\ leopoldensis}$	1.15	-	-	1.05
Erythromycin	HC5	21.3	16.18	28.76	5
	$PNEC_{Lowest}$	0.62 *	-	-	0
	$PNEC_{A.\ flos\text{-}aquae}$	2.9	-	-	0
	$PNEC_{S.\ leopoldensis}$	3.1	-	-	0
Azithromycin	HC5	3.15	2.11	5.03	5
	$PNEC_{Lowest}$	0.15 *	-	-	0
	$PNEC_{A.\ flos\text{-}aquae}$	1.02	-	-	0
	$PNEC_{S.\ leopoldensis}$	0.19	-	-	0

Table 2. 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 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. $PNEC_{A.\ flos\text{-}aquae}$ 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.

Antibiotic	Measured concentration	Concentration (µg/L)	Proportion of cyanobacteria affected (%)
Cefazolin	Median	0.15 (10)	0.95
	Highest	42.9 (Lin *et al.*, 2008)	60
Cefotaxime	Median	0.033 (16)	0.25
	Highest	41.9 (Lin *et al.*, 2008)	96
Ampicillin	Median	0.021 (15)	0.85
	Highest	27.1 (Mutiyar and Mittal, 2013)	44
Erythromycin	Median	0.050 (533)	0
	Highest	7.8 (Lin and Tsai, 2009)	0
Azithromycin	Median	0.054 (255)	0
	Highest	2.8 (Jones-Lepp *et al.*, 2012)	3

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-aque*. 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

581 the case of macrolides, the SSDs showed that an AF of 10 might be at a level that
582 is suitable for the adequate population protection of cyanobacteria. We show
583 that in some of the more polluted environments with antibiotics, based on the
584 highest published MECs in the literature, up to 60% of cyanobacteria populations
585 may be affected in these specific environments.

586 ### 4.1. Chemical analysis, fate and behavior in the cultures

587 Our analytical results showed considerable variation in the fate of the antibiotics
588 in our assays. Generally speaking, reductions in the measured concentrations
589 over the exposure period were high for the β-lactams, likely due to
590 photodegradation (Wang and Lin, 2012 {Arsand, 2018 #177), and for
591 azithromycin possibly due to adsorption to the culture vessel materials and
592 cellular or extracellular matter given its high partition-coefficient (LogP, 4.02)
593 and adsorption coefficient (K_d, 3100) (National Center for Biotechnology
594 Information, 2018). Erythromycin and the sulfonamides were more stable in the
595 assay system.
596
597 Generally, the presence of the cyanobacteria resulted in a reduced amount of
598 antibiotic in the culture medium (measured at the end of the exposure) likely as
599 a consequence of adsorption and/or uptake into the bacterial cells and/or
600 biodegradation by the cyanobacteria. Biodegradation is considered the most
601 likely factor influencing the measured levels between the species studied for any
602 one antibiotic, particularly notable in the β-lactams, as differences in surface
603 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).

627 Porins differ between bacterial clades and cyanobacteria specifically do not
628 appear to have the same porin families as those typically found in other bacteria
629 (Flores *et al*., 2006). Gram-negative bacteria, for example, generally have smaller
630 outer membrane porins but with higher channel conductance than cyanobacteria
631 allowing more molecules to enter into the cell (Hoiczyk and Hansel, 2000). It is
632 hypothesised that, as autotrophs, cyanobacteria synthesise the large organic
633 molecules they require (Hoiczyk and Hansel, 2000; Kowata *et al*., 2017), whilst
634 non-autotrophic bacteria need to uptake more (and larger) molecule types from
635 outside of the cell. It is therefore reasonable to hypothesise that cyanobacteria
636 may not be as susceptible as Gram-negative bacteria to larger antibiotics that
637 require larger porin channels. Since ERA only uses one species of cyanobacteria
638 to represent all primary producer diversity, if sensitivity is, at least in part,
639 driven by uptake due to their outer membrane porins, other bacterial clades
640 such as Gram-negative bacteria that differ in their membrane structure and
641 porins may not be well represented.
642
643 In addition to uptake, efflux and β-lactamase enzymes may have key roles in
644 determining the sensitivity of bacteria to antibiotics. Efflux rates of the
645 antibiotics in cyanobacteria studied are not known and thus conclusions cannot
646 be drawn, but our data do indicate the possibility of biodegradation for all the β-
647 lactams tested. For cefazolin this (potential) biodegradation was greatest for
648 *S. elongates* and *Synechococcus sp*., which were also the least sensitive species
649 tested based on growth inhibition. This is in accordance with findings that the
650 Gram-negative *Enterobacteriaceae* family showed interspecies variability in
651 sensitivity to β-lactams, ranging by between one and two orders of magnitude,

652 which were attributed to differences in chromosomal β-lactamases (Stock,
653 2005).
654
655 ***Sulphonamides:*** Growth inhibition of cyanobacteria following exposure to
656 sulfonamides was generally limited and in some species the inhibitory effect
657 plateaued with increasing antibiotic concentration, which may suggest the
658 initiation of a possible resistance mechanism. The results are in accordance with
659 a recent meta-analysis where cyanobacteria were found to be less sensitive to
660 sulfonamides compared to microalgae and macrophytes (Le Page *et al*., 2017). A
661 possible explanation for their insensitivity could be that cyanobacteria contain a
662 protein (*slr0642* identified in Synechocystis) that may act as a folate transporter
663 and which allows the uptake of folates from the environment. This in turn
664 overcomes the effect of the targeting of this drug on the folate synthesis pathway
665 (de Crécy-Lagard *et al*., 2007; Klaus *et al*., 2005). It should be highlighted that the
666 growth rate was lower than the controls and thus there appears to be some
667 fitness consequence to this resistance mechanism.
668
669 ***Macrolides:*** Responses to the macrolides were more consistent across the
670 cyanobacteria compared with the β-lactam antibiotics; the EC10s and EC50s for
671 the eight species differed by less than an order of magnitude. Uptake and efflux
672 may also influence the differences in cyanobacteria sensitivity to macrolides.
673 Indeed, (Stock, 2005) hypothesized that Gram-negative bacteria species specific
674 differences are, at least in part, driven by differences in outer membrane
675 hydrophobicity. Due to macrolides large size, uptake is generally thought to be
676 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_{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

750 for such interspecies sensitivity differences. For the two macrolides however, the
751 PNEC was protective of all cyanobacteria regardless of the species from which
752 the PNEC was derived. These data indicate that under current ERA procedures
753 (of using a single test species) the choice of species is critical in establishing a
754 protection limit and the MoA can be an important factor in this consideration.

755 The large interspecies variability observed between cyanobacteria exposed to
756 cell membrane synthesis inhibitors causes the PNEC to have a higher probability
757 of being under protective because the assessment factor of 10 is likely
758 inadequate as highlighted in our previous meta-analysis (Le Page *et al.*, 2017).
759 For other MoAs, such as the macrolides, the smaller interspecies variability
760 means an assessment factor of 10 is sufficient to cover the entire SSD no matter
761 which species is selected on which to base the PNEC.

762 The limited sensitivity of cyanobacteria to sulfonamide antibiotics confirmed the
763 findings from the previous meta-analysis (Le Page *et al.*, 2017) expressing
764 concern that cyanobacteria may not be suitable for the estimation of
765 environmental protection limits. Furthermore, in some cases microalgae and
766 macrophytes may be more sensitive than cyanobacteria to this class of
767 antibiotics (Le Page *et al.*, 2017) but under current ERA framework for
768 pharmaceuticals neither microalgae nor macrophytes would be tested, although
769 the revised ERA currently under consultation for the European Medicine Agency
770 does require a microalgae in addition to two cyanobacteria species and an
771 invertebrate (EMA, 2018).
772

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

797 macrolides. The HC5 95% confidence intervals suggest some uncertainty
798 (although this is relatively small) but given that these estimates incorporate the
799 error around the original EC_{10}s via the use of the 95% confidence limits as
800 censored data, this might be expected. The wider HC5 95% confidence limits for
801 cefazolin may be due to higher variability observed between replicates in the
802 microplate assay.

803 The HC5 has been suggested as a protection limit under the premise that
804 functional redundancy (where multiple species are capable of performing the
805 same ecological functions) in the ecosystem will compensate for some small
806 effects on the most sensitive species (Solomon and Sibley, 2002). However, the
807 magnitude of functional redundancy is not clear, especially in bacterial
808 communities (Antwis *et al*., 2017). Further investigation is required to explore
809 the hypothesis that 5% of species can be affected beyond their EC_{10} without
810 adverse effects upon environmental communities and ecosystem function. Such
811 studies are best undertaken using semi-field test designs, as conducted, for
812 example, by (Rico et al., 2014). These authors found disruptions to the nitrogen
813 cycle occurred in mesocoms exposed to enrofloxacin that resulted from reduced
814 numbers of ammonia-oxidising bacteria and archaea leading to higher ammonia
815 and lower nitrate concentrations. In order to better estimate the effects of
816 antibiotics on ecosystem functioning, additional endpoints that better represent
817 functions of interest might usefully be included, for example oxygen evolution
818 (as a proxy for photosynthetic rate) and pigment content (Guo et al., 2016b).

819 The selection of species for use in an SSD is important (Verdonck *et al*., 2003).
820 Our analysis reflects only cyanobacteria sensitivity, and even here we studied

821 only a small selection of classes of cyanobacteria that grew adequately in the
822 assay method adopted. Thus, a more diverse range of bacteria should be
823 included since sensitivity differences between taxonomic clades could be large,
824 even spanning several orders of magnitude. Furthermore, it should be
825 emphasised that non-bacterial taxa including certain macrophytes (Le Page et al.,
826 2017) and diatoms (Guo et al., 2016a) have been shown to be more sensitive to
827 some antibiotics (e.g. sulphonamides and trimethoprim) than cyanobacteria. It is
828 likely that some other bacterial taxa (i.e. not cyanobacteria) could be equally or
829 more sensitive than all eukaryotes and thus it should be possible to select an
830 appropriate diversity of bacteria for ERA testing of antibiotics that provide
831 appropriate limits for the protection of all prokaryotes and eukaryotes.

832 A protection limit also needs to consider the extrapolation from the laboratory to
833 the field. Previous authors have concluded that large safety factors are not
834 considered necessary for extrapolation between the laboratory and field
835 (Chapman *et al*., 1998). Indeed, biofilms in the field may provide resilience to
836 chemical toxicity due to the protective nature of complex biofilm communities
837 and extracellular substances (Harrison *et al*., 2007) and interspecies competition
838 for resources may lower sensitivity to chemical contaminants (Rico et al., 2018).
839 On the other hand, environmental conditions could significantly increase the
840 sensitivity of bacteria to antibiotics due to chemical mixtures or as a result of
841 different biotic and abiotic factors (e.g. competition, predation, temperature, pH
842 (Rohr *et al*., 2016)). As such, in the absence of conclusive evidence
843 demonstrating the safe concentrations in mixtures or in a variety of
844 environmental conditions, it may be prudent to take a protective approach and

845 continue to include an assessment factor to compensate for this, as is required in
846 some regulatory guidance (EFSA, 2013; TGD, 2003). Using an assessment factor
847 with the HC5 or $HC5_{(2.5)}$ to establish a protection limit may appear to undermine
848 the benefits of conducting a more accurate, reliable and robust SSD but more
849 confidence can be applied to an empirically derived HC5/$HC5_{(2.5)}$ with a smaller
850 assessment factor (of less than 10, although further investigation is required as
851 these are still largely arbitrary) and for which error can be quantified.
852 Furthermore, a HC5 has greater certainty compared with the current PNEC and
853 thus a reduced likelihood of underestimating the PNEC where interspecies
854 variability is high and overestimating the PNEC where interspecies variability is
855 low. Finally, a SSD based on an EC_x avoids the criticisms of the NOEC that is
856 flawed and dependent of experimental design.

857 The SSDs highlight that for the majority of MECs there is a limited general effect
858 on cyanobacteria in the natural systems (potentially affected proportions of
859 <1%) from all antibiotics based on the median MEC, which was based on data
860 where the non-detects were excluded and thus a worse case scenario. However,
861 60, 96 and 44% of cyanobacteria may be affected when exposed to the highest
862 cefazolin, cefotaxime and ampicillin environmentally relevant MECs recorded in
863 the UBA database (Umwelt bundesamt, 2018). Our analysis therefore suggests
864 that there are some cyanobacterial communities that may be severely affected by
865 antibiotic pollution with potential consequences on the ecosystem functions that
866 they provide. Equally, however, our data suggests that these effects are likely to
867 be restricted to a small number of highly contaminated locations. In order to
868 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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