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COMPARING THE SENSITIVITY OF CHLOROPHYTES, CYANOBACTERIA AND DIATOMS TO MAJOR-USE ANTIBIOTICS

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COMPARING THE SENSITIVITY OF CHLOROPHYTES, CYANOBACTERIA AND DIATOMS TO MAJOR-USE ANTIBIOTICS

Running title: Algal sensitivity to antibiotics

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Abstract: The occurrence of antibiotic residues in the aquatic environment is an emerging concern. In contrast to daphnia and fish, algae are known to be particularly sensitive to antibiotic exposure. However, to date, a systematic evaluation of the sensitivity of different algal species to antibiotics has not been performed. The aim of the present study was therefore to explore the sensitivity of a battery of algal species towards antibiotic exposures. The present study investigated the growth inhibition effects of three major-use antibiotics, tylosin, lincomycin and trimethoprim, on seven algal species from the chlorophyte, cyanobacteria and diatom groups. Based on EC50 values, cyanobacteria (EC50 = 0.095-0.13 µmol/L) were found to be the most sensitive group to lincomycin followed by chlorophytes (EC50 = 7.36-225.73 µmol/L) and diatoms (EC50 >225.73 µmol/L). Cyanobacteria were also the most sensitive group to tylosin (EC50 = 0.09-0.092 µmol/L) but, for this compound, diatoms (EC50 = 1.33-5.7 µmol/L) were more sensitive than chlorophytes (EC50 = 4.14-81.2 µmol/L). Diatoms were most sensitive to trimethoprim (EC50 = 7.36-74.61 µmol/L) followed by cyanobacteria (EC50 = 315.78-344.45 µmol/L) and chlorophytes (EC50 >344.45 µmol/L) for trimethoprim. While the results of our study partly support the current approach to regulatory environmental risk assessment where cyanobacterial species are recommended for use on antibiotic compounds, they indicate that for some antibiotics this group might not be the most appropriate test organism. We would therefore advocate that environmental risk assessments consider data on three algal groups (chlorophytes, cyanobacteria and diatoms) and use test species from these groups that are consistently found to be the most sensitive (Pseudokirchneriella subcapitata, Anabaena flos-aquae and Navicula pelliculosa). This article is protected by copyright. All rights reserved

Keywords: Species sensitivity, Antibiotics, Algae, Growth inhibition, Risk Assessment

INTRODUCTION

Antibiotics have a selective toxic mode of action on bacteria or other single-celled microorganisms [1]. Following their use in human and veterinary medicine, these substances are emitted into the aquatic environment via a range of pathways [2]. The occurrence of antibiotic residues (unaltered antibiotics) has been reported in surface waters across the globe [3], where their exposure to non-target organisms may occur. While antibiotics are designed to interact with receptors in pathogenic bacteria, the fact that similar receptors and/or pathways might also be conserved in non-target organisms means that the presence of antibiotics in the natural environment could result in potential adverse effects on ecosystems [2].

Studies have demonstrated that, compared to fish and daphnia, algal species exhibit higher sensitivity towards antibiotics [4, 5]. Available data on toxicity of antibiotics to chlorophytes (primarily P. subcapitata and D. subspicatus) show EC50 values generally occur at the mg/L level [6]. Effects of antibiotics on cyanobacteria have also been reported and these organisms have been found to be particularly sensitive to antibiotics with EC50 values reported at the µg/L level [6]. A limited amount of data are also available on toxicity of antibiotics to diatoms with reported EC50 values in the mg/L range. As a consequence of the observed high sensitivity of cyanobacteria to antibiotics, blue green algal species are recommended as one of the test species that should be used in the environmental risk assessment of antibiotics as part of the marketing authorisation process [7].

In instances where data are available on the toxicity of a single antibiotic to a range of algal and cyanobacterial species, large differences can be observed in the EC50 values for the different species

tested. These differences could be attributed to four potential reasons: 1) differences in antibiotic bioavailability, which is related to the pKa of the chemical and pH values in the test medium during the test period [8]; 2) the characteristics of binding sites in the primary targets, where highly conserved antibiotic ligand-binding pockets in some algal species may result in a higher sensitivity [9]; 3) Elimination process (enzymatic inactivation) in the various algal species that could reduce the impacts of different antibiotics by direct degradation or modification of their structure [10]; or 4) the presence of efflux pumps, which are the transport proteins used to extrude intracellular toxic substrates, including antbiotics. Differences in efflux pumps present in the various algal species could contribute to their different responses to antibiotic exposures [11].

While the differences in sensitivity of algae to antibiotics are recognised, our understanding of these differences is limited with data being available for only a handful of species and groups [8, 12-14]. There is therefore a need for investigations examining the sensitivity of a battery of algal species, from a range of groups (e.g. chlorophytes, cyanobacteria and diatoms) to a range of antibiotics. Data from these types of studies could be invaluable in informing the development of more intelligent environmental risk assessment strategies for antibiotic compounds.

In the present study, therefore we present the results of a systematic study into the sensitivity of algal/ cyanobacterial species to three major-use antibiotics, tylosin, lincomycin and trimethoprim, with contrasting mechanisms of action. These substances have been highly ranked in a recent prioritisation study of pharmaceuticals in the natural environment where they all demonstrated risk scores greater than one, based on ecotoxicity to algae [6]. Tylosin is an antibiotic administrated as a veterinary prophylactic (intestinal and respiration infections) and growth enhancer [15, 16]. The primary mode of action is

inhibiting bacterial protein synthesis by binding to the 50S ribosome. Lincomycin is a veterinary lincosamide antibiotic and its side effect on algae is thought to occur through the inhibition of the synthesis of the D1 protein in photosystem II, which handles the algal recovery ability from light-inhibition [15]. Trimethoprim is used for the treatment of urinary tract infections, uncomplicated pyelonephritis and mild acute prostatitis [17]. It is a dihydrofolate reductace inhibitor, binding to susceptible bacteria and influencing folate synthesis (Table 1). The three antibiotics have beendetected in the surface waters of the US and elsewhere with concentrations ranging from 0.05 to 0.7 µg/L(Table 1).

Six algal species recommended in the OECD 201 guideline [18] including chlorophytes (Pseudokirchneriella subcapitata, Desmodesmus subspicatus and Chlorella vulgaris), cyanobacteria (Synechococcus leopoliensis and Anabaena flos-aquae) and a diatom (Navicula pelliculosaand Phaeodactylum tricornutum) were chosen for use in the ecotoxicity studies. All these seven species are ecologically relevant and their distribution have been widely reported in five continents (Asia, Europe, Africa, North America and Oceania) [19]. The hypothesis for the present study was that cyanobacteria would be more sensitive than chlorophytes and diatoms, and that the two cyanobacterial species would exhibit similar sensitivities.

MATERIALS AND METHODS

Chemicals

Tylosin tartrate (referred to as tylosin, 86.4%) (CAS-no. 1405-54-5), lincomycin hydrochloride (referred to as lincomycin, ≥95%) (CAS-no. 859-18-7), trimethoprim (≥98%) (CAS-no. 738-70-5) and potassium dichromate (≥99.8%; used as reference substance) were purchased from Sigma-Aldrich. Ammonium acetate and formic acid (≥95%) as analytical reagent grade were purchased from Fisher

Scientific UK and Sigma-Aldrich, respectively. Acetonitrile, methanol and water (HPLC Gradient grade) were purchased from Fisher Scientific UK.

Algal cultures

Algal toxicity tests were conducted using three chlorophytes: P. subcapitata (CCAP 278/4), D. subspicatus (CCAP 258/137) and C. vulgaris (CCAP 211/11b); two cyanobacteria: S. leopoliensis (CCAP 1405/1) and A. flos-aquae (CCAP 1403/13A); two diatoms N. pelliculosa (CCAP 1050/9) and P. tricornutum (CCAP 1052/1b) obtained from the Institute of Freshwater Ecology (Culture Collection of Algae and Protozoa, UK). P. subcapitata, D. subspicatus and C. vulgaris were cultured in Kuhl medium, pH 6.8 [20]; S. leopoliensis and A. flos-aquae were grown in Jaworski's Medium (JM), pH 7.8 [21]; N. pelliculosa and P. tricornutum were grown in Enriched Seawater-Artificial Water (ESAW) plus f/2 medium, pH 8.2 [22].

Cultures of algae were grown at 20 °C under gentle and continuous shaking (100 cycles per minute (cpm)) in a culture chamber, with a controlled temperature (20 ± 2 °C) and a constant illumination (76 µmol $m^{-2}s^{-1}$). Triplicate cultures were prepared in conical flasks (250ml) containing 100 ml of medium and 1 ml algal cells. To avoid contamination, the flasks were washed in Decon, rinsed with hydrochloric acid (50mM) and then autoclaved (at 121 °C for 30 min) before use. The algal numbers for the cultivation phase were counted daily with a hemacytometer under a microscope, and growth curves (cell numbers over time) were plotted to identify the logarithmic phases (usually over 2-4 days cultivation). The algal stocks were subcultured on a weekly basis.

Procedures for the growth inhibition test

Growth inhibition tests were undertaken following the OECD 201 Guideline for freshwater alga

and cyanobacteria, growth inhibition tests [18] for the study antibiotics and the reference toxicant (potassium dichromate). The inhibition experiments were conducted in two steps: range-finding and EC50 determination. Range-finding was used to estimate the EC50, and then at least six selected concentrations (maximum 93.79, 225.73 and 344.45 μmol/L for tylosin, lincomycin and trimethoprim, respectively) of samples (triplicates each) around the predicted EC50 in geometric series were used for the definitive EC50 test. The concentration-response curve based on growth (cell density) over t days (t=1, 2, 3, 4) was then generated based on the definitive data.

Prior to use, all glassware and stoppers used in the tests were autoclaved at 121 °C for 30 min. The antibiotics in the media were prepared and filtered into a 25 mL vial, using a 0.2 μm sterilized syringe filter. The pre-cultured algal inocula, taken from logarithmic growing cultures, were diluted to 15 mL with the prepared antibiotic solutions in a 25 mL vial. The initial algal concentrations for P. subcapitata and D. subspicatus were set at 5000 cells/mL, 2×10^4 cells/mL for C. vulgaris and A. flos-aquae, 1×10^4 cells/mL for N. pelliculosa and P. tricornutum and 5×10^5 cells/mL for S. leopoliensis. The test vials were then capped with air-permeable stoppers made of cotton and muslin. All the operations were performed on a sterilized bench.

The prepared vials were put in the culture chamber under the same conditions as used for the culturing. Bioassays lasted for 96 h, and the cell numbers were measured every 24 h using UV-Vis spectrophotometry. Cell density was calculated from a calibration curve of known cell density counted by a haemocytometer against adsorption (turbidity) measured by an ultraviolet and visible (UV-Vis) spectrophotometry for each species (R^2>0.999).Measurement of turbidity (adsorption) using a spectrophotometer with an appropriate selected wavelength is a reliable method to determine cell density

[23]. Each algal culture was diluted and scanned between the 600-800 nm ranges. The wavelengths with the highest absorbance were selected for experiments. The wavelength for absorption measurement was 750 nm for P. subcapitata, 720 nm for C. vulgaris, 682 nm for D. subspicatus, N. pelliculosa, P. tricornutum, A. flos-aquae and S. leopoliensis.

The prepared concentration of tested samples before the test was confirmed by chemical analysis. Samples with the highest and lowest concentrations were analysed again after the test to determine the antibiotic stability. In several algal toxicity tests, the recoveries of antibiotics in the highest and lowest test concentrations were less than 80% after 4d test. In these cases, the first-order degradation reaction (Equation 1) was used to estimate a dissipation rate constant (k). The k was then applied in Equation 2 to estimate the time-weighted average concentration (TWAC) over t days (where t=1, 2, 3, 4). By comparing the TWAC with the nominal concentration, a correction factor was then obtained for use in the concentration response analyses. Observations from the low concentration recovery tests were used for correcting the three lowest concentrations used in the ecotoxicity study while concentrations for the high concentration recovery were used for correction of the three highest concentrations.

$$C_t = C_0 \text{ X } e^{-kt} \qquad \text{Equation 1}$$

$$C_{avet} = C_0 \text{ X } (1- e^{-kt}) / kt \qquad \text{Equation 2}$$

Where C_0: initial concentration (µmol/L); Ct: concentration at the t day (µmol/L); C_{avet}: average concentration over t days (µmol/L); k: rate constant (/day) t: time (day) [24].

Antibiotic analyses

Samples were analysed by High Performance Liquid Chromatography (HPLC) using an Agilent 1100 with C18 Supelco Discovery column (15 cm × 4.6 mm × 5 µm). Tylosin and trimethoprim were

analysed using a 24 min gradient method. The mobile phase consisted of methanol (A) and a buffer (B) (50 mM ammonium acetate plus 0.01% formic acid, pH 6.5 adjusted with 2.5% ammonium solution). The gradient was as follows: 5 minute equilibration at a 10:90 ratio (A:B); 2 minutes at 50:50; 20 minutes at 90:10; and 2 minutes at 10:90. A retention time of 13 min with a flow rate of 1 ml/min and detection wavelength of 280 nm was used for tylosin and 6.4 min, 1 mL/min, 238 nm was used for trimethoprim. Lincomycin was analysed by an isocratic method using 0.1% formic acid plus acetonitrile at a ratio 75:25 with a retention time of 4 min, flow rate of 1.2 mL/min and a detection wavelength of 196 nm. A range of antibiotic standards was prepared to derive calibration curves for each of the analytical methods. A linear relationship between concentrations and peak areas was obtained for each analyte (R^2> 0.999); the mean recovery was more than 98% for tylosin and trimethoprim and 95% for lincomycin. The limit of detection (LOD) of tylosin, trimethoprim and lincomycin in the nutrient medium were 0.44, 0.55 and 1.15 µmol/L, respectively. The limit of quantification (LOQ) value of three above antibiotics was each 1.41, 1.86 and 3.86 µmol/L.

For measuring low concentration solutions (less than 0.28 µmol/L) of tylosin and lincomycin (less than 0.68 µmol/L) for the cynobacterial tests, solid phase extract (SPE) was used to concentrate the samples prior to analysis. Oasis HLC 3cc extraction cartridges were used purchased from Waters (UK). The SPE procedures were as follows: cartridge conditioning was undertaken by adding 6 mL methanol followed by 6 mL water. The sample (100 ml) was then loaded onto the SPE. The cartridges were then rinsed with 6 mL water and eluted using 6 mL methanol. Eluates were then concentrated, by evaporation with nitrogen in a fume hood, to dryness before being taken up in 1 mL methanol. The mean SPE recovery for tylosin and lincomycin were 119% and 138%, respectively.

Statistical methods

The data were analysed with Sigma-plot software. The concentration response curve was obtained by fitting regression analysis of sigmoidal functions (sigmoid, logistic, weibull, gompertz, hill and chapman equations) embedded in the Sigma plot software version 12.0. The best fitting model (highest coefficient of determination (R^2)) was used for EC50, EC10 and EC5 calculation. Significant differences between inhibition percentages calculated based on the cell density in treatments and controls were determined using the Dunnett test with a p value <0.05 taken as being statistically significant. NOEC, LOEC values were derived from this statistic analysis.

To explore whether pH in the three different algal media (Kuhl, 6.8; JM, 7.8; ESAW+f/2, 8.2) were significantly different, pH values of controls (n=3) in each algal test were compared using Tukey's test (p value <0.05).

RESULTS AND DISCUSSION

Chemical analyses

At the high test concentrations, decreases in antibiotic levels over the 4 d study period were observed for tylosin (C. vulgaris 74.4%, A. flos-aquae 74.8%, S. leopoliensis 53.14%) and trimethoprim (P. subcapitata 37%). Measured concentrations of unaltered antibiotics for most other antibiotic/algal combinations remained within 80 - 120% of the initial concentration (Figure 1). For the low test levels, decreases in concentration were observed for tylosin (A. flos-aquae 27.2%, S. leopoliensis 15.54%), lincomycin (N. pelliculosa 66.86%, P. tricornutum 64.18%) and trimethoprim (P. subcapitata 48.11%, A. flos-aquae 43.55%, S. leopoliensis 42.83%; Figure 1). The reductions in concentrations could be due to a range of processes including abiotic (photolysis, hydrolysis) or biotic (i.e. metabolism by the algae)

degradation or due to sorption or uptake to/into the algal cells.

The three study compounds are known to be hydrolytically stable [25-27]. However, the photolysis of the three antibiotics has demonstrated previously. The photolysis of tylosin under simulated sunlight has been reported by Werner et al. [28], where tylosin underwent a rapid decrease in the first 4 min of the study followed by photochemical loss at a slower time scale over 120 min. Tylosin equilibrated to approximately one-half of the original concentration for over 48 h and importantly, photochemical equilibrium was independent of initial concentration and pH value. In a photolysis study of trimethoprim in two matrices (distilled water and sea water) under simulated sunlight, 50% of the original trimethoprim concentration disappeared after 780 min of exposure [29]. However, a longer half-life was observed in the sea water solution due to the influence of salt content [29]. Direct photolysis of lincomycin has been studied by Paola et al. [30], They found that parent compound with initial concentration 49.2 μmol/L dropped 40% after 5h exposure to UV light. This evidence indicated that photolysis of antibiotics may occur in algal tests during the 4d study period but this degradation is dependent on media type and the concentration of the antibiotic.

While studies on biodegradation of three antibiotics in algal species are rare, information on their biodegradation in activated sludge have been well established. All three antibiotics show a high resistance to biodegradation in activated sludge in several studies, and they were classified as non-biodegradable compounds [31-33]. The losses of antibiotics in our studies were therefore unlikely due to biodegradation in algae.

While no significant difference in pH values in JM's (7.8) and ESAW+f/2 (8.2) media used for culturing cyanobacteria and diatoms was found, the pH in Kuhl medium used for culturing chlorophytes

(6.8) was significantly different from others. The pH of the exposure medium for all the treatments varied slightly over the study period (Figure 2). For chlorophytes D. subspicatus and C. vulgaris the rise of pH was within 1 unit and for diatoms N. pelliculosa and P. tricornutum the variation of pH values were within 0.9 units. These variances were within the scope of OECD 201 guideline. However, no evident pH increases were observed for the tests on P. subcapitata, A. flos-aquae and S. leopoliensis with changes < 0.2 units. The low pH increases for these species is believed to be due to their relative low growth rates compared to other species [12]. The pH variations agreed with published work e.g. Halling-Sorensen et al. [8] investigated the effects of eight antibiotics including tylosin on the growh of cyanobacteria Microcystis aeruginosa with a initial pH 8.1–8.3, where almost no increase in pH was observed for M. aeruginosa due to a lower growth rate. Kolar et al. [34] explored the influence of trimethoprim on chlorophyte P. subcapitata and cyanobacteria A. flos-aquae, where the pH values were in the range of 7.6-8.3 and 7.1-7.4, respectively.

The reference substance, potassium dichromate, has previously been tested on the three chlorophytes with the EC50 values in the range of 1.33-4.86 µmol/L for D. subspicatus [35], 0.54-2 µmol/L for C. vulgaris [36] and 1.29-8.87 µmol/L for P. subcapitata [35]. In the present study, EC50 values for D. subspicatus and P. subcapitata were 4.59 µmol/L and 5.23 µmol/L respectively. For C. vulgaris a higher EC50 value 8.29 µmol/L was obtained, the discrepancy might be due to the differences in the selection of algal strain. No toxicity data of potassium dichromate on cyanobacteria and diatoms have been reported with which to compare our data. In the present study EC50s were found within the range from 15.94 µmol/L to 33.99 µmol/L and greater than 33.99 µmol/L for cyanobacteria and diatom species, respectively.

Toxicity tests analysis

All three antibiotics were found to inhibit the growth of selected algal species after 4 day exposure (Tables 2-4; Figure 3). Lincomycin inhibited the growth of all seven test species with EC50 values ranging from 0.095 µmol/L to 225.73 µmol/L; Tylosin inhibited the growth of selected species with EC50 values ranging from 0.09 µmol/L to 81.2 µmol/L; The EC50 values of seven species exposed to trimethoprim ranged from 7.36 µmol/L to 344.45 µmol/L (Tables 2-4). Here a wide range of algal toxicity values (as much as 4 orders of magnitude) was found for these compounds. While clear stimulation effects (hormesis) in the lower range of test concentrations were observed in some algal tests such as N. pelliculosa/ tylosin and P. triconutum for trimethoprim, most of the negative growth inhibition observed in the present study were around 20% or less. Low dose stimulation effects were therefore ignored in EC50 calculation [18].

Slopes of the concentration-effect curves are of importance in algal tests. It is assumed that chemicals with the same "mode of action" have a comparable slope for a particular species [37]. While no universal measure for slope of a concentration-response curve exists, it can be defined as a ratio between two EC values (e.g. the EC50/EC5 ratio), which has been reported in a range of literatures [38]. Most of the EC50/EC5 ratios in the present study ranged from 1.77 to 18, which agreed with the average value (7.2) in bioassay of algae [37]. However, no clear trend in slope variance was observed for chlorophytes, cyanobacteria and diatoms (Tables 2-4).The toxicological effects of the test antibiotics on selected algal species have been reported previously (Table 5). For tylosin, three studies have been reported on P. subcapitata with 72h EC50 ranging from 0.0083 µmol/L to 1.51 µmol/L [8, 39]. EC50 values for two of the studies are within an order of magnitude of the EC50 of 4.14 µmol/L we obtained

for tylosin. The EC50 of 0.0083 µmol/L reported by van den Grinten et al. [39] is surprisingly low in comparison to our study. Halling-Sorensen [8] reported the effects of tylosin on the cyanobacteria Microcystis aeruginosa with a 72h EC50 value of 0.037 µmol/L (Table 5). This value is lower than the EC50s for A. flos-aquae and S. leopoliensis in the current study of 0.092 µmol/L and 0.09 µmol/L respectively.

For lincomycin, 72h EC50 previously reported EC50s for P. subspicata are within an order of magnitude of the value we obtained (Table 5). Data are also available for toxicity to S. leopoliensis and a diatom species [40]. Our 96h EC50 0.095 µmol/L for S. leopoliensis was around a factor of 4 lower than the previously reported value. For diatoms we saw no inhibition effects for either diatom species (EC50 >225.73 µmol/L), for N. pelliculosa and P. tricornutum) whereas Andreozzi et al. [40], obtained an EC50 of 4 µmol/L although it is important to recognize this was a different species Cyclotella meneghiniana than we used.

For trimethoprim previously reported EC50s for chlorophytes ranged from > 31 µmol/L to 444.34 µmol/L (Table 5), whereas we obtained an EC50 >344.45 µmol/L. For blue green algae, our lowest 96h EC50 value was 315.78 µmol/L for A. flos-aquae which is similar to a previously reported value for this species of 871.45 µmol/L.

Toxicity data for three earlier time points are summarized in Tables S1-3 (Supplemental Data). In most cases no evident algal toxicities were observed at the maximum test concentration over the first 2 days of the exposure. While the toxicity effects of antibiotics to algal species were continuously increasing from 3d to 4d exposure, the EC50 values were very similar. For example, over 3d and 4d exposure of N. pelliculosa to trimethoprim, EC50 values only decreased from 9.4 to 7.36 µmol/L (Figure

4).

Hypothesis-based no effect concentration (NOEC) and low effect concentration (LOEC) are common statistical approaches used to summarize ecotoxicological effects. However, the use of NOEC data has been criticized as experiments conducted using poor laboratory practice would report larger variability [41]. Therefore, the difference between the control and treatments would have to be larger in order to be significant different. Instead of using NOEC, a range of studies have called for using regression-based effect concentration (ECx) value as an alternative (e,g. EC10) [42]. In the present study therefore, in addition to determining the NOEC and LOEC values, we also have derived the EC10 value for each algal test (Tables 2-4). Most of the NOEC and EC10 data are within an order of magnitude of each other.

Species sensitivity comparisons towards antibiotics at EC50 level

Sensitivities of the seven algal species exposed to the three antibiotics at EC50 level were assessed. For the three chlorophytes, P. subcapitata was slightly more sensitive to tylosin and lincomycin exposure than D. subspicatus, while C. vulgaris was not sensitive at the highest concentrations tested (Tables 2-4). For the cyanobacteria, while A. flos-aquae was slightly more sensitive to trimethoprim exposure than S. leopoliensis, sensitivities of the two cyanobacteria to tylosin and lincomycin exposures based on EC50 values were of the same order of magnitude (Tables 2-4). The two diatom species were not affected by lincomycin at the highest concentration tested. But based on data for tylosin and trimethoprim, N. pelliculosa was more sensitive than P. tricornutum (Tables 2-4).

In general, cyanobacteria were more sensitive than chlorophytes to lincomycin with the EC50 ranging from 0.095 µmol/L to 0.13 µmol/L. No effects of lincomycin were seen on diatoms (Tables 2-4).

The result of sensitivity across algal classes agreed with the literature. For example, Andreozzi et al. [40] found the 4d EC50 value of lincomycin on the growth of cyanobacteria S. leopoliensis were around eight times lower than that for P. subcapitata.

Cyanobacteria were also found to be most sensitive algae tested to tylosin with EC50 values ranging from 0.09 µmol/L to 0.092 µmol/L which was more than 5 times lower than EC50 values for chlorophytes and diatoms (Tables 2-4). The sensitivities of chlorophytes and diatoms towards tylosin were similar (Tables 2-4). These results are consistent with the findings of Halling-Sorensen [8], who observed that the cyanobacteria M. aeruginosa was ten times more sensitive to tylosin than the chlorophyte P. subcapitata.

For trimethoprim, no effects were seen on the growth of chlorophyte and cyanobacteria species at the maximum test concentration (344.45 µmol/L) whereas the diatom species were found to be much more sensitive to trimethoprim exposure with EC50 values ranging from 7.36 µmol/L to 74.61 µmol/L.

The differences in the sensitivities within and across algal classes to the antibiotics tested might be attributed to a number of explanations, including: differences in antibiotic uptake; differences in the binding pockets in the primary targets; differences in antibiotic elimination; and differences in active efflux pumps. These are discussed below.

In the present study, the tests were performed in different media with different pH values. It has long been recognised that the pH of a system can affect the toxicity of ionisable compounds such as the study antibiotics. The initial pH values of culture media for chlorophyte, cyanobacteria and diatom species were different: 6.82 (Kuhl medium for chlorophyte), 7.8 (JM medium for cyanobacteria) and 8.2 (ESAW+f/2 medium for diatoms), respectively. For acidic antibiotics such as tylosin and lincomycin,

which have pKa values ranging from 7 to 8 (Table 1), media with higher pH values would promote ionisation of the antibiotics which would reduce uptake into the cells [8]. Species tested in lower pH media might therefore be expected to accumulate more antibiotic than higher pH media and hence toxicity, expressed based on the concentration of the antibiotic in water, would be increased. In instances where the pH of the test system changes significantly over time, this will also affect uptake. Based on the pH of the test media, uptake of tylosin and lincomycin by chlorophyte would be expected to be greater than by cyanobacteria and diatoms based on the proportion of substance present in the neutral form (Tables 2-4). As the chlorophyte were never the most sensitive group to lincomycin and tylosin, it seems that the observed differences in toxicity are not explained by differences in uptake alone. For the weak base trimethoprim, a higher pH would increase the percentage of neutral compound. The neutral percentage of trimethoprim increased from 32.37% in Kuhl medium to 82.72% in JM medium, and reached 92.32% in ESAW+f/2 medium. The higher neutral percentage of trimethoprim in ESAW+f/2 medium may therefore contribute to a higher toxicity observed for the diatom species (Tables 2-4).

The toxicity of antibiotics in the non-target organisms is most frequently due to interactions with the specific drug target [43]. While orthologous drug targets (protein) are evolutionarily conserved in different species, they are likely to bind to the same exogenous chemicals by binding the same or similar endogenous ligands [9]. Well-conserved targets in a given species might, therefore, increase the risk of pharmacological effects in aquatic organisms after exposure to pharmaceuticals [43]. Though currently no studies have reported the conservation of pharmaceutical ligand-binding sites in the algal species, the pockets of endocrine disrupting chemicals (EDCs) have already been found to be highly conserved in aquatic toxicity testing organisms such as amphibians and fish [9].

The sensitivity of algal species to antibiotics may also be attributed to differences in antibiotic elimination (enzymatic inactivation) by direct degradation or modification of compounds [10]. Some organisms (e.g. bacteria) could produce enzymes that degrade the antibiotics and further inactivate them. A wide range of antibiotics have hydrolytically susceptible chemical bonds (e.g. esters and amides), the integrity of which are important for biochemicalactivity. However, for some compounds such as beta-lactam antibiotics (e.g. penicillin), the beta-lactam ring could be cleaved by beta-lactamases. Macrolide esterase hydrolyses the macrolide antibiotic (e.g. erythromycin) by opening the ring [10]. Other antibiotic resistant enzymes are the group transferases, which impair target binding by structural alteration. A wide range of enzymes such as chloramphenicol acetyltransferases and streptogramin acetyltransferases inactivate antibiotics by this pathway [10]. While the above antibiotic elimination has been only reported in bacteria, the potential occurrence in the algal species may result in different sensitivities towards antibiotics.

The different sensitivity of algal species towards antibiotics may be due to differences in active efflux pumps. Efflux pumps are transport proteins used to extrude intracellular toxic substrates including antibiotics to the extracellular environment [11]. Several efflux pumps covering a variety of substrates were found in prokaryotic bacteria, and they are believed to lead to acquired bacterial antibiotic resistance due to the broad variety of substrates they recongnise [11]. In eukaryotic cells, some efflux pumps were found to modulate the accumulation of antibiotics in phagocytic cells [44]. As efflux pumps are specific for one substrate or multiple classes of antibiotics, differences in efflux pumps included in each organism might explain their sensitivities towards antibiotic exposures [11]. Though no antibiotic

efflux studies have been reported in the algae, the potential appearance of different efflux pumps in the algal species may determine their sensitivities to antibiotic exposure.

The observations of differences in species sensitivity seen in the present study are probably due to a combination of these factors. We would therefore advocate that more work be done to assess the toxicokinetics and toxicodynamics of antibiotics in different algal species, and other pharmaceuticals, in order to provide a better understanding of the key drivers of species sensitivity.

Implication for environment risk assessment

As can be seen from Table 5, previously reported toxicity data for antibiotics for algal species have been predominately available for chlorophytes and cyanobacteria. The observed sensitivity of cyanobacteria to antibiotics has resulted in these organisms being recommended for use in assessing the environmental risks of antibiotics as part of the Market Authorisation process for new antibiotics [45]. This conclusion is partly supported by our present toxicity results for lincomycin and tylosin. However, trimethoprim appears to be significantly more toxic to diatoms than the chlorophytes and cyanobacteria (Tables 2-4) so the assumption that cyanobacteria are the most sensitive species does not seem to hold true for all antibiotics. The current EMEA regulation [45] on the risk assessment of antibiotics by only considering chlorophyte and cyanobacteria as indicators might, therefore, underestimate the influence on diatoms. For the purpose of risk assessment of antibiotics on the algal species in the aquatic environment and based on the OECD 201 guideline, we recommend that the inhibition effects of antibiotics on the growh of at least three species, one from each algal class, be investigated. It would make sense that these tests are done on the species from each class that appear to be consistently most sensitive to antibiotic exposure i.e. P. subcapitata, A. flos-aquae and N. pelliculosa. It is also important to recognise that we

have only worked with a selection of indicator species from three classes. Further work on other antibiotic classes and other species is warranted to better inform the development of risk assessment approaches.

CONCLUSIONS

The present study explored the effects of lincomycin, tylosin and trimethoprim on a battery of algal species using a standard test procedure. The results showed that algal sensitivity to antibiotics varied with EC50 values ranging from < 1 µmol/L level to > 344.45 µmol/L for three antibiotics. For lincomycin, cyanobacteria were found to be the most sensitive group followed by chlorophytes and then diatoms. For tylosin, cyanobacteria were found to be the most sensitive group, but diatoms were more sensitive than chlorophytes. Chlorophytes and cyanobacteria were not sensitive to trimethoprim at the top concentration tested (344.45 µmol/L) but diatoms were found to be sensitive with EC50 values ranging from 7.36 µmol/L to 74.61 µmol/L. It is concluded that the ecotoxicological information of antibiotics on model algal species (e.g. P. subcapitata and D. subspicatus) may not generalize to other algal groups in light of variations in species sensitivity. We would, therefore, recommend that future risk assessment of antibiotics in the aquatic compartment should include at least three species from different algal classes.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.xxxx.

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Data availability—Data, associated metadata, and calculation tools are available from the corresponding

author (alistair.boxall@york.ac.uk).

REFERENCES

1. Morton IK, Hall JM. 1999. Antibiotics. In Morton IKM, eds, Concise Dictionary of Pharmacological Agents: Properties and Synonyms, 1st ed. Springer, London, UK, pp 23.
2. Boxall ABA. 2004. The environmental side effects of medication - How are human and veterinary medicines in soils and water bodies affecting human and environmental health? Embo R 5:1110-1116.
3. Monteiro SC, Boxall ABA. 2010. Occurrence and Fate of Human Pharmaceuticals in the Environment. Rev Environ Contam T 202: 53-154.
4. Crane M, Watts C, Boucard T. 2006. Chronic aquatic environmental risks from exposure to human pharmaceuticals. Sci Total Environ 367:23-41.
5. Santos LHMLM, Araujo AN, Fachini A, Pena A, Delerue-Matos C, Montenegro MCBSM. 2010. Ecotoxicological aspects related to the presence of pharmaceuticals in the aquatic environment. J Hazard Mater 175:45-95.
6. Guo J, Boxall A, Selby K. 2015. Do pharmaceuticals pose threat to primary producers? Crit Rev Env Sci Tec 45: 2565-2610.
7. EMEA. 2008. Revised guideline on environmental impact assessment for veterinary medicinal products. [cited 2015 August 10]. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/10/WC500004389.pdf.
8. Halling-Sorensen B. 2000. Algal toxicity of antibacterial agents used in intensive farming.

Chemosphere 40:731-739.

9. McRobb FM, Sahagun V, Kufareva I, Abagyan R. 2014. In Silico Analysis of the Conservation of Human Toxicity and Endocrine Disruption Targets in Aquatic Species. Environ Sci Technol 48:1964-1972.

10. Wright GD. 2005. Bacterial resistance to antibiotics: Enzymatic degradation and modification. Adv Drug Deliver Rev 57:1451-1470.

11. Webber MA, Piddock LJV. 2003. The importance of efflux pumps in bacterial antibiotic resistance. J Antimicrob Chemoth 51:9-11.

12. Luetzhoft HCH, Halling-Sorensen B, Jorgensen SE. 1999. Algal toxicity of antibacterial agents applied in Danish fish farming. Arch Environ Con Tox 36:1-6.

13. Eguchi K, Nagase H, Ozawa M, Endoh YS, Goto K, Hirata K, Miyamoto K, Yoshimura H. 2004. Evaluation of antimicrobial agents for veterinary use in the ecotoxicity test using microalgae. Chemosphere 57:1733-1738.

14. DeLorenzo ME, Fleming J. 2008. Individual and mixture effects of selected pharmaceuticals and personal care products on the marine phytoplankton species Dunaliella tertiolecta. Arch Environ Con Tox 54:203-210.

15. Hagenbuch IM, Pinckney JL. 2012. Toxic effect of the combined antibiotics ciprofloxacin, lincomycin, and tylosin on two species of marine diatoms. Water Res 46:5028-5036.

16. De Liguoro M, Cibin V, Capolongo F, Halling-Sorensen B, Montesissa C. 2003. Use of oxytetracycline and tylosin in intensive calf farming: evaluation of transfer to manure and soil. Chemosphere 52:203-212.

17. Drugbank. 2013. Open Data Drug and Drug Target Database. [cited 2013 December 13]. Available from: http://www.drugbank.ca.

18. OECD. 2011. OECD guidelines for the testing of chemicals, Freshwater Alga and Cyanobacteria, Growth Inhibition Test. [cited 2015 March 16]. Available from: http://www.oecd-ilibrary.org/environment/test-no-201-alga-growth-inhibition-test_9789264069923-en.

19. AB. 2015. AlgaeBase. [cited 2015 August 16]. Available from: http://www.algaebase.org/search/species/.

20. Kuhl A, Lorenzen H. 1964. Handling and culturing of Chlorella. In Prescott DM, eds, Methods of cell physiology, 1st ed, Vol 1. Academic Press, London, UK, pp 159-187.

21. CCAP. 2014. Jaworski's Medium (JM) recipe. [cited 2014 May 15]. Available from: http://www.ccap.ac.uk/media/documents/JM.pdf.

22. Berges JA, Franklin DJ, Harrison PJ. 2004. Evolution of an artificial seawater medium: Improvements in enriched seawater, artificial water over the last two decades. J Phycol 37: 1138-1145.

23. ABO. 2013. Industrial Algae Measurements, Algae Biomass Organisation. [cited 2013 May 15]. Available from: http://algaebiomass.org/wp-content/gallery/2012-algae-biomass-summit/2010/06/IAM-6.0.pdf.

24. Boesten J, Helweg A, Businelli M, Bergstrom L, Schaefer H, Delmas A, Kloskowski R, Walker A, Travis K, Smeets L, Jones R, Vanderbroeck V, Van Der Linden A, Broerse S, Klein M, Layton R, Jacobsen O, Yon D. 1997. Soil persistence models and EU registration. [cited 2015 October

10]. Available from: http://ec.europa.eu/food/plant/pesticides/guidance_documents/docs/wrkdoc11_v1997.pdf.

25. Lam MW, Young CJ, Brain RA, Johnson DJ, Hanson MA, Wilson CJ, Richards SM, Solomon KR, Mabury SA. 2004. Aquatic persistence of eight pharmaceuticals in a microcosm study. Environ Toxicol Chem 23:1431-1440.

26. Loftin KA, Adams CD, Meyer MT, Surampalli R. 2008. Effects of ionic strength, temperature, and pH on degradation of selected antibiotics. J Environ Qual 37:378-386.

27. Mitchell SM, Ullman JL, Teel AL, Watts RJ. 2015. Hydrolysis of amphenicol and macrolide antibiotics: Chloramphenicol, florfenicol, spiramycin, and tylosin. Chemosphere 134:504-511.

28. Werner JJ, Chintapalli M, Lundeen RA, Wammer KH, Arnold WA, McNeill K. 2007. Environmental photochemistry of tylosin: Efficient, reversible photoisomerization to a less-active isomer, followed by photolysis. J Agric Food Chem 55:7062-7068.

29. Sirtori C, Aguera A, Gernjak W, Malato S. 2010. Effect of water-matrix composition on Trimethoprim solar photodegradation kinetics and pathways. Water Res 44:2735-2744.

30. Di Paola A, Addamo M, Augugliaro V, Garcia-Lopez E, Loddo V, Marci G, Palmisano L. 2006. Photodegradation of lincomycin in aqueous solution. Int J Photoenergy 2006: 1-6.

31. Prado N, Ochoa J, Amrane A. 2009. Biodegradation and biosorption of tetracycline and tylosin antibiotics in activated sludge system. Process Biochem 44:1302-1306.

32. Kim HY, Jeon J, Yu S, Lee M, Kim TH, Kim SD. 2013. Reduction of toxicity of antimicrobial compounds by degradation processes using activated sludge, gamma radiation, and UV. Chemosphere 93:2480-2487.

33. Halling-Sorensen B, Lutzhoft HCH, Andersen HR, Ingerslev F. 2000. Environmental risk assessment of antibiotics: comparison of mecillinam, trimethoprim and ciprofloxacin. J Antimicrob Chemoth 46:53-58.

34. Kolar B, Arnus L, Jeretin B, Gutmaher A, Drobne D, Durjava MK. 2014. The toxic effect of oxytetracycline and trimethoprim in the aquatic environment. Chemosphere 115:75-80.

35. Pattard M. 2009. Range of Reference Tests in Aquatic Tests. In Moser H, Rombke J, eds, Ecotoxicological Characterization of Waste, 2nd ed, Springer, New York, USA, pp 61-7.

36. ECB 2005. European union risk assessment report. [cited 2015 July 05]. Available from: http://echa.europa.eu/documents/10162/a880b869-5ce0-45ef-8444-379733a0c340.

37. Smit MGD, Hendriks AJ, Schobben JHM, Karman CC, Schobben HPM. 2001. The variation in slope of concentration-effect relationships. Ecotox Environ Safe 48:43-50.

38. Brosche S, Backhaus T. 2010. Toxicity of five protein synthesis inhibiting antibiotics and their mixture to limnic bacterial communities. Aquat Toxicol 99:457-465.

39. Van der Grinten E, Pikkemaat MG, van den Brandhof E-J, Stroomberg GJ, Kraak MHS. 2010. Comparing the sensitivity of algal, cyanobacterial and bacterial bioassays to different groups of antibiotics. Chemosphere 80:1-6.

40. Andreozzi R, Canterino M, Lo Giudice R, Marotta R, Pinto G, Pollio A. 2006. Lincomycin solar photodegradation, algal toxicity and removal from wastewaters by means of ozonation. Water Res 40:630-638.

41. Warne SJM, Van Dam R. 2008. NOEC and LOEC data should no longer be generated or used. Australas J Ecotoxicol 14:1-5.

42. Iwasaki Y, Kotani K, Kashiwada S, Masunaga S. 2015. Does the Choice of NOEC or EC10 Affect the Hazardous Concentration for 5% of the Species? Environ Sci Technol 49:9326-9330.

43. Gunnarsson L, Jauhiainen A, Kristiansson E, Nerman O, Larsson DGJ. 2008. Evolutionary conservation of human drug targets in organisms used for environmental risk assessments. Environ Sci Technol 42:5807-5813.

44. Van Bambeke F, Balzi E, Tulkens PM. 2000. Antibiotic Efflux Pumps. Biochem Pharmacol, 60:13.

45. EMEA. 2006. Guideline on the environmental risk assessment of medicinal products for human use. [cited 2015 July 15]. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/10/WC500003978.pdf

46. HSDB. 2015. Hazard substances database. [cited 2015 September 10]. Available from: http://toxnet.nlm.nih.gov/cgi-bin/sis/search2/f?./temp/~32J1jZ:1.

47. Sigma-Aldrich. 2015. Material safety data sheet (MSDS). [cited 2015 September 10]. Available from: http://www.sigmaaldrich.com/united-kingdom.html.

48. EPA. 2013. Environment Protection Agency EPI suite. [cited 2013 September 15]. Available from: http://www.epa.gov/oppt/exposure/pubs/episuitedl.htm.

49. Kim SC, Carlson K. 2007. Temporal and spatial trends in the occurrence of human and veterinary antibiotics in aqueous and river sediment matrices. Environ Sci Technol 41:50-57.

Figure 1. The residual percentage (%) of the three antibiotics in growth inhibition cultures of the seven algal species (samples in lowest and highest concentration for each biotest). Data represent mean ± standard deviation (n=3). PS, P. subcapitata; DS, D. subspicatus; CV, C. vulgaris; NP, N. pelliculosa; PT, P. tricornutum; AF, A. flos-aquae; SL, S. leopoliensis. LIN, lincomycin; TYN, tylosin; TMP, trimethoprim.

Figure 2. Changes in pH during 4 days of exposure to antibiotics. Data represent mean ± standard deviation (n=21). PS, P. subcapitata; DS, D. subspicatus; CV, C. vulgaris; NP, N. pelliculosa; PT, P. tricornutum; AF, A. flos-aquae; SL, S. leopoliensis. LIN, lincomycin; TYN, tylosin; TMP, trimethoprim.

Figure 3. The 4d concentration-response curves for seven algal species towards single exposure.

Figure 4. Toxicity comparison (EC_{50} μmol/L) of three antibiotics to selected algal species based on 3 day and 4 day measurement. PS, P. subcapitata; DS, D. subspicatus; NP, N. pelliculosa; PT, P. tricornutum; AF, A. flos-aquae; SL, S. leopoliensis. LIN, lincomycin; TYN, tylosin; TMP, trimethoprim.

Table 1. Structures, properties, mode of action and occurrence in the aquatic environment for the three study antibiotics.

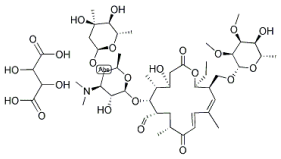
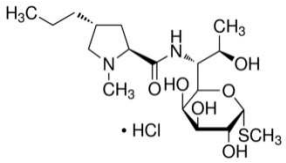
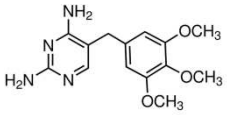
	Tylosin tartrate	Lincomycin hydrochloride	Trimethoprim
CAS-no.	1405-54-5	859-18-7	738-70-5
Structure			
Molecular weight (g/mol)	1066.19	443	290.32
Pka	7.73 [46]	7.6 [46]	7.12 [46]
Solubility in H_2O	Very soluble (50000 mg/L) [47]	Soluble (3.02 mg/L) [47]	Slightly soluble (0.4mg/L) [48]
Mode of action	Inhibit bacterial protein synthesis by binding to 50S ribosome [47]	Inhibit bacterial protein synthesis by forming crosslinks within the peptidyl transferase loop region of the 23S rRNA [47]	Inhibit dihydrofolate reductase [17]
Maximum Occurrence in surface water (µg/L)	0.05 (USA) [49]	<0.001 - 0.73 (US) [3]	0.71 (US) [3]

Table 2. Summary of the effects of tested antibiotics in 4d ecotoxicological biotests. Toxicity data derived from testing lincomycin and potassium dichromate. All toxicity values are in µmol/L (values in parenthesis are the range of 95% confidence limits). Seven algal species are *P. subcapitata* (PS), *D. subspicatus* (DS), *C. vulgaris* (CV), *N. pelliculosa* (NP), *P. tricornutum* (PT), *A. flos-aquae* (AF) and *S. leopoliensis* (SL)

Species	Lincomycin								Potassium dichromate
	EC50	EC10	EC5	NOEC	LOEC	Slope (EC50/EC5)	Model, R^2	Neutral fraction (%)	EC50
PS	7.36 (4.88-11.98)	0.88	0.57	1.35	4.06	12.91	Weibull, 0.93	86.32	5.23 (3.37-n.a.)
DS	16.07 (11.2-23.72)	0.19 (n.a.-0.77)	0.13	<1.35	1.35	123.62	Weibull 0.93	86.32	4.59 (3.84-5.88)
CV	>225.73	n.a	n.a	225.73	>225.73	n.a.	n.a.	86.32	8.29 (n.a.-12.92)
NP	>225.73	35.66 (13.77-66.78)	16.07 (n.a-41.43)	121.89	180.59	14.05	Gompertz 0.64	20.08	>34
PT	>225.73	n.a.	n.a	121.9	180.59	n.a.	n.a	20.08	>34
AF	0.13 (0.11-0.15)	0.03	0.017	0.045	0.14	7.65	Weibull 0.971	38.69	15.94 (13.05-19.61)

SL	0.095 (0.076-0.13)	0.02	0.013	<0.14	0.14	7.31	Hill 0.93	38.69	>34

n.a. not available.

Table 3. Summary of the effects of tested antibiotics in 4d ecotoxicological biotests. Toxicity data derived from testing tylosin. All toxicity values are in µmol/L (values in parenthesis are the range of 95% confidence limits). Seven algal species are *P. subcapitata* (PS), *D. subspicatus* (DS), *C. vulgaris* (CV), *N. pelliculosa* (NP), *P. tricornutum* (PT), *A. flos-aquae* (AF) and *S. leopoliensis* (SL)

Species	Tylosin								
	EC50	EC10	EC5	NOEC	LOEC	Slope (EC50/EC5)	model	Neutral (%)	fraction
PS	4.14 (3.4-5.06)	0.91 (0.45-1.37)	0.4	0.56	1.69	10.35	Gompertz 0.963	89.49	
DS	12.19 (10.57-15.42)	4.05 (1.95.-7.33)	3	<9.38	9.38	4.06	Chapman 0.955	89.49	
CV	>81.2	n.a.	n.a	>81.2	>81.2	n.a	n.a.	89.49	
NP	1.33 (1.14-1.76)	0.83 (0.6-1.06)	0.75	0.56	1.13	1.77	Chapman 0.916	25.31	
PT	5.7 (3.67-9.6)	0.21 (n.a-0.43)	0.08	0.28	0.56	71.25	Hill 0.89	25.31	
AF	0.092 (0.073-0.12)	0.02	0.012	0.037	0.074	7.67	Hill 0.96	45.98	
SL	0.09 (0.068-0.13)	0.011	0.005	0.009	0.026	18	Chapman 0.95	45.98	

n.a. not available.

Table 4. Summary of the effects of tested antibiotics in 4d ecotoxicological biotests. Toxicity data derived from testing trimethoprim. All toxicity values are in µmol/L (values in parenthesis are the range of 95% confidence limits). Seven algal species are *P. subcapitata* (PS), *D. subspicatus* (DS), *C. vulgaris* (CV), *N. pelliculosa* (NP), *P. tricornutum* (PT), *A. flos-aquae* (AF) and *S. leopoliensis* (SL)

Species	Trimethoprim							
	EC50	EC10	EC5	NOEC	LOEC	Slope (EC50/EC5)	model	Neutral fraction (%)
PS	>218.28	n.a	n.a	218.28	>218.28	n.a	n.a.	32.37
DS	>344.45	n.a	n.a	>344.45	>344.45	n.a	n.a.	32.37
CV	>344.45	n.a	n.a	>344.45	>344.45	n.a	n.a.	32.37
NP	7.36 (6.74-8.28)	4.55 (3.65-5.5)	4	4.13	6.89	1.84	Chapman 0.96	92.32
PT	74.61 (55.47-105.23)	17.19 (7.62-30.59)	11.44	20.67	62	6.52	Chapman 0.894	92.32
AF	315.78 (285.16-n.a.)	63.13	32.5	46.79	137.78	9.72	logistic 0.9	82.72
SL	>344.45	97.58	28.67	206.67	275.56	12	Sigmoid 0.74	82.72

n.a. not available.

Table 5. Ecotoxicity data of tested antibiotics to algal growth in literature

Species	Test duration	EC50 (μmol/L)	Reference
Lincomycin			
P. subcapitata	4 d	3.71	[40]
Cyclotella meneghiniana	4 d	4	[40]
S. leopoliensis	4 d	0.49	[40]
Tylosin			
P. subcapitata	3 d	0.0083	[39]
P. subcapitata	3 d	1.51	[8]
P. subcapitata	3 d	0.38	[13]
Microcystis aeruginosa	3 d	0.037	[8]
Trimethoprim			
P. subcapitata	3 d	>31	[39]
P. subcapitata	3 d	276.59	[13]
P. subcapitata	3 d	444.34	[34]
A. flos-aquae	3 d	871.45	[34]

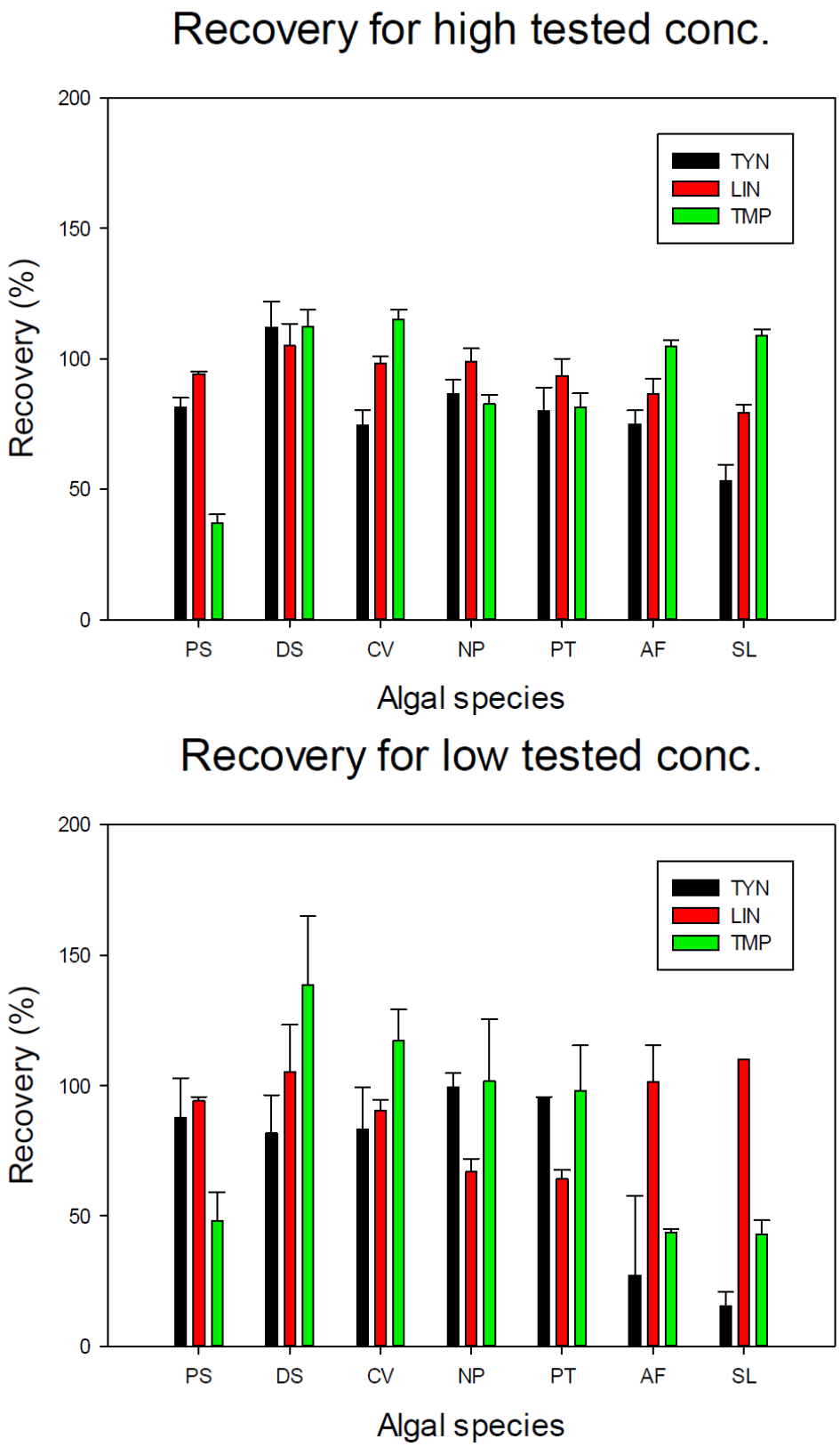


Figure 1

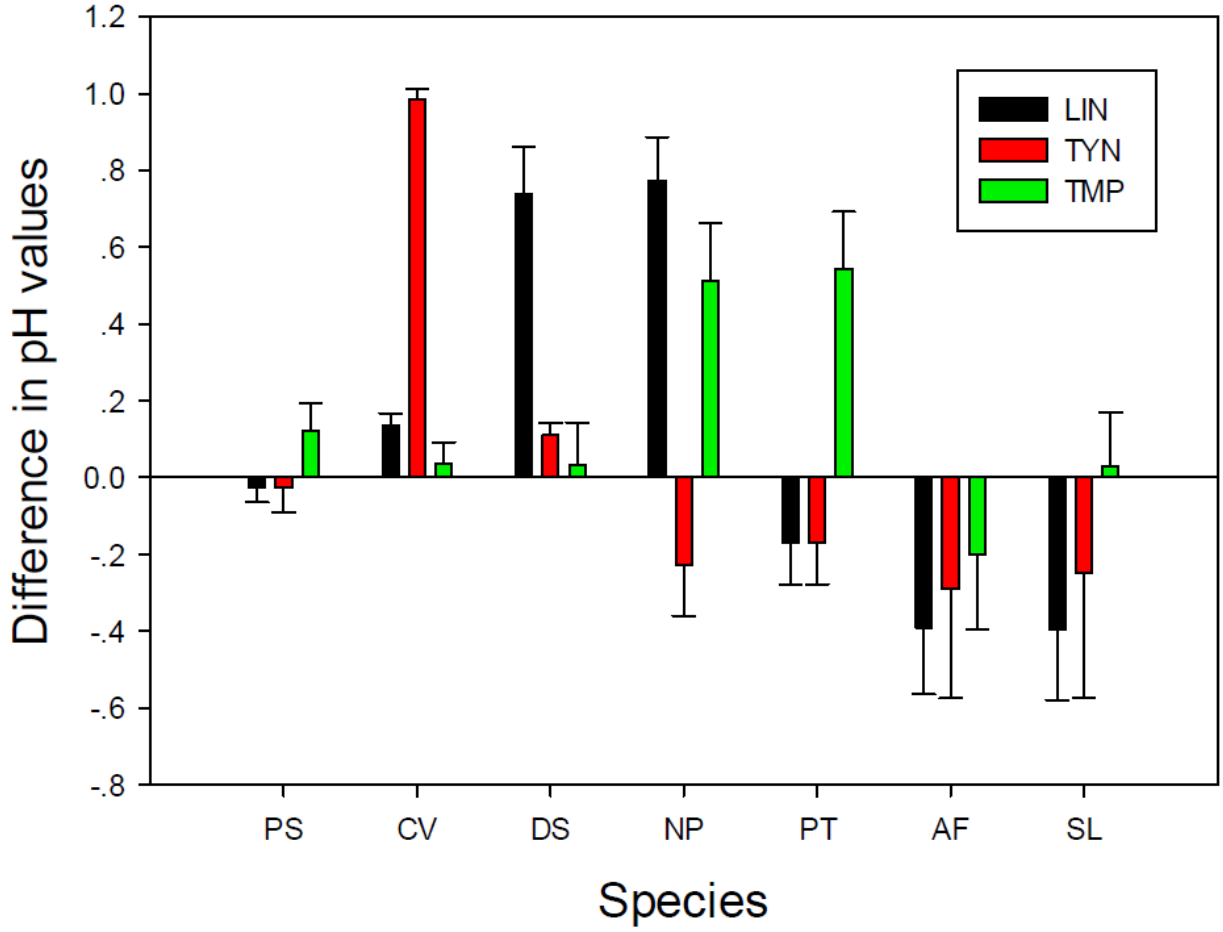


Figure 2

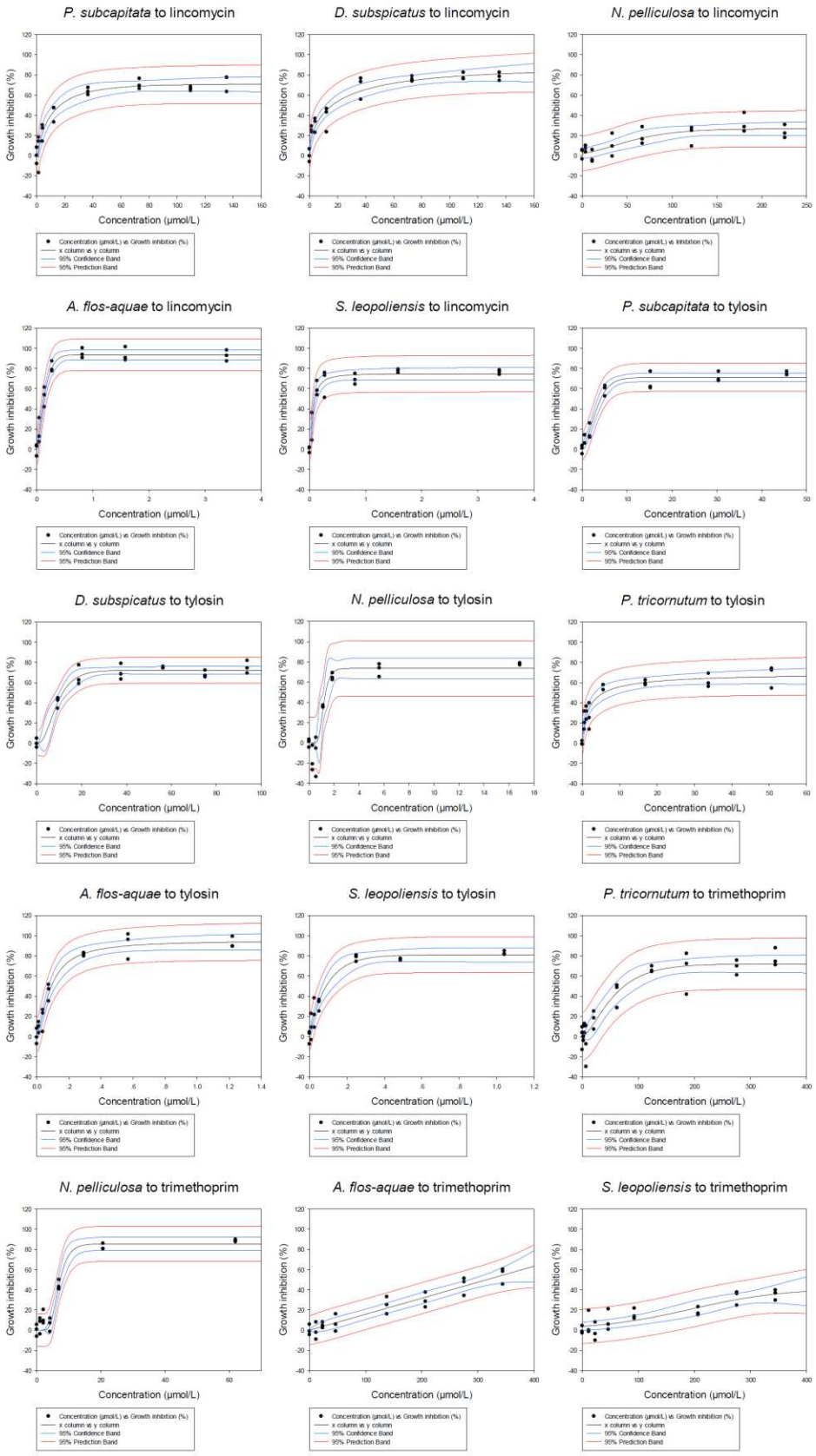


Figure 3

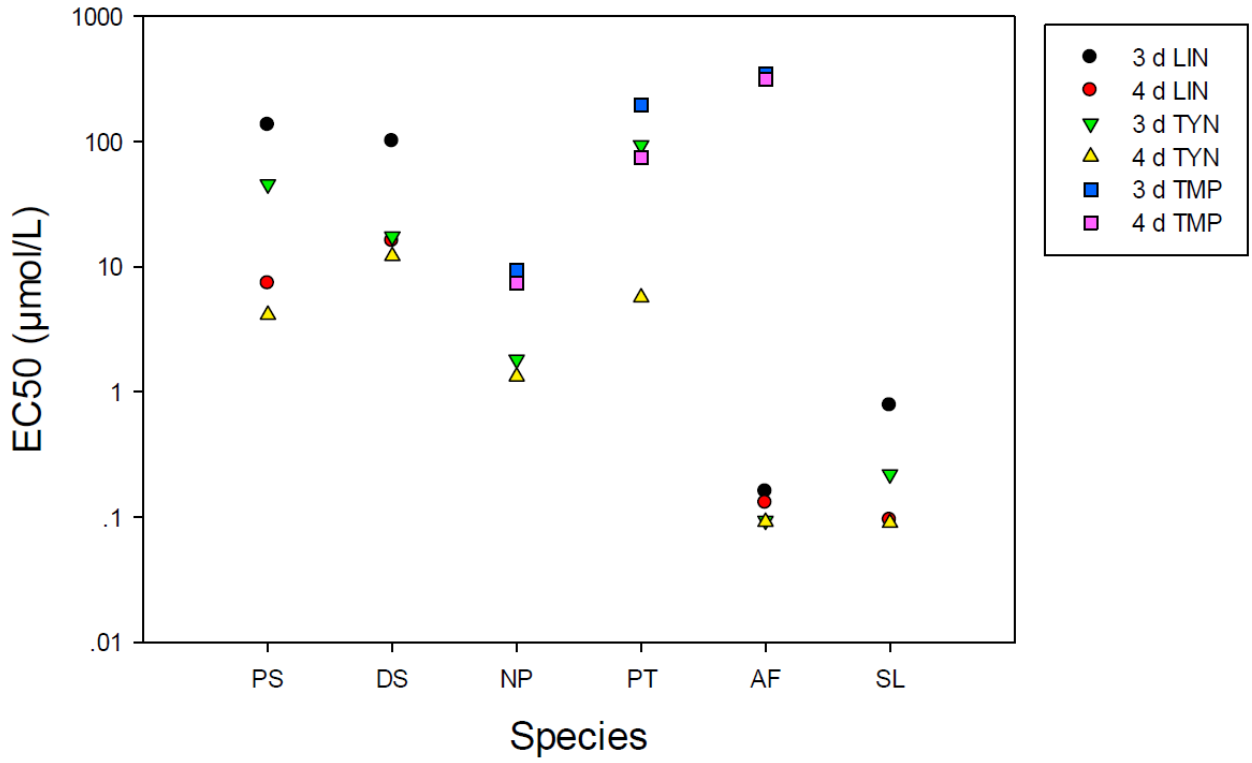


Figure 4