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Assessment of the Risks of Mixtures of Major Use Veterinary Antibiotics in European Surface Waters

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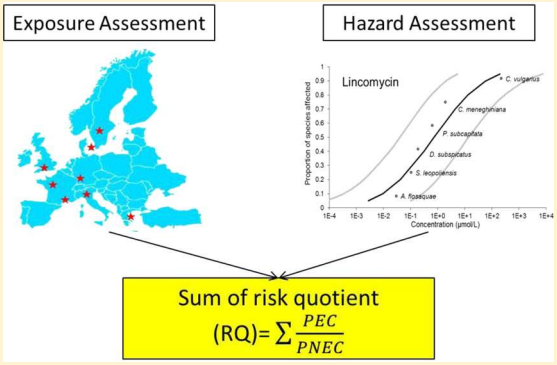
Supporting Information

ABSTRACT: Effects of single veterinary antibiotics on a range of aquatic organisms have been explored in many studies. In reality, surface waters will be exposed to mixtures of these substances. In this study, we present an approach for establishing risks of antibiotic mixtures to surface waters and illustrate this by assessing risks of mixtures of three major use antibiotics (trimethoprim, tylosin, and lincomycin) to algal and cyanobacterial species in European surface waters. Ecotoxicity tests were initially performed to assess the combined effects of the antibiotics to the cyanobacteria *Anabaena flos-aquae*. The results were used to evaluate two mixture prediction models: concentration addition (CA) and independent action (IA). The CA model performed best at predicting the toxicity of the mixture with the experimental 96 h EC50 for the antibiotic mixture being 0.248 μmol/L compared to the CA predicted EC50 of 0.21 μmol/L. The CA model was therefore used alongside predictions of exposure for different European scenarios and estimations of hazards obtained from species sensitivity distributions to estimate risks of mixtures of the three antibiotics. Risk quotients for the different scenarios ranged from 0.066 to 385 indicating that the combination of three substances could be causing adverse impacts on algal communities in European surface waters. This could have important implications for primary production and nutrient cycling. Tylosin contributed most to the risk followed by lincomycin and trimethoprim. While we have explored only three antibiotics, the combined experimental and modeling approach could readily be applied to the wider range of antibiotics that are in use.



1. INTRODUCTION

Antibiotics are used widely in veterinary medicine to treat bacterial disease and protect the health of livestock.[1] Following treatment, antibiotics can be directly excreted to soil and surface water or enter the environment when animal manures and slurries are applied to land as a fertilizer. Antibiotics emitted to the soil environment can subsequently be transported to surface waters through runoff and subsurface drainage.[2]

Low levels of veterinary antibiotics have been reported in surface waters across the globe with concentrations ranging from nmol/L to μmol/L levels.[3] Effects of antibiotics on aquatic organisms have also been reported. For examples, Isidori et al.[4] demonstrated that the antibiotics erythromycin, oxytetracycline, sulfamethoxazole, ofloxacin, lincomycin, and clarithromycin can cause immobilization of the crustacea *Daphnia magna* and *Ceriodaphnia dubia* with 48 h median effective concentration values (EC50) ranging from 13.9 μmol/L to 87.9 μmol/L, but they showed no effects on fish *Danio rerio*. Halling-Sorensen[5] studied the inhibitory effects of seven antibiotics penicillin G, chlortetracycline, spiramycin, streptomycin, tetracycline, tiamulin, and tylosin on the cyanobacteria *Microcystis aeruginosa* and green algae *Pseudokirchneriella subcapitata*, and obtained 72 h EC50s ranged from 0.018 μmol/L to 0.2 μmol/L and 0.23 μmol/L to 6.47 μmol/L, respectively. This evidence indicates that algal species, especially cyanobacteria, are more likely to be affected by antibiotics than other aquatic organisms such as fish and crustacea. While the mechanisms for these particular observations are not totally understood, the high sensitivity of cyanobacteria is likely due to that fact that these organisms have receptor systems similar to those targeted by antibiotics in bacteria.[6] As algal and cyanobacterial species play a critical role in key ecosystem functions such as primary production and nutrient transformation, antibiotics could be adversely impacting aquatic ecosystems.[6]

Agricultural surface waters are unlikely to be exposed to single antibiotics as some antibiotic products contain mixtures of active substances (e.g., sulfonamides and trimethoprim are often used in combination) and a number of different antibiotics are likely to be in use in a catchment at any one time.[7,8] When assessing the environmental risks of antibiotics, it is therefore important to consider the potential combined effects of these compounds. A number of studies have explored the effects of pharmaceutical mixtures, including antibiotics, on

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aquatic organisms. Some of these have also explored the utility of well-established empirical models including the concentration addition (CA) and independent action (IA) models to estimate the joint effects of pharmaceuticals. The CA model is applicable to toxicants acting on the same biological site by the same mode of action, while IA is applicable to toxicants with different modes of toxic action.[9] For examples, Cleuver[10] assessed the joint toxicity of clofibric acid and carbamazepine on the green algae *Desmodesmus subspicatus*, and showed that the mixture toxicity could be predicted using the IA model. Christensen et al.[11] investigated the effects of binary mixtures of citalopram, fluoxetine, fluvoxamine, paroxetine, and sertraline on algae and daphnids, and showed that the combined toxicity of the compounds could be predicted by CA. However, most of these studies have not explored the implications of pharmaceutical mixtures in terms of risk to ecosystems.

Methods for assessing the risks of mixtures of chemicals to the natural environment have been proposed.[8] For example REACH (Regulation No 1907/2006) presents a tiered approach for assessment of industrial chemical mixtures.[12] At tier 1, a conservative approach, based on CA, is applied. Risk quotients (RQ) for individual mixture components are determined from predicted environmental concentrations (PECs) and predicted no effect concentrations (PNECs) and are then summed to determine the RQ of the mixture (RQ_{mix}).[8] A similar approach could be used to evaluate the risks of antibiotic mixtures.

In this study, we therefore present an approach combining experimental ecotoxicity studies with modeling approaches to characterize the risks of mixtures of veterinary antibiotics to algal and cyanobacterial communities in surface waters in Europe. We apply the approach to three major-use antibiotics (tylosin, lincomycin and trimethoprim) which have been detected in surface water worldwide (e.g., UK,[13] U.S.[14]) and which represent active ingredients from the third and fourth most widely used antibiotic classes in the UK.[15] Trimethoprim acts by inhibiting dihydrofolate reductase (DHFR),[16] while tylosin and lincomycin are designed to interact with bacterial protein synthesis by binding to the 50s ribosome.[17] While we only explore three antibiotics, we believe that the strategy used could be applied more widely in the assessment of risks of antibiotics, and other active ingredients, used in veterinary medicine.

2. MATERIALS AND METHODS

The study was performed in two phases. In Phase 1, experimental studies were performed on a mixture of the antibiotics and the results used to evaluate the CA and IA models for estimating effects of mixtures of the study compounds. In Phase 2, the best performing mixture model was used alongside exposure models and scenarios for European surface waters and species sensitivity distribution analyses to establish the combined risks of the study antibiotics to algal and cyanobacterial communities in surface waters across Europe.

2.1. Evaluation of Mixture Toxicity Models Using Experimental Data. An experimental investigation was performed into the effects of a mixture of the study antibibiotics on the cyanobacteria *Anabaena flos-aquae*. *A. flos-aquae* was selected as our previous work has demonstrated that this species is more sensitive to tylosin and lincomycin than other cyanobacterial, diatom and chlorophyte species.[17]

2.1.1. Chemicals. Tylosin tartrate (hereafter referred to as tylosin, 86.4%) (CAS-no. 1405−54−5), lincomycin hydrochloride (referred to as lincomycin, ≥ 95%) (CAS-no. 859−18−7), and trimethoprim (≥98%) (CAS-no. 738−70−5) were purchased from Sigma-Aldrich. Analytical Reagent grade ammonium acetate and formic acid (≥95%) were purchased from Fisher Scientific UK and Sigma-Aldrich, respectively. HPLC grade acetonitrile, methanol and water were purchased from Fisher Scientific UK. These chemicals were used as the mobile phase for high performance liquid chromatography (HPLC).

2.1.2. Algae Culture. *A. flos-aquae* (CCAP 1403/13A) was obtained from the Institute of Freshwater Ecology (Culture Collection of Algae and Protozoa, UK) and grown in Jaworski's Medium (JM), pH 7.8[18] at 20 °C ± 2 °C under gentle and continuous shaking (100 cycles per minute (cpm)) under constant illumination (76 μmol $m^{-2}s^{-1}$). Cultures were maintained in conical flasks (250 mL) containing 100 mL of medium and 1 mL algal cells. Prior to use, all the flasks were washed in Decon 90 and rinsed with hydrochloric acid (50 mM) and then autoclaved at 121 °C for 30 min. The cell number was counted with a hemacytometer under a microscope, the growth curve (cell density versus days) was plotted to identify the logarithmic phases (usually after 2−4 days). The algal stock was subcultured on a weekly basis.

2.1.3. Selection of Test Concentrations for Use in the Mixture Toxicity Study. Exposure models described in the CVMP Guidance Document[19] were used to define relative concentrations, on a molar basis, of the three study compounds for use in the mixture toxicity study (See eq 1 in Supporting Information (SI)). The CVMP exposure models estimate concentrations based on information on the dose applied to the animal, the treatment duration, application rates for manure and slurry, and the sorption behavior of the substance. Metcalfe et al.[20] demonstrated reasonable agreement between the predicted environmental concentration (PEC) calculated using the models and measured environmental concentration (MEC) for veterinary pharmaceuticals in surface waters. Based on the exposure modeling a mixture comprising 1 part tylosin to 4.31 parts trimethoprim and 4.18 parts lincomycin was selected for testing.

2.1.4. Assessment of the Toxicity of the Mixture. The 96 h EC50 values for the single study compounds were determined in the work described in Guo et al.[17] (Table 1; Table S1 in SI). Therefore, the EC50 determination for the mixture was conducted without a range-finding step. Thirteen concentrations of the mixture in a geometric series around the lowest EC50 of the study compounds (i.e., tylosin) were used in the EC50 test. The dose−response curve based on growth (cell density) was then generated based on the definitive data.

The ecotoxicity test followed the OECD 201 Guideline for freshwater alga and cyanobacteria, growth inhibition tests.[21] All glassware and stoppers used in the tests were autoclaved at 121 °C for 30 min prior to use. The antibiotics in the media were prepared and filtered into a 25 mL vial using a 0.2 μm sterilized syringe filter. The precultured algal inocula, taken from logarithmic growing precultures, were diluted into 15 mL of the prepared antibiotic solutions in the vials. The initial cell density was set at 2×10^4 cells/ml. The test vials were capped with air-permeable stoppers made of cotton and muslin. All the operations were performed on a sterilized bench. Afterwards, the prepared vials were put into an incubator with the same shaking and physical conditions as used for the culturing.

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Table 1. Best Fitting Concentration-Response Models, EC05, EC50 and EC50/EC05 Ratio of the Tested Antibiotics and the Mixture

substance	model	EC05 (μmol/L)	EC50 (μmol/L)	EC50/EC05
trimethoprim	three-parameter sigmoid	<1.56[a]	285.95 (246.88- n.a.)[a]	183
tylosin	three-parameter hill	0.025[a]	0.13 (0.09−0.18)[a]	5.2
lincomycin	three-parameter hill	0.036[a]	0.14 (0.11−0.15)[a]	3.89
mixture	three-parameter hill	0.05	0.248 (0.22−0.29)	5
CA	calculated	<0.061	0.21	3.44
IA	calculated	<0.12	0.34	2.83

[a]data obtained from Guo et al.[17]

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 hemocytometer against adsorption (turbidity) measured by an ultraviolet and visible (UV−vis) spectrophotometer for *A. flos-aquae* (R^2 > 0.999). Measurement of turbidity (adsorption) using a spectrophotometer with an appropriate selected wavelength is a reliable method to determine cell density.[22] Algal cultures were diluted and scanned between the 600−800 nm ranges. The wavelengths with the highest absorbance were selected for experiments (682 nm). The pH values of 13 tested exposure solutions were measured at the start and the end of the exposures.

Concentrations of the antibiotics in the exposure solutions were confirmed using high performance liquid chromatography (HPLC) using an Agilent 1100 with C18 Supelco Discovery column (15 cm × 4.6 mm × 5 μm) using methodologies described in Guo et al.[17] Recoveries for tylosin, lincomycin and trimethoprim were 122 ± 16% (mean ± standard deviation), 191 ± 37% and 80 ± 24%, respectively. As the chemical recovery of lincomycin was far above 100%, measured concentration of lincomycin was used to modify the mixture ratio (i.e., tylosin: trimethoprim: lincomycin, 1:4.31:6.65) in the mixture model evaluation work.

Results from ecotoxicity test were analyzed using Sigma-plot 12.0 software. The experimental concentration−response curve was obtained by fitting experimental results to a sigmoidal regression, where the *x*-axis was the molar sum of each component (μmol/L) and the *y*-axis was growth inhibition (%).

2.1.5. Evaluation of Mixture Models against the Experimental Data. The CA and IA were evaluated against the experimental data by plotting the concentration response curve from the experimental mixture study against concentration−response curves based on predictions, using the CA and IA models, of the toxicity for each mixture exposure concentration (the single compound data were taken from Guo et al.[17]). The 5% effective concentration values (EC05) and median effect concentration values (EC50) with approximate 95% confidence intervals were also estimated for the experimentally derived and modeled concentration response curves. Modeled and experimental-derived EC50/EC05 ratios, which provide a measure of slope, were then compared to evaluate the predictive capability of the two models−the assumption being that the mixture model resulting in a slope closest to the slope for the experimental data worked best.

2.2. Assessment of Risks of Mixtures of the Study Antibiotics to European Surface Waters. *2.2.1. Modeling Exposure to the Three Antibiotics in European Surface Waters.* Concentrations of the study antibiotics in representative surface waters in agricultural areas in Europe were estimated using models and scenarios recommended by the Forum for Pesticide Fate Models (FOCUS).[23] The application rate, which is a required input for the models, was estimated based on recommended dosages and treatment frequencies and durations for each antibiotic, obtained from the Compendium of Data Sheets for Animal Medicines 2012,[24] using the approach recommended by the European Medicines Agency.[19] For each antibiotic, the maximum application rate, the average application rate and the minimum application rate of all products and indications were used for the FOCUS modeling. The medical products used to derive the maximum application rates were Synutrim (trimethoprim) and Pharmasin (tylosin) used for the treatment of broilers, and Lino-spectin 100 (lincomycin) for treating pigs.[24] The medical products used to derive the minimum application rates were Trimacare injection (trimethoprim) and TYLAN 200 (tylosin) used for the treatment of cattle, and Lincocin Premix (lincomycin) for pig treatment.

Modeling of the eight scenarios (five covering systems with soil drainage: D1, D2, D4, D5, D6; and three systems that are vulnerable to runoff: R1, R3, R4) and different watercourse types (ditch, pond and stream) was performed assuming winter wheat as the crop. The eight scenarios have predefined soil properties and weather data and are geographical representatives of agricultural areas in the EU (the distribution of these scenarios are shown in SI Figure S1). While the data of the scenarios are taken from specific fields in the area, they have been manipulated to mimic the characteristics of the broader area (detailed information on the soil properties and weather data for each scenario can be found in FOCUS).[23]

To run the models, the ground incorporation method of application was selected and inputs from spray drift were set at zero. No uptake by plants was assumed.[19] Physico-chemical properties of the antibiotics, needed for the modeling, were derived from a variety of sources and are given in Supporting Information (Table S2 in SI). Detailed estimation procedures can be found in the FOCUS model manual.[25] The 4 day time-weighted averaged exposure concentrations (TWAEC) in the water layer for each scenario and antibiotic were obtained and used in the risk characterization work.

2.2.2. Derivation of Predicted No Effect Concentrations. PNECs were obtained from species sensitivity distributions (SSDs) for toxicity for each antibiotic. SSDs are models of the variation in sensitivity of species to a particular stressor (here an antibiotic). Data on the toxicity of the three antibiotics to algal and cyanobacterial species were obtained from our previous work,[17] the literature and databases (e.g., EPA ECOTOX).[26] For trimethoprim, 18 toxicity data values were obtained from 11 algal species. For tylosin and lincomycin, SSDs were plotted based on seven toxicity data points obtained from five algal species and eight toxicity data from seven algal species, respectively (Table S3 in SI).

The preferred end point used to develop the SSDs was the concentration causing a 10% inhibition (EC10) of biomass (growth/cell density). Where EC10 values were not available, no observed effect concentration (NOEC) values were used.

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SSD curves were fitted with the Excel macro "SSD generator V1" developed by the US EPA.[27] Where multiple data were available for one species, the geometric mean was used as the input value for the SSD calculation. The hazardous concentration affecting 5% of the species (HC5) was estimated from each SSD and this was divided by an assessment factor (AF) of 5 to derive a PNEC for each antibiotic.[28]

2.2.3. Mixture Risk Assessment for the Three Antibiotics. CA was used as the basis for the risk characterization for the mixtures of the three antibiotics for each of the FOCUS scenarios. Initially, a risk quotient (PEC/PNEC) for each veterinary antibiotic was calculated based on the concentration estimated for the antibiotic in each scenario. The risk quotient for the mixture (RQ_{mix}) of antibiotics for a scenario was then obtained by summing up the PEC/PNEC ratios for the individual antibiotics.[8] If the RQ_{mix} was lower than one then the risk of the mixture to algae was deemed to be acceptable.[19]

3. RESULTS AND DISCUSSION

3.1. pH Variation. With an increase in the exposure concentration, the pH in the mixture studies decreased gradually from 7.99 to 6.96. While a pH variation (1 unit) was observed, it was within the validity range of the OECD 201 guideline (less than 1.5 units). A drift in pH can be caused by CO_2 mass transfer from the surrounding air to the test solution.[21] The variation in pH was consistent with our previous study into the effects of the single antibiotics.[17]

3.2. Mixture Toxicity Analysis and Model Evaluation. Concentration−response curves, based on the experiments as well as CA and IA predictions, are shown in Figure 1. The

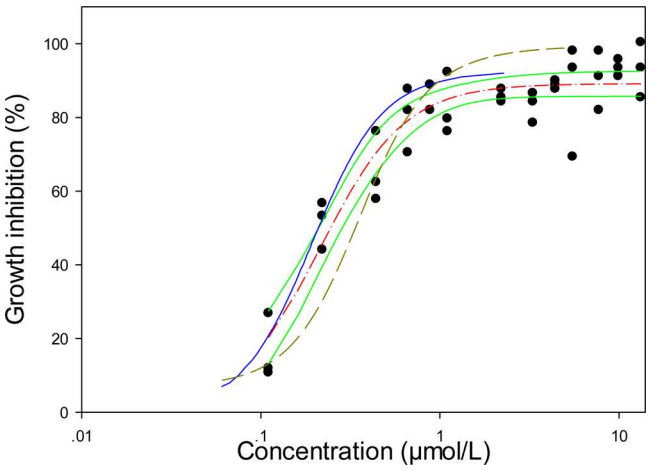

Figure 1. Predicted and observed toxicity for the mixture of the study antibiotics. Solid line (blue) = prediction according to concentration addition (CA); dashed line (dark yellow) = prediction according to independent action (IA); dashed-dotted line (red) = fit to the experimental mixture ecotoxicity data; Solid line (green) = 95% confidence band; solid symbols= treated samples. *X* axis (C_{mix}) is the sum concentrations of three antibiotics. Molar ratio of tylosin: trimethoprim: lincomycin =1:4.31:6.65.

experimental 4 day EC50 for the mixture was 0.248 μmol/L (trimethoprim 0.089 μmol/L, tylosin 0.021 μmol/L, and lincomycin 0.138 μmol/L) (Table 1). While both the CA and IA concepts provided good estimations of the combined effects of the different mixtures of tylosin, lincomycin and trimethoprim (Table 1; Figure 1), the CA model better predicted the toxicity of the mixture. The IA predicted an EC50 of 0.34 μmol/L which was 37.1% higher than the observed EC50, while CA predicted a slightly higher toxicity (EC50 0.21 μmol/L) which was within 15.3% of the observed EC50. This finding was consistent with other publications investigating combination effects of pharmaceuticals and other contaminants such as pesticides. For examples, Cleuvers[10] reported that the toxic effect of a binary mixture of pharmaceuticals ibuprofen and diclofenac on the chlorophyte *D. subspicatus* could be predicted well using the CA concept. The binary mixture toxicity of three selective serotonin reuptake inhibitors (SSRIs) citalopram, fluoxetine and sertraline to freshwater algae *P. subcapitata* was also shown to be predictable by CA model.[11] The CA model has also been shown to work for a mixture of more than 10 antibiotics with dissimilar modes of action[7] and to be an appropriate model for estimating effects of pesticides on algal species.[29]

The fact that the CA model works well is probably explained by the modes of action of the three antibiotics as well as the relative concentrations. Trimethoprim acts by inhibiting dihydrofolate reductase (DHFR),[16] while tylosin and lincomycin act by inhibiting bacterial protein synthesis by binding to 50s ribosome.[16,30] The relative concentrations mean that tylosin and lincomycin, which act by the same mode of action, are the two components within the mixture that dominate toxicity (both EC50s are 1000 times lower than that of trimethoprim; Table 1).

Steepness is important in determining the predictability of CA and IA models. 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/EC05 ratio).[9,31] Brosche and Backhaus[9] reported that with an EC50/EC05 ratio of 13.5, CA and IA models will predict quantitatively identical toxicity despite their mutually exclusive conceptual ideas. CA will predict a lower EC50 (higher toxicity) for the mixture than IA if the ratio for the concentration−response curve of the mixture is lower. In this study the EC50/EC05 ratio of 5 indicated a high steepness of the observed concentration−response curve for the mixture (Table 1; Figure 1).[9] The steepness of the mixture curve was within the range of slope for each single component, e.g. the ratio of EC50/EC05 ranged from 3.89 for tylosin up to 183 for trimethoprim. The steepness of the CA model was closest to the experimental steepness compared to the IA model (Table 1). The application of CA to a mixture tested on algae would therefore result in a slight overestimation of the mixture toxicity and IA predicted higher toxicity value (Table 1). As CA predicted more accurately the combined effect of three antibiotics on *A. flos-aquae*, this model was used as a basis for the risk assessment work.

3.3. Estimation of Exposure Concentrations. The maximum 4d TWAECs for trimethoprim in three different waterbodies (pond, ditch and stream) were 0.016 μmol/L, 0.026 μmol/L and 0.084 μmol/L, respectively. For tylosin, the maximum 4d TWAECs for pond, ditch and stream were 0.0061 μmol/L, 0.011 μmol/L and 0.073 μmol/L. For lincomycin, the 4d TWAECs reached 0.0004 μmol/L, 0.00075 μmol/L and 0.011 μmol/L for three waterbodys (Table S4 in SI). While the 4d TWAECs for trimethoprim and tylosin were nearly within an order of magnitude, estimations of exposure for lincomycin were much lower than the other compounds (Table S5−S7 in SI). These particular results can be explained by differences in the application rates for the antibiotics to soils, where the maximum application rate (A_{max}) of lincomycin was only 0.46 kg/ha in contrast with 6.65 kg/ha and 8.45 kg/ha for

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trimethoprim and tylosin, respectively (Table S2 in SI). The maximum occurrence of three substances were found in scenario R3 in stream systems (Figure 2). R3 is a southern Europen scenario considering the superficial loading from runoff to surface water, where runoff is determined by annual rainfall and slope. The R3 stream scenario had a higher annual rainfall (800−1000 mm) than the other runoff scenarios (600−800 mm), and a slope of 4−10% in comparison with the intermediate case 2−4% of scenario R1.[23]

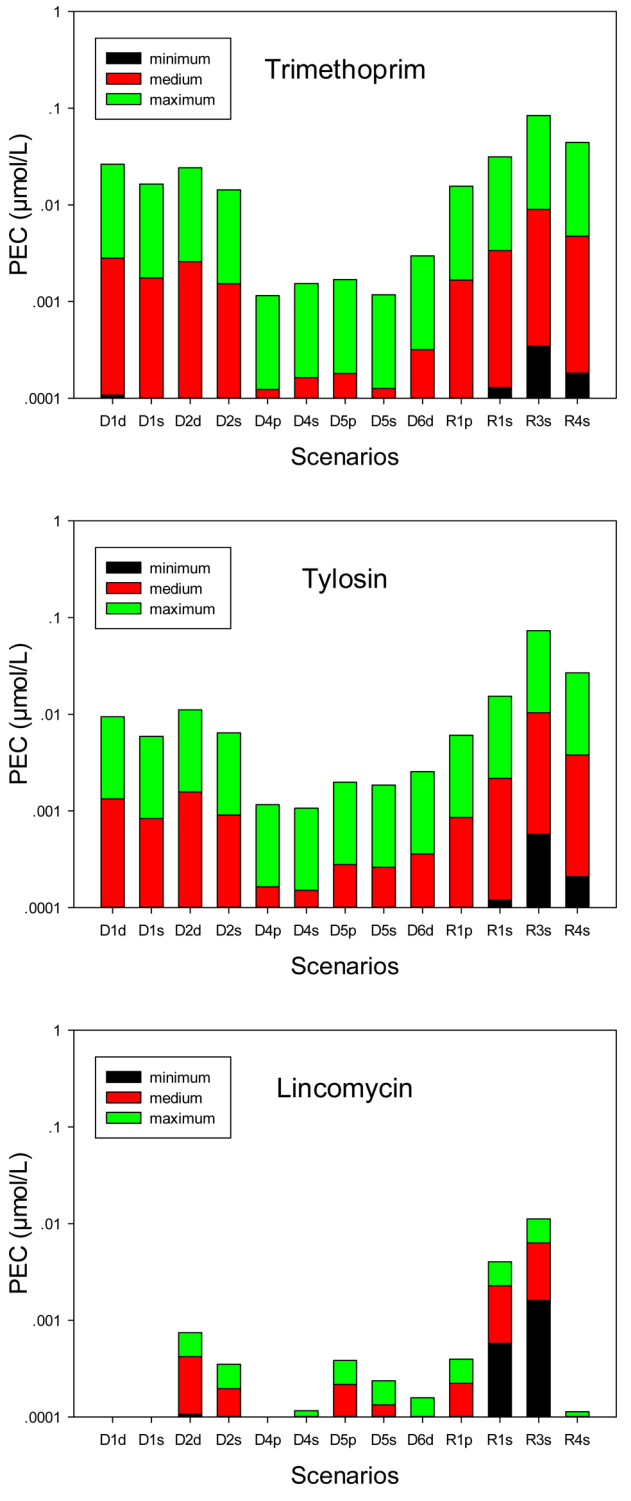

Figure 2. Predicted environmental concentrations for each antibiotic for the different FOCUS surface water scenarios. Values were estimated based on maximum, medium and minimum application rate. *d* = ditch; *s* = stream; and *p* = pond.

The occurrence of three antibiotics has been reported from studies in different regions of the World. Measured concentrations of trimethoprim range from less than 3.4×10^{-5} µmol/L in UK surface waters[13] to 0.0061 µmol/L (U.S.)[14] in U.S. While very limited information on the occurrence of tylosin and lincomycin in surface waters was available, the presence of lincomycin in surfacewater has been recorded from less than 2.46×10^{-6} µmol/L to 0.0018 µmol/L (U.S.).[32] The maximum occurrence of tylosin was found at 5.46×10^{-5} µmol/L downstream of agricultural land in U.S.[3] All these reported concentrations for the antibiotics are within the range of the predicted concentrations in this study (Figure 2) which gives some confidence in the model predictions.

3.4. Species Sensitivity Distributions. Data were available on the toxicity of the study antibiotics to both chlorophyte and cyanobacterial species. SSDs for the three antibiotics are shown in Figure 3. The sensitivity of test organisms to tylosin and lincomycin varied by around 3 orders of magnitude with cyanobacteria found to be more sensitive than chlorophytes. In contrast, effects endpoints for trimethoprim were within an order of magnitude of each other indicating similar sensitivity. The observed differences in sensitivity are likely explained by a range of factors including the mode of action of the antibiotic, the presence/absence of the antibiotic target receptor in the study organisms and differences in uptake and metabolism by the test organisms. We discuss these factors in detail elsewhere.[17] Using the SSDs, PNECs of 2.55 µmol/L, 0.0002 µmol/L, and 0.0006 µmol/L were obtained for trimethoprim, tylosin and lincomycin respectively (see Table S8 in SI). While it is recommended[28] that AFs varying from 1 to 5 can be applied in derivation of PNEC from SSDs, we selected five, that is, the most conservative, because the number of ecotoxicity data sets for tylosin and lincomycin were limited.[28]

3.5. Risk Assessment for Single Antibiotics and Antibiotic Mixtures. In terms of single component solutions, trimethoprim was found to pose an acceptable risk to aquatic systems in Europe at maximum application rates as the maximum risk quotient (RQ) was 0.033 (R3 stream scenario). Maximum RQ values for tylosin and lincomycin were 367 and 18.68, respectively, indicating that both of these compounds pose an unacceptable risk to the European aquatic environment. For tylosin, an unacceptable risk was observed from all the exposure scenarios across the EU with the RQ values based on maximum application rate ranging from 5.33 to 367 (Table S5 in SI). For lincomycin, the unacceptable risk occurred at the scenarios of D2 ditch, R1 stream and R3 stream with the RQ values of 1.24, 6.71 and 18.68, respectively (SI Table S5). Three exposure scenarios geographically represent the agriculture in western, middle and south Europe (SI Figure S1). These risk characterization results for single antibiotics agreed with other risk assessments or risk based prioritisation studies. For example, the maximum RQ of trimethoprim was 0.15 in a risk assessment study performed in Norway[33] while RQs of 39.81 and 62.46 were obtained for tylosin and lincomycin in a study assessing risks to the UK environment.[6]

Risk quotients for mixtures, estimated based on medium application rates, exceeded one for most exposure scenarios, that is, D1, D2, D5, D6, R1, R3, and R4. The RQ values of the antibiotic mixture, estimated based on the three application rate

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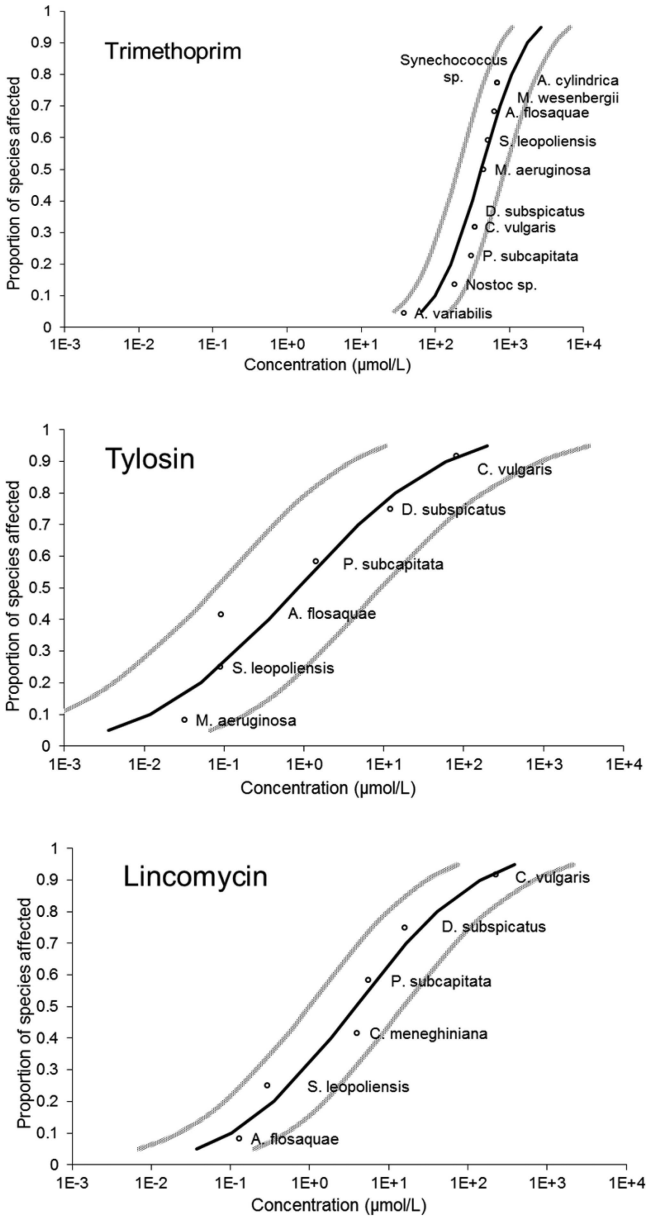

Figure 3. Species senstivity distribution curves for the three antibiotics. Black lines are the best SSD model fit based on EC10/NOEC values and gray lines represent the 95% confidence intervals.

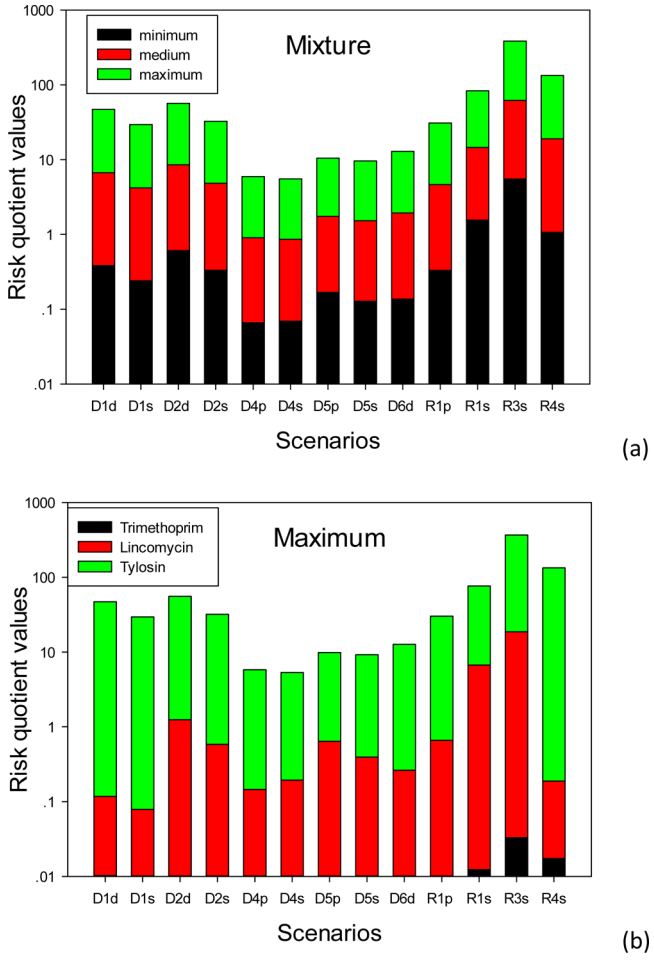

Figure 4. Risk quotients (PEC/PNEC) for the different FOCUS scenarios for: (a) a mixture of three antibiotics estimated for the maximum, medium and minimum application rates; and (b) the three antibiotics estimated for maximum application rate. *d*, ditch; *s*, stream; and *p*, pond.

scenarios, ranged from 0.066 to 385 (Figure 4a). While the 4d TWAECs for trimethoprim and tylosin were within an order of magnitude, compared to tylosin, lower RQs of trimethoprim were observed from all the exposure estimation scenarios. Differences in RQs were due to the hazard assessment. The PNEC of trimethoprim derived from the SSD was 2.55 μmol/L, which was 4 orders of magnitude higher than tylosin (0.0002 μmol/L). The RQs of lincomycin were the lowest in the antibiotic mixture on account of the exposure assessment (Figure 2), though the derived PNECs of lincomycin (0.0006 μmol/L) and tylosin (0.0002 μmol/L) were comparable. Therefore, while the RQ values for this antibiotic mixture indicated a high potential risk to the aquatic environment, the risk was dominated by tylosin (Figure 4b).

Given that an unacceptable risk was observed for the mixture of the three antibiotics for surface water scenarios covering different regions of Europe, primarily due to the effects from tylosin and lincomycin, we recommend that target monitoring of these antibiotics in the European surface water should be performed to gather data for a more realistic risk assessment and that biological monitoring be performed to see whether effects on algae are occurring in reality. A range of indicators based on algae could be applied for biomonitoring of chemical pollution. For examples, variations in algal species diversity and bioaccumulation of chemicals have been observed and demonstrated to relate to changes in water quality.[34]

As aquatic organisms are more likely to be exposed to mixtures of veterinary antibiotics in surface water, assessing biological effects of mixtures is considered to be more realistic (and protective) than assessments that consider only single substances. This study illustrates a combined experimental and modeling-based strategy to assess the risk of mixtures. The proposed method has been demonstrated for a selection of antibiotics and algal species. In the future, we recommend that the approach be applied to the wider range of antibiotics in use and other exposure scenarios such as emissions of antibiotics from wastewater treatment plants.

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.6b01649.

Equations for predicted environmental concentration (PEC); concentration−response curves for *A. flos-aquae* for each antibiotic; input parameters for the FOCUS modeling; algal toxicity values used for developing species sensitivity distributions; exposure concentrations and risk quotients for each veterinary antibiotic based on maximum, medium and minimum application rate (PDF)

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Notes

The authors declare no competing financial interest.

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