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Toxicity of five antibiotics and their mixtures towards photosynthetic aquatic organisms: Implications for environmental risk assessment

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ABSTRACT

The individual and combined toxicities of amoxicillin, erythromycin, levofloxacin, norfloxacin and tetracycline have been examined in two organisms representative of the aquatic environment, the cyanobacterium *Anabaena* CPB4337 as a target organism and the green alga *Pseudokirchneriella subcapitata* as a non-target organism. The cyanobacterium was more sensitive than the green alga to the toxic effect of antibiotics. Erythromycin was highly toxic for both organisms; tetracycline was more toxic to the green algae whereas the quinolones levofloxacin and norfloxacin were more toxic to the cyanobacterium than to the green alga. Amoxicillin also displayed toxicity to the cyanobacterium but showed no toxicity to the green alga. The toxicological interactions of antibiotics in the whole range of effect levels either in binary or multicomponent mixtures were analyzed using the Combination Index (CI) method. In most cases, synergism clearly predominated both for the green alga and the cyanobacterium. The CI method was compared with the classical models of additivity Concentration Addition (CA) and Independent Action (IA) finding that CI could accurately predict deviations from additivity. Risk assessment was performed by calculating the ratio between Measured Environmental Concentration (MEC) and the Predicted No Effect Concentration (PNEC). A MEC/PNEC ratio higher than 1 was found for the binary erythromycin and tetracycline mixture in wastewater effluents, a combination which showed a strong synergism at low effect levels in both organisms. From the tested antibiotic mixtures, it can be concluded that certain specific combinations may pose a potential ecological risk for aquatic ecosystems with the present environmentally measured concentrations.

1. Introduction

Antibiotics are biologically active molecules with an increasing use in both human and veterinary medicine. These pharmaceuticals play a major role in livestock industries and modern agriculture, which use them as therapeutics and growth promoters in livestock production, as feed additives in fish farms and to prevent crop damage induced by bacteria

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(Sarmah et al., 2006); although since 2006 antibiotics have been forbidden as growth promoters in animal feed in the EU. From the antibiotics administered to animals, a large proportion is excreted without metabolizing and is dispersed with the manure used for soil amendment, eventually reaching surface waters (Elmund et al., 1971). A large portion of the antibiotics administered in fish farms (70–80%) has been reported to reach the environment (Schneider, 1994; Hektoen et al., 1995).

The major pathway whereby residual antibiotics from human use enter the environment is the effluent of wastewater treatment plants (WWTP) as conventional plants are relatively inefficient in completely removing pharmaceuticals (McArdell et al., 2003; Göbel et al., 2005; Xu et al., 2007; Rosal et al., 2010b). Antibiotic contamination of the natural environment has been reported in many countries in Europe, North America and Asia, all classes of antibiotics having been detected in river water (Kolpin et al., 2002, 2004; Batt et al., 2006; Xu et al., 2007; Yang et al., 2008; Ginebreda et al., 2009; Tamtam et al., 2009; Watkinson et al., 2009), seawater (Gulkowska et al., 2007; Xu et al., 2007), groundwater (Hirsch et al., 1999; Lindsey et al., 2001; Sacher et al., 2001), drinking water (Zuccato et al., 2000), sediments (Kerry et al., 1996; Kim and Carlson, 2007), agricultural soils due to dispersion of sewage sludge and manure (Sarmah et al., 2006), biota (Dolliver et al., 2007; Kong et al., 2007),and WWTP effluents (Costanzo et al., 2005; Batt et al., 2006; Watkinson et al., 2009; Gros et al., 2010; Rosal et al., 2010b). Although recorded environmental levels are usually low, at ng L^{-1} to μg L^{-1} in waters (see Table S1 under Supplementary Information) and μg kg^{-1} to mg kg^{-1} in soils and sediments, antibiotics are considered to be "pseudopersistant" contaminants due to their continued release into the environment and permanent presence (Daughton and Ternes, 1999; Hernando et al., 2006).

Although the major concern of antibiotics is associated with the development of resistance mechanisms by bacteria and its implications in human health, their sustained release to different environmental compartments and their bioactive properties also raise serious concerns about the toxicity of antibiotics to non-target organisms (Migliore et al., 1997'Halling-Sørensen et al., 1998, 2000; Baguer et al., 2000). Algae and cyanobacteria play a crucial role in aquatic ecosystems. They are primary producers which supply nutrients for the rest of the aquatic biota (Greenberg et al., 1992). Cyanobacteria, in addition, are prokaryotes and are thus considered as sensitive organisms to antibiotics (Maul et al., 2006). In fact, the European Medicines Evaluation Agency (EMEA) explicitly recommends the use of cyanobacteria for effect testing of antimicrobials due to their sensitivity (EMEA, 2006).

Different classes of antibiotics have been detected simultaneously in environmental compartments (Kolpin et al., 2004; Li et al., 2009; Watkinson et al., 2009; Suzuki and Hoa, 2012). Therefore, aquatic organisms may be exposed to mixtures of antibiotics. While the individual concentrations of antibiotics in aquatic environments may be low, the combined concentrations could result in significant toxicity to aquatic organisms. Besides, as chemicals in a mixture may either not interact or interact synergistically or antagonistically, it is essential to investigate the potential interactions in mixtures of antibiotics of different classes (Cleuvers, 2003; Teuschler, 2007; Rodea-Palomares et al., 2010). This issue has important implications in terms of environmental toxicity and risk assessment strategies, which are often carried out considering the individual effect and additive behavior. This may severely underestimate the risk associated with antibiotic mixtures as well as mixtures of antibiotics with other pharmaceuticals and anthropogenic contaminants (Kolpin et al., 2002).

The aim of the present study was to evaluate the individual and combined toxicity to the green alga *Pseudokirchneriella subcapitata* and the recombinant bioluminescent filamentous cyanobacterium *Anabaena* CPB4337 of five antibiotics from different classes: amoxicillin (β-lactam), erythromycin (macrolide), tetracycline (tetracycline) and the quinolones norfloxacin and levofloxacin. All of them have a variety of uses in human and veterinary medicine (Table S1). In order to identify and quantify the nature of the interactions between the antibiotics, binary and multicomponent mixtures of them were prepared and tested. Results were analyzed by the method of the Combination Index (CI)-isobologram equation which we have previously used to study pollutant interactions (Rodea-Palomares et al., 2010; Rosal et al., 2010a; Boltes et al., 2012; Rodea-Palomares et al., 2012). In this paper, we also report a first approach of risk assessment of the antibiotic mixtures for the aquatic environment based on currently available exposure data (Table S1) and our toxicity results.

2. Materials and methods

2.1. Chemicals

The antibiotics used in this study belong to different classes and were selected based on their use and occurrence in the aquatic environment (Table S1). The following five antibiotics were selected: amoxicillin (AMO), erythromycin (ERY) and levofloxacin (LEV), purchased from TCI Chemicals; tetracycline (TET) purchased from Sigma–Aldrich; and norfloxacin (NOR) purchased from Fluka. Amoxicillin is a β-lactam with a bactericidal action, it inhibits bacterial cell-wall synthesis. Erythromycin is a macrolide which exerts its antibiotic effects by binding irreversibly to the 50S subunit of bacterial ribosome interfering with bacterial protein synthesis. Tetracycline also inhibits bacterial protein synthesis by preventing the association of aminoacyl-tRNA with the bacterial ribosome. Norfloxacin and levofloxacin are quinolones, which inhibit bacterial DNA gyrase, preventing DNA replication.

The purity of each antibiotic was AMO ≥ 90%, ERY ≥ 98%, LEV ≥ 98%, TET ≥ 98%, NOR ≥ 98%. Except ERY, which was dissolved in methanol, stock solutions of antibiotics were made in high purity water obtained from a Milipore Mili-Q system with a resistivity of at least 18 MΩ cm at 25 °C. The antibiotics were stored in the dark and 4 °C. The dilutions of each antibiotic and their corresponding mixtures were freshly prepared before each experiment with a careful monitoring of solution pH. It was ensured that pH did not change significantly during the experiments. For the case of ERY, the final concentration of methanol in the assay media was always below 0.005% (v/v), which did not result in any

significant effect on the bioluminescence of *Anabaena* CPB4337 or the growth rate of *P. subcapitata*.

2.2. Stability of antibiotics

Initial concentrations and stability of antibiotics under bioassay conditions were examined according to the (OECD, 2008) guidance. Analyses have been performed in assay media both in the absence of cells and in the presence of either the cyanobacterium or the green alga in a course-time study up to 72-h exposure. The stability assessment was performed using HPLC-diode array liquid chromatography and TSQ Quantum LC–MS triple quadrupole as follows. HPLC analyses were used to determine the exposure concentration of TET, LEV and NOR and were performed using an HPLC Agilent Technologies equipped with a diode array detector. The column used was a reversed phase Kromasil 5u 100A C18 analytical column. The mobile phase was a mixture of acetonitrile and acidified water with H_3PO_4 (20:80), adjusted to pH 3 with NaOH 2 M. UV detection was as follows: LEV, 272 nm, NOR and TET, 292 nm. LC-MS QqQ was used to determine the exposure concentration of ERY using an Ascentis express C18 5 cm × 2.1 mm × 2.7 μm analytical column. The mobile phase was acetonitrile and water acidified with 0.1% formic acid (50:50).

No significant differences were found between the nominal and measured exposure concentrations for ERY, NOR and LEV either in the presence or absence of the green alga and the cyanobacterium. Therefore, throughout the present study, their nominal concentrations were used for data analyses. AMO was previously reported to be highly unstable in aqueous solutions due to hydrolysis involving the opening of the β-lactam ring (Perez-Parada et al., 2011). Immediately after being put in solution, within a period of minutes, AMO degraded completely and for that reason we used the nominal concentration. This behavior has already been observed by others (Pérez-Parada et al., 2011). The hydrolysis probably produced a set of intermediary products that are not characterized but might also be toxic to aquatic organisms. For TET, compared to the nominal value, and expressed as percentage, the average measured concentrations (in abiotic conditions) were 53.6, 28.6 and 14.3 after 24, 48 and 72 h, respectively. The exposure concentrations were calculated as follows:

$$D^{TET} = \int_0^{72\,h} c_{TET}\, dt \quad (1)$$

In what follow, D^{TET} was used instead of nominal concentrations, C_{TET}, according to the OECD guidance (OECD, 2008). The integral of Eq. (1) was solved using a numerical approximation fed with C_{TET} values recorded every 24 h.

2.3. Toxicity bioassays

The antibiotic concentrations tested ranged from 0.01 to 1500 mg/L for AMO; 0.01–100 mg/L for NOR; 0.01–200 mg/L for LEV; 0.01–100 mg/L for TET and 0.001–10 mg/L for ERY.

Anabaena sp. PCC 7120 strain CPB4337 (hereinafter *Anabaena* CPB4337), which bears in the chromosome a Tn5 derivative with *luxCDABE* from the luminescent terrestrial bacterium *Photorhabdus luminescens* (formerly *Xenorhabdus luminescens*), was used in this study as a bioreporter of antibiotic toxicity. This strain shows a high constitutive self-luminescence with no need to add exogenous aldehyde (Fernandez-Pinas and Wolk, 1994). The toxicity bioassays using *Anabaena* CPB4337 are based on the inhibition of constitutive luminescence caused by the presence of a toxic substance; this endpoint is related to metabolic activity of the organism as toxicants affecting the metabolism of the bacterium reduce luminescence. *Anabaena* CPB4337 was routinely grown at 28 °C in the light, ca. 65 μmol photons m^2 s^{-1} on a rotary shaker in 150 mL AA/8 (Allen and Arnon, 1955) (Table S2), supplemented with nitrate (5 mM) (hereinafter AA/8 + N) in 250 ml Erlenmeyer flask for 3 days. The strain was grown in liquid cultures with 10 μg/mL of neomycin sulfate (Nm). Toxicity assays were performed as follows: cyanobacterial cultures grown as described were centrifuged, washed twice and resuspended in fresh medium at $OD_{750nm} = 2.5$. 640 μL of the corresponding antibiotic solution were added to transparent sterile 24-well microtiter plates, followed by 160 μL of fivefold concentrated AA/8 + N and 200 μL of *Anabaena* CPB4337, prepared as described, were added to the wells to reach a final $OD_{750nm} = 0.5$. The 24-well microtiter plates were kept at room temperature (28 °C) and light ca. 65 μmol photons m^2 s^{-1} on a rotary shaker during 72 h of exposure. For luminescence measurements, 100 μL of each sample were transferred to an opaque white 96-well microtiter plate. Luminescence was recorded in a Centro LB 960 luminometer during 10 min. Three independent experiments with quadruplicate samples were carried out for all *Anabaena* toxicity assays. Copper sulfate ($CuSO_4$) was selected as reference toxicant for calibration in all assays. The calibration allows one to calculate the mean EC_{50} values (the median effective antibiotic concentration that causes a 50% effect with respect to a non-treated control) of copper in order to refuse or accept the experiment if those EC_{50} values fall in or out of the 95% of confidence limits previously fixed for this reference toxicant (USEPA, 1994, 2002). To achieve this, five copper dilutions were tested by quadruplicate in control wells of each assay.

Algal beads of *P. subcapitata* and growing media (Table S3) were purchased from MicroBioTest Inc. (Belgium). The determination of multigenerational exposure toxicity was performed following the algal growth inhibition test described in OECD TG 201 open system (OECD, 2006). The de-immobilization of algal cells was conducted according to the manufacturer's recommendations. Algal cells were first cultured in 25 mL shaken flasks in which growth was assessed by following optical density at 670 nm. The prescribed amount of cells (final cellular density of 0.1) was then transferred to 96-well clear disposable microplates and was exposed to pollutants during the logarithmic growth phase. The total volume occupied was 200 μL. *P. subcapitata* growth was monitored for 72 h, measuring chlorophyll fluorescence, which excitation/emission wavelengths are 450/672 nm, in a Fluoroskan Ascent EL (Thermo Scientific) microplate reader. Plates were incubated in a growing chamber at 22 ± 2 °C under continuous light. Each concentration was replicated four times in three independent series of assays.

2.4. Experimental design of antibiotic mixtures

Solutions of antibiotics were used singly and in binary and four- or five-antibiotic mixtures. Algal and cyanobacterial cells were treated with serial dilutions of each chemical individually and in combinations prepared with a fixed constant ratio (1:1) based on the individual EC_{50} values (mg/L). Five to seven dilutions (serial dilution factor = 2) of each antibiotic and combination plus one control were tested in three independent experiments with quadruplicate samples as described elsewhere (Rodea-Palomares et al., 2010).

2.5. Median-effect and Combination Index (CI)-isobologram equations for determining individual and combined toxicities

The response to toxic exposure in *Anabaena* CPB4337 and in *P. subcapitata* tests was estimated using the median-effect equation based on the mass action law (Chou and Talalay, 1984):

$$\frac{f_a}{f_u} = \left(\frac{D}{D_m}\right)^m \tag{2}$$

where D is the dose, D_m is the dose for 50% effect (EC_{50}), f_a is the fraction affected by dose D (e.g., 0.75 if cell bioluminescence/growth is inhibited by 75%), f_u is the unaffected fraction (therefore, $f_a = 1 - f_u$), and m is the coefficient of the sigmoidicity of the dose–effect curve: $m = 1$, $m > 1$, and $m < 1$ indicate hyperbolic, sigmoidal, and flat sigmoidal dose–effect curve, respectively. Therefore, the method takes into account both the potency (D_m) and shape (m) parameters. Eq. (2) may be rearranged as follows:

$$D = D_m\left(\frac{f_a}{1-f_a}\right)^{1/m} \tag{3}$$

The D_m and m values for each individual compound or mixture were determined by the median-effect plot: $x = \log(D)$ versus $y = \log(f_a/f_u)$ which is based on the logarithmic form of Eq. (2). In the median-effect plot, m is the slope and $D_m = 10^{-(y-\text{intercept})/m}$. The conformity of the data to the median-effect principle can be ready assessed by the linear correlation coefficient (r) of the fitting to Eq. (3) (Chou, 2006).

These parameters were then used to calculate doses of individual compound and their mixtures required to produce various effect levels according to Eq. (2). For each effect level, Combination Index (CI) values were then calculated according to the general Combination Index equation for n-chemical combination at x% inhibition (Chou, 2006):

$$(CI)_x = \sum_{j=1}^{n}\frac{(D)_j}{(D_x)_j} = \sum_{j=1}^{n}\frac{(D_x)_{1-n}\left\{\dfrac{[D]_j}{\sum_1^n [D]}\right\}}{(D_m)_j\left\{\dfrac{(f_{ax})_j}{\left[1-(f_{ax})_j\right]}\right\}^{1/mj}} \tag{4}$$

where $^n(CI)_x$ is the Combination Index for n chemicals at x% inhibition; $(D_x)_{1-n}$ is the sum of the dose of n chemicals that exerts x% inhibition in combination, $\{[D_j]/\sum_1^n [D]\}$ is the proportionality of the dose of each of n chemicals that exerts x % inhibition in combination; and $(D_m)_j\{(f_{ax})_j/[1-(f_{ax})_j]\}^{1/mj}$ is the dose of each drug alone that exerts x% inhibition. From Eq. (4), $CI < 1$, $CI = 1$ and $CI > 1$ indicates synergism, additive effect and antagonism, respectively.

2.6. Analysis of results

A non-linear fitting was performed to derive individual and mixture dose–effect parameters using the Levenberg–Marquardt algorithm to Eq. (2). To compute EC_{10} (the median effective antibiotic concentration that causes a 10% effect with respect to a non-treated control) and EC_{20} (the median effective antibiotic concentration that causes a 20% effect with respect to a non-treated control) in *P. subcapitata* assays for which the inhibition did not reach the median effect value (D_m in Eq. (2)), a reparametrization was deemed necessary to reduce multicollinearity. For it, we substituted D_m in Eqs. (2) and (3) by EC_{20} or EC_{10} in the following modified forms of Eq. (2):

$$\frac{f_a}{f_u} = \frac{1}{9}\left(\frac{D}{EC_{10}}\right)^m \tag{5}$$

$$\frac{f_a}{f_u} = \frac{1}{4}\left(\frac{D}{EC_{20}}\right)^m \tag{6}$$

This procedure allowed reaching efficient non-linear regression with adequate standard deviations for the estimators. The calculation of EC_{50} (the median effective antibiotic concentration that causes a 50% effect with respect to a non-treated control) for *P. subcapitata* exposed to TET, the data for which lie outside the range of validity of the median effect equation (Eq. (2)), was performed using a linear interpolation method that did not assume any particular dose–effect model (USEPA, 2002). Not special transformations or reparametrization of Eq. (2) were needed for estimation of dose–effect relationship parameters for *Anabaena* CPB4337 since whole dose–effect curves were obtained for the tested antibiotics and their mixtures. The computer program CompuSyn (Chou, 2005) was used for calculation of the EC_x (the median effective antibiotic concentrations that cause x% effect with respect to a non-treated control) and Combination Index (CI) values of the different mixtures in the whole range of effect levels.

2.7. Mixture toxicity predictions based on CA, IA and CI equations

Experimental toxicity of the antibiotic mixtures were computed based on the predictive equations of the two most widely used definitions of additivity, that is, Concentration Addition (CA) (Eq. (7)) and Independent Action (IA) (Eq. (8)) (Faust et al., 2001; Altenburger et al., 2004). CA is based on the assumption that mixture components have the same sites and similar mode of action (MOA), and is computed by equation (Altenburger et al., 2004):

$$ECx_{mix} = \left(\sum_i^n \frac{p_i}{EC_{xi}}\right)^{-1} \tag{7}$$

where ECx_{mix} is the effect concentration of the mixture provoking x% effect, EC_{xi} is the concentration of the component i

provoking the same effect (x%) as the mixture when applied individually, and p_i is the molar ratio of the ith component in the mixture.

IA is based on the assumption that mixture components have dissimilar MOA. The following equation applies for IA (Altenburger et al., 2004).

$$E(c_{mix}) = 1 - \prod_{i=1}^{n}(1 - E(c_i)) \quad (8)$$

where c_{mix} and $E(c_{mix})$ are the total concentration and total effect of the mixture, respectively, and $E(c_i)$ is the effect of the ith component with the concentration c_i in the mixture. The c_i in Eq. (8) can be replaced by ($p \times c_{mix}$). The $E(c_i)$ can be calculated from the function that described the concentration–response curve of the ith component (Altenburger et al., 2004; Qin et al., 2010).

The predictive equation based on CI (that is considering deviations from additivity as CI values) was computed as follows:

$$ECx_{mix} = \left(\sum_{i}^{n} \frac{p_i}{EC_{xi} \times CI_{x\ comp}}\right)^{-1} \quad (9)$$

where ECx_{mix} is the effect concentration of the mixture provoking x% effect, EC_{xi} is the concentration of the component i provoking the same effect (x%) as the mixture when applied individually, p_i is the molar ratio of the ith component in the mixture and CI_x comp is the computed Combination Index value for the mixture at the x level of effect (x% or f_a) from the experimental toxicity curve of the mixture. Note that Eq. (9) is easily derived from Eq. (7) since CI and CA methods share the same definition of additivity (Loewe additivity) (Chou, 2006).

2.8. Risk quotients assessment of antibiotic mixtures

Risk quotients (RQs) try to estimate the actual potential ecological risk (probability of an expected effect, i.e. potential danger, caused by an environmental concentration) of a pollutant. This quotient is calculated as the ratio between Predicted Environmental Concentrations (PEC) or Measured Environmental Concentrations (MECs) and Predicted No Effect Concentrations (PNECs) (Sanderson et al., 2003; Von der Ohe et al., 2011).

The lowest predicted no effect concentrations (PNECs) for single compounds and mixtures were combined with the highest available measured environmental concentrations (MECs) from the literature (Table S1). Risk quotients (RQs) were then estimated as follows (Sanderson et al., 2003; Von der Ohe et al., 2011):

$$RQ = \frac{MEC}{PNEC} \quad (10)$$

A PNEC value for a given antibiotic mixture was derived dividing the EC_{50} values by an assessment factor of 1000 (Sanderson et al., 2003; EMEA, 2006; Von der Ohe et al., 2011). Highest MEC values (Table S1) converted to μmol/L were summed up to give a corresponding mixture concentration. RQ higher than one suggests that an ecological impact is expected for the given antibiotic mixture.

3. Results and discussion

3.1. Toxicity of individual antibiotics

Table 1 shows EC_{10}, EC_{20} and EC_{50} values of the five antibiotics tested individually and in binary and complex mixtures. After an exposure of 72 h to AMO, the green alga *P. subcapitata* showed no effect at concentrations up to 1500 mg/L (less than 10% growth inhibition), indicating that this antibiotic was not toxic to the green alga. For ERY, a clear concentration–response relationship was obtained and demonstrated to be highly toxic with an $EC_{50} = 0.35 \pm 0.03$ mg/L and a very low EC_{10}, in the range of μg/L. In the case of the quinolones, NOR and LEV displayed flat and incomplete concentration–response curves (Fig. 1). This represents a plateau with respect to cell growth rate, the reduction of which did not progressed for drug dosage above 40 mg/L approximately. The reason for this behavior is more probably due to the role played by antibiotic transporters which regulate the influx, efflux and intracellular concentration of antibiotics (Van Bambeke et al., 2003b). In both cases, the fitting to the dose–effect equations was performed only for the lower effect levels. The data shown in Table 1 correspond to the fitting of the lower exposures and were calculated using Eqs. (5) and (6). Based on EC_{10} and EC_{20} values, LEV was more toxic than NOR to the green alga; however, EC_{50} was >80 mg/L for NOR and >120 mg/L for LEV. TET proved very toxic for the green alga with a exposure concentration (D^{TET} as indicated in Eq. (1)) as low as 32 ± 8 μg/L producing a measurable effect (EC_{10}); EC_{50} for TET, calculated as indicated before, was 3.31 ± 0.96 mg/L.

With regards to the cyanobacterium, complete concentration–response relationships could be recorded for the five tested antibiotics after an exposure of 72 h (Fig. 2). The EC_{50} values ranged between 0.022 mg/L and 56.3 mg/L with an order of toxicity as follows: ERY > LEV > NOR > TET > AMO. It should be noticed that, like in the case of the green alga, ERY showed a measurable effect at a concentration as low as 5 μg/L (EC_{10}). The results indicated that the cyanobacterium, most probably due to its prokaryotic nature, is in general, more sensitive than the green alga to the tested antibiotics. Several other researchers found similar results with unicellular cyanobacteria and green algae (Harrass et al., 1985; Lutzhoft et al., 1999; Halling-Sørensen et al., 2000). Although the green alga is a non-target organism for antimicrobials, the observed toxicity exerted by some antibiotics could be, at least in part, due to the cyanobacterial nature of the chloroplasts which makes these plastids susceptible as potential antibiotic targets (McFadden and Roos, 1999); the antiplastidic effects of several antibiotics has been quantified at concentrations well above 1 mg/L (Ebringer, 1972; Nicolas, 1981). Furthermore, an effect on mitochondria might also account for part of the observed toxic effect in the green alga.

From the tested antibiotics, ERY for both the alga and the cyanobacterium, could be classified as "very toxic to the aquatic life" (Regulation EC No. 1272/2008 on classification, labelling and packaging of substances and mixtures, Category I, $EC_{50} < 1$ mg/L). In fact, this antibiotic shows measurable effects in both organisms at concentrations in the μg/L, close to measured environmental concentrations (see Table S1).

Table 1 – Dose–effect parameters and main Combination Index (CI) values of: amoxicillin (AMO), erythromycin (ERY), levofloxacin (LEV), norfloxacin (NOR) and tetracycline (TET); individually and of their binary and multi-component combinations for *Pseudokirckneriella subcapitata* and *Anabaena* sp. CPB4337 tests.

	Pseudokirckneriella subcapitata						*Anabaena* sp. CPB4337					
	Dose–effect parameters (mg/L)			CI values			Dose–effect parameters (mg/L)			CI values		
	EC_{10}	EC_{20}	EC_{50}	$f_a = 0.10$	$f_a = 0.20$	$f_a = 0.50$	EC_{10}	EC_{20}	EC_{50}	$f_a = 0.10$	$f_a = 0.20$	$f_a = 0.50$
AMO	>1500	>1500	>1500	–	–	–	6.16 ± 3.5	15.1 ± 3.2	56.3 ± 2.5	–	–	–
ERY	0.036 ± 0.016	0.082 ± 0.023	0.35 ± 0.03	–	–	–	0.005 ± 0.004	0.009 ± 0.003	0.022 ± 0.003	–	–	–
LEV	0.93 ± 0.30	4.5 ± 0.6	>120[c]	–	–	–	1.1 ± 0.4	1.9 ± 0.4	4.8 ± 0.4	–	–	–
NOR	10.9 ± 1.2	20.6 ± 1.0	>80[d]	–	–	–	1.2 ± 0.5	2.1 ± 0.6	5.6 ± 0.5	–	–	–
TET	0.032 ± 0.008	0.10 ± 0.01	3.31 ± 0.96	–	–	–	2.5 ± 0.7	3.5 ± 0.7	6.2 ± 0.8	–	–	–
ERY + LEV	1.3 ± 0.3	23 ± 0.3	> 20.4[e]	2.5	1.2	0.42	0.87 ± 0.3	1.3 ± 0.3	2.5 ± 0.2	1.4	1.2	0.95
ERY + NOR	3.0 ± 0.6	5.8 ± 0.7	18.2 ± 1.5	0.82	0.70	0.54	0.96 ± 0.5	1.7 ± 0.6	4.5 ± 0.7	1.1	1.2	1.2
ERY + TET	0.0046 ± 0.0024	0.021 ± 0.007	0.27 ± 0.04	0.33	0.40	0.60	0.28 ± 0.11	0.67 ± 0.13	3 ± 0.3	0.23	0.36	0.79
LEV + NOR	1.1 ± 0.4	2.9 ± 0.7	15.0 ± 1.3	0.28	0.24	0.25	1.3 ± 0.5	2.0 ± 0.5	4.3 ± 0.4	1.1	0.98	0.8
LEV + TET	0.34 ± 0.10	0.89 ± 0.14	4.6 ± 0.5	0.84	0.52	0.24	0.37 ± 0.12	0.63 ± 0.24	1.6 ± 0.3	0.21	0.24	0.28
NOR + TET	1.9 ± 0.5	3.5 ± 0.3	9.2 ± 0.9	1.2	0.59	0.22	1.9 ± 0.4	2.7 ± 0.4	5.1 ± 0.5	1.2	1	0.87
4 AB[a]	1.7 ± 0.4	3.9 ± 0.5	16.0 ± 2.0	1.1	0.69	0.37	–	–		–	–	–
AMO + ERY	–	–	–	–	–	–	9.4 ± 5.2	16.2 ± 5.5	41 ± 3.5	2	1.4	1
AMO + NOR	–	–	–	–	–	–	8.9 ± 4.0	14.5 ± 4.1	33.1 ± 3.3	2	1.5	1
AMO + LEV	–	–	–	–	–	–	7.7 ± 2.3	11.4 ± 2.1	22.4 ± 1.5	1.5	1	0.5
AMO + TET	–	–	–	–	–	–	1.8 ± 0.6	2.9 ± 0.5	6.5 ± 0.5	0.33	0.25	0.2
5 AB[b]	–	–	–	–	–	–	5.2 ± 1.2	7.2 ± 0.8	12.4 ± 0.8	1.5	1.1	0.7

a ERY + LEV + NOR + TET.
b ERY + LEV + NOR + TET + AMO.
c $f_a = 0.42$ at 120 mg/L.
d $f_a = 0.39$ at 80 mg/L.
e $f_a = 0.488$ at 20.4 mg/L (20.0 mg LEV/L, 0.4 mg ERY/L).

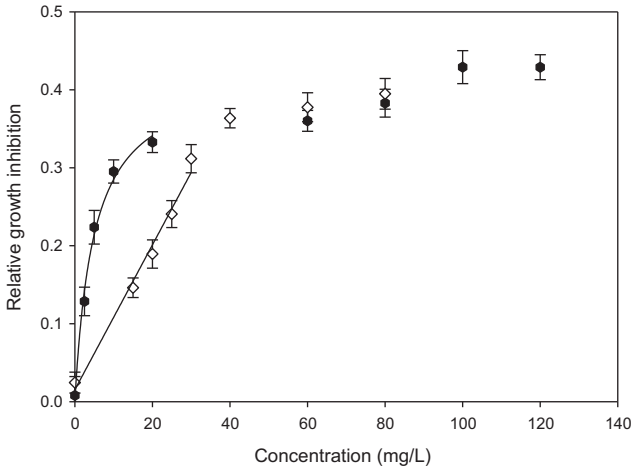

Fig. 1 – Dose–response curves of levofloxacin (◆) and norfloxacin (●) for *Pseudokirchneriella subcapitata*. Solid lines represent the fitting to the median effect equation. Error bars correspond to 95% confidence intervals.

Most authors have found that β-lactams antibiotics such as AMO do not affect green algae (Lutzhoft et al., 1999; Halling-Sørensen et al., 2000; Eguchi et al., 2004), most probably due to the mode of action of this antibiotic which specifically inhibits bacterial cell wall synthesis. Studies on ERY toxicity to the aquatic environments report EC_{50} values ranging between 0.02 mg/L for green algae (Eguchi et al., 2004; Isidori et al., 2005; similar to the one found for the cyanobacterium in this report), and 1000 mg/L for the fish *Danio rerio* with EC_{50} values around 20 mg/L for rotifers, crustacean and invertebrates (Isidori et al., 2004). For LEV, an EC_{50} (after 7 days of exposure) of 7.9 mg/L was reported for the unicellular cyanobacterium *Microcystis aeruginosa* (Robinson et al., 2005), which is slightly higher than the one found for *Anabaena* in this study. A similar value was also reported for the green alga *P. subcapitata* (Robinson et al., 2005). In this report, a much higher EC_{50} was obtained for LEV in the same alga (>80 mg/L). There are not

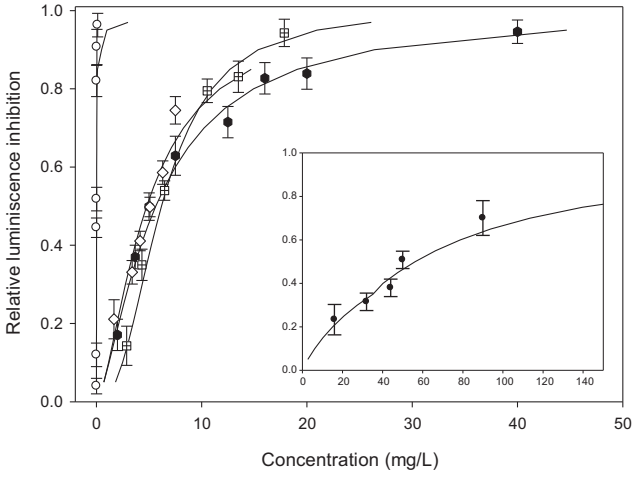

Fig. 2 – Dose–response curves of amoxicillin (●; inset), norfloxacin (●), levofloxacin (◆), tetracycline (⊞) and erythromycin (○) for *Anabaena* CPB4337. Solid lines represent the fitting to the median effect equation.

many published data on NOR toxicity; an EC_{50} value of 22 μg/L has been reported for the marine bacterium *Vibrio fischeri* (Backhaus et al., 2000) and EC_{50} values of 16.6 mg/L and 10.4 have been reported for the green algae *P. subcapitata* and *Chlorella vulgaris*, respectively (Eguchi et al., 2004). Again, these are significantly lower than those reported here. For TET, EC_{50} values of 0.09 mg/L and 2.2 mg/L have been found for the unicellular cyanobacterium *M. aeruginosa* and the green alga *P. subcapitata*, respectively (Halling-Sørensen et al., 2000), which, for the cyanobacterium, is lower than the one reported in this study.

From the results in previous reports (Halling-Sørensen et al., 2000; Robinson et al., 2005) except for LEV, the unicellular cyanobacterium *M. aeruginosa* appears to be more sensitive to antibiotics than the filamentous *Anabaena* CPB4337, although due to the scarcity of data, more research is needed. However, it should be noted that both the exposure time (7 days for the unicellular vs. 3 days for *Anabaena*) and the toxicity endpoint (growth vs. bioluminescence) were different. In the case of the green alga, the observed discrepancies between our results and those published could be due to the different endpoints used to measure growth which might influence the outcome; we used chlorophyll fluorescence while Eguchi et al. (2004) and Isidori et al. (2005) used cell counts; besides, in the case of the quinolones it could also be due to the flat and incomplete dose–response curves obtained for both quinolones in this work; nevertheless, quite discrepant results have also been reported for LEV toxicity to *Lemna* species with EC_{50} ranging from 0.8 mg/L to 51 mg/L after 7 days of exposure measuring different endpoints such as frond number, growth rate or pigment content (Brain et al., 2004; Robinson et al., 2005).

3.2. Toxicological interactions of the tested antibiotics in mixtures

Table 1 shows the effective concentrations EC_{10}, EC_{20} and EC_{50} as well as predicted CI values at these effect levels of binary, four-antibiotic (for the green alga, as AMO was not toxic, it was not included in the mixture assays) and five-antibiotic (for the cyanobacterium) mixtures. As shown in the table, the binary mixture ERY-TET displayed a considerable toxic effect, particularly for the green alga and at environmentally relevant concentrations (Table S1). In fact, in binary as well as in multi-antibiotic mixtures, ERY and to a lesser extent TET, were assayed at near environmental concentrations (see Table S1, also Table S4 for information of individual concentrations of antibiotics in the mixture effective concentrations). The data show that both antibiotics, when present in mixtures, may exhibit a toxic effect even at very low concentrations.

The Combination Index (CI) quantifies the nature of the interaction between antibiotics at any effect level. Fig. 3 shows the f_a-CI plots of binary (Fig. 3a) and multi-antibiotic mixtures (Fig. 3b). The f_a-CI plot depicts the CI value versus f_a (the effect level or fraction of luminescence/growth inhibited with respect to the control). Due to the fact that complete concentration–response relationship could not be recorded for the green alga for some antibiotics, the maximum effect level (f_a) shown in the figures of the green alga is 0.5. Average Combination Index (CI) values for the three representative EC values (EC_{10}, EC_{20} and EC_{50}) are shown in Table 1.

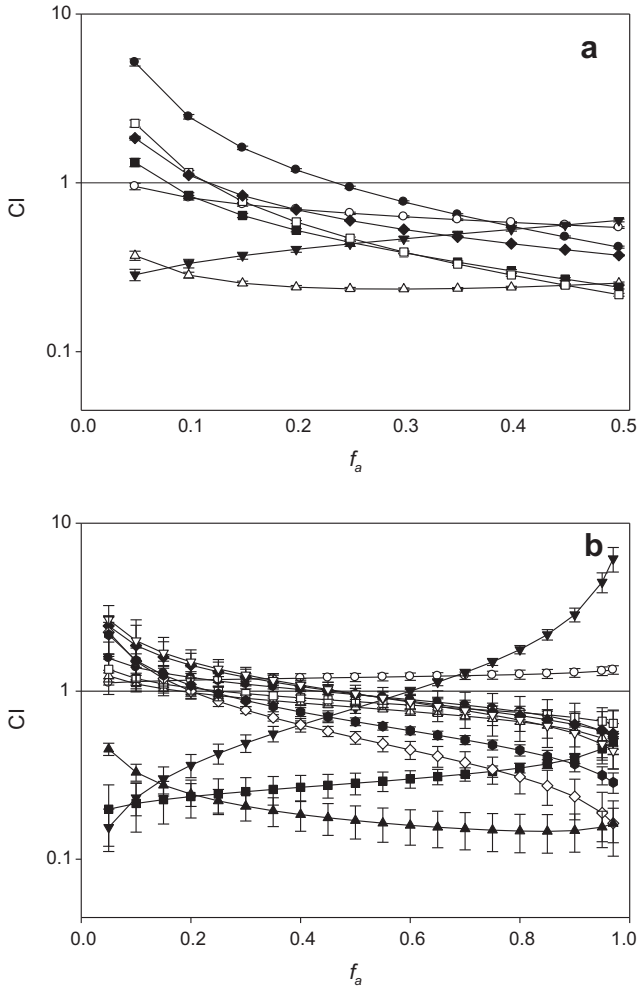

Fig. 3 – Combination index Plot (f_a- *CI plot*) for binary and multi-antibiotic mixtures for *Pseudokirchneriella subcapitata* test (a) and *Anabaena* CPB4337 test (b): ERY+LEV (—●—), ERY+NOR (—○—), ERY+TET (—▼—), LEV+NOR (—△—), LEV+TET (—■—), NOR+TET (—□—), AMO+ERY (—◇—), AMO+LEV (—●—), AMO+TET (—▽—), AMO+NOR (—▲—), ERY+LEV+NOR+TET (—◆—), ERY+LEV+NOR+TET+AMO (—◆—). CI values are plotted as a function of the fractional inhibition of bioluminescence/growth (f_a) by computer simulation (CompuSyn). CI < 1, = 1 and > 1 indicates synergism, additive effect and antagonism, respectively. At least three independent experiments with two replicates were used. The vertical bars indicate 95% confidence intervals for CI values based on SDA (Sequential Deletion Analysis) (Chou and Martin, 2005). AMO = Amoxicillin; TET = Tetracycline; ERY = Erythromycin; LEV = Levofloxacin; NOR = Norfloxacin.

For the green alga (Fig. 3a), the ERY-LEV combination showed a clear antagonism at low f_a levels, becoming synergistic at f_a values above 0.3. The ERY-NOR combination was nearly additive in the whole range of effect levels. The ERY-TET mixture showed a clearly synergistic interaction, particularly at very low f_a values, becoming slightly less synergistic at higher values. The LEV-TET combination was nearly additive at very low effect levels becoming clearly synergistic at f_a values above 0.1. The LEV-NOR mixture was clearly synergistic in the whole range of f_a levels while the NOR-TET combination showed antagonism at very low to low f_a values which changed into synergism at higher effect levels. The four-antibiotic mixture was nearly additive at very low effect levels and clearly synergistic at effect levels above 0.1. The global picture of antibiotic interactions in the green alga is that synergism clearly predominated.

For the cyanobacterium (Fig. 3b), a somewhat more heterogeneous pattern of interactions emerged. The ERY-LEV combination showed antagonism at very low to low effect levels which turned into a near additive behavior at f_a levels above 0.4. The ERY-NOR combination was nearly additive in the whole range of effect levels. The ERY-TET combination was clearly synergistic at very low effect levels which turned into a strong antagonism above a f_a value of 0.6. The NOR-TET combination showed a pattern similar to the one of ERY-LEV combination with slight antagonism at very low to low effect levels, approaching additivity at medium effect levels and slightly synergistic at the higher effect levels. The LEV-NOR combination showed a tendency of interactions similar to that of NOR-TET although at very low to low effect levels it approached the additivity line. The LEV-TET combination showed a strong synergistic interaction in the whole range of f_a levels. The AMO-ERY and AMO-NOR combinations showed a similar pattern of interactions with strong antagonism at f_a values below 0.6 which became slightly synergistic at higher effect levels; the AMO-LEV combination also showed strong antagonism at low effect levels but became slightly synergistic at f_a levels above 0.2. The AMO-TET combination was strongly synergistic in the whole range of f_a values. The five-antibiotic mixture followed a pattern of interactions quite similar to the one found for the four-antibiotic mixture in the green alga with antagonism at low effect levels which turned into synergism at f_a values above 0.25. The global pattern of interactions that emerged in the cyanobacterium showed that at very low effect levels, antagonism predominated in most binary mixtures with the exception of three mixtures with tetracycline: TET-AMO, TET-ERY and TET-LEV, which were synergistic. At higher effect levels the tendency was similar to that observed in the green alga and synergism became the predominant interaction. It should be noticed that in both cell systems, the ERY-TET combination showed strong synergism at very low to low effect levels, emphasizing again that this could be a quite toxic combination in the environment, even when both antibiotics are present at very low concentrations as already discussed. For the cyanobacterium, two other combinations could be potentially dangerous for their synergistic interactions: the AMO-TET and LEV-TET. For the green alga, the LEV-NOR combination could also be dangerous due to the synergistic interactions found at low effect levels. For both organisms, the multi-antibiotic mixtures were also synergistic at relatively low effect levels (above $f_a = 0.10$ for the green alga and $f_a = 0.25$ for the cyanobacterium), indicating increased toxicity towards both aquatic organisms when all the tested antibiotics co-occur.

An important feature of the observed antibiotic interactions in both organisms is that the nature of the interaction change with the effect level and with the test organism. We have

already found this behavior with other pollutants interactions (Rodea-Palomares et al., 2010, 2012; Rosal et al., 2010a). A possible explanation of this phenomenon might be that at low effect levels, cells are less affected by the pollutants and antagonism/synergism may be explained by specific modes of action; thus, antagonism could be due to competition for uptake/same binding sites or suppression of the toxic effect of one drug by another while synergism could be explained by mechanisms of drug combinations such as "facilitating actions" meaning that secondary actions of one drug enhances the activity or level of another drug in the mixture or alternatively "complementary actions" when drugs act at the same target at different sites, at overlapping sites or at different targets of the same pathway (Jia et al., 2009); at high effect levels, cells may be heavily affected and toxicity may be increased by an unspecific way of action not probably related to the pharmacological mechanism; at high effect levels, cells membranes may be damaged allowing the bulk entry of toxicants; under these situations, synergism, considered as the combined action of pollutants which increase toxicity by unspecific mode of action, may predominate as we have previously found for the combined action of heavy metals (Rodea-Palomares et al., 2009) or fibrates (Rodea-Palomares et al., 2010).

The CI method allows quantitative determination of synergism or antagonism but does not give information on the mechanism by which the interactions occur (Chou, 2006). However, it should be taken into account that toxicological interactions may be influenced not only by the toxic mode of action but also by pharmacokinetic properties and biological sensitivity (already discussed under toxicity of individual antibiotics). Research on pharmacokinetic properties of antibiotics (absorption, accumulation, distribution and elimination) have mostly focused in invertebrates and fish (Samuelsen, 2006; Li et al., 2009) with no similar studies been conducted with algae or cyanobacteria; antibiotic efflux pumps found in prokaryotic and eukaryotic cells play a fundamental role in the modulation of absorption, cellular accumulation and elimination of antibiotics (Van Bambeke et al., 2003a,b); drug efflux pump genes are present in the genomes of sequenced cyanobacteria (http://genome.kazusa.or.jp/cyanobase/); similarly, multiple antibiotic resistance genes have been found in chloroplasts (Conte et al., 2009); further research is needed to understand the role of these systems in the pharmacokinetics of antibiotics in both algae and cyanobacteria.

The individual mechanisms of action of the five tested antibiotics towards their target organisms, prokaryotes, are well known and may be useful to try to explain, at least, some of the observed interactions in the cyanobacterium. However, in the green alga, a eukaryotic organism, the mechanisms of toxicity are probably unrelated to the known mechanism of action in prokaryotes, although, as already mentioned, direct effects on chloroplasts and mitochondria cannot be discarded.

For the cyanobacterium, the nearly additive behavior of the LEV-NOR combinations at f_a levels below 0.5 (Fig. 3b) could perhaps be explained by their identical specific mechanism of action: inhibition of bacterial DNA gyrase. Similarly, Backhaus et al. (2000) found that the mixture toxicity of 10 quinolones to *V. fischeri* followed the concept of Concentration Addition. Christensen et al. (2006) also reported that the combination of two quinolones, flumequine and oxolinic acid, behaved additively in activated sludge microorganisms. In the green alga, the LEV-NOR combination was strongly synergistic in the whole range of effect levels. Based on these antibiotics' mechanisms of action, the interaction should have been additive as in the case of *Vibrio*, sludge microorganisms and *Anabaena*. Interestingly, Yang et al. (2008) also found synergism as the interaction of a norfloxacin–ciprofloxacin binary mixture at an effect level of 0.5 in *P. subcapitata*. The authors indicate that, as the green alga is not a target organism, it is very difficult to explain the interaction. Whatever the mechanism, their results and ours indicate that quinolones appear to interact synergistically in this green alga and this could be environmentally relevant.

The observed strong synergistic interaction between ERY and TET (at f_a levels below 0.6) (Fig. 3b) in the cyanobacterium could be explained by the complementary action of both antibiotics in prokaryotic protein synthesis as indicated by Jia et al. (2009) in their extensive review of mechanisms of drug interactions. Synergism was also found for this antibiotic combination in the green alga (Fig. 3a). Perhaps an effect on the chloroplast and mitochondrial ribosomes may explain the observed interaction. Christensen et al. (2006) also found synergistic effects of a similar mixture, oxytetracycline and erythromycin, in this green alga. The fact that, as reported before, both antibiotics when applied individually, both in the cyanobacterium and the green alga, provoked measurable toxicity at concentrations close to measured environmental concentrations indicates that the observed synergism might represent an ecological risk when the two antibiotics co-occur in aquatic environments.

The patterns of interactions found in the rest of binary mixtures cannot be easily explained in terms of the known mechanisms of antibiotic action. The multi-antibiotic mixtures interactions cannot be explained in terms of mechanisms either. Besides, there are not many reports in the literature which have studied the nature of interactions of this kind of complex mixtures. Yang et al. (2008) studied the combined effects of 12 antibacterial agents on *P. subcapitata* finding that antagonistic effects predominated at certain antibiotic concentrations. Here, we report antagonism of the multi-component mixtures at very low to low effect levels, with synergism clearly predominating at higher effect levels (Fig. 3a). As discussed earlier, this may have important implications in the aquatic environment.

Regarding multi-component mixtures like the four- and five-antibiotic mixtures of this work, an interesting feature of the CI method is that knowledge on the component–component type of interaction is not required to assess the overall interaction resulting of a complex mixture (Chou, 2006), however more complex studies can be performed based on the CI method in order to identify the component–component interaction contribution to the overall resulting interaction; for example, when analyzing a ternary mixture (A + B + C), it can be considered as a binary mixture composed by (A + B) + C and similarly with other combinations (Chou, 2006). This problem could also be assessed by regression analysis where the CI values of the multi-component mixture of interest are regressed against the CI values of the different binary mixtures (Rodea-Palomares et al., 2010, 2012).

3.3. *Experimental and predicted toxicity of the antibiotic mixtures under CA, IA and CI methods*

In order to validate the antibiotic interactions predicted by the Combination Index (CI) in the different antibiotic mixtures, we have generated predicted dose–response curves of the antibiotic mixtures based on the classical models used in toxicology to predict the expected toxicity of mixtures under additivity: Concentration Addition (CA) and Independent Action (IA). We have also generated predicted dose–response curves of the different mixtures based on the CI values obtained at the different f_a levels of the mixtures. Fig. 4 shows predicted dose response curves under CA, IA and CI models together with experimental values for four representative antibiotic mixtures of both the cyanobacterium (Fig. 4a–d) and the green alga (Fig. 4e–f). Experimental and predicted toxicity values under CA, IA and CI models for all tested mixtures can be found in supplementary Information (Table

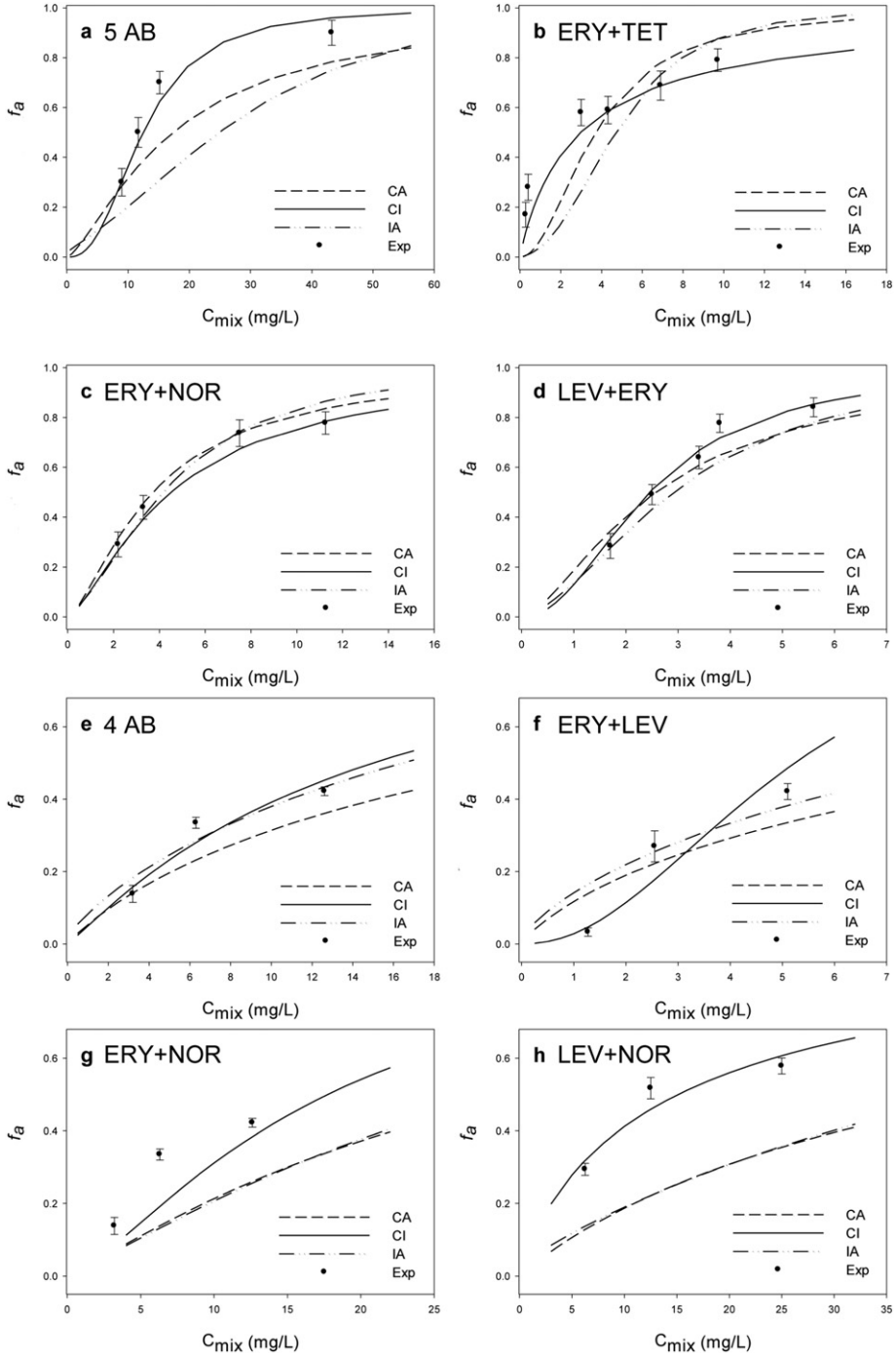

Fig. 4 – Experimental toxicity values (Exp) and predicted dose–response curves of representative antibiotic mixtures based on concentration addition (CA), Independent Action (IA) and Combination Index (CI) models for *Anabaena* CPB4337 test (a, b, c and d) and *Pseudokirchneriella subcapitata* test (d, e, f and g). 5 AB = ERY + LEV + NOR + TET + AMO, 4 AB = ERY + LEV + NOR + TET. Exp = mean ± SD.

S5). As can be seen in the figure, in general the prediction of CI method seems to fit more accurately the real experimental toxicity values for both organisms in those effect levels (f_a) of mixtures where CI method predicted departures from additivity (Fig. 3); for example, analyzing the five AB mixture in the cyanobacterium (Fig. 4a), at effect levels above 0.5, and the ERY + TET mixture (Fig. 4b) at low effect levels ($f_a < 0.5$), it can be seen that CA and IA did not predict the observed experimental deviation from additivity (towards synergism) which was accurately predicted by CI. A similar pattern can be observed in the ERY + NOR and LEV + NOR combinations for the green algae (Fig. 4g and h, respectively). When analyzing the predicted toxicity under CA, IA and CI at mixture effect levels where CI method predicted additivity (CI values near to 1; Fig. 3), the three methods CA, IA and CI offered a close and very similar predictions of the expected toxicity of the mixtures, as can be seen in the ERY + NOR and ERY + LEV combinations in the cyanobacterium (Fig. 4c,d, respectively), and at the lowest effect levels in the four AB and ERY + NOR mixtures in the green algae (Fig. 4e, g, respectively).

When comparing the experimental and predicted toxicities of all the tested mixtures based on these three models (CA, IA and CI) (Supplementary Information Table S5), it can be seen that, in general, the predicted toxicity of the mixtures according to CI is closer than that of CA and IA to that of experimental toxicity values in those mixtures where CI method predicted departures from additivity, specially in synergistic interactions (Fig. 3). However, it has been detected that in some of the mixtures of the cyanobacterium the three methods have underestimated the actual synergism of the mixtures: LEV + NOR and NOR + TET combinations are even more synergistic than that expected based on CI calculations, and the mixture NOR + TET, classified as near additive by the CI method was in fact synergistic as experimental toxicity values are above those predicted by the CI model. Therefore the CI method predicted with a very low error rate (only 1, the NOR-TET combination in the cyanobacterium, of 18 mixtures was wrongly evaluated as near additive when in fact was synergistic, see Supplementary Information Table S5) deviations from additivity of mixtures with different degrees of complexity. Furthermore, by taking in to account the estimated CI values of the different antibiotic mixtures, the CI method improved the predictive power of the classical additivity models CA and IA. The possibility of using CI values to get accurate predictions of toxicity in synergistic mixtures is a promising tool for assessing the real toxicity of mixtures of chemicals. In fact, Cedergreen et al. (2008) performed an extensive statistical analysis of the accuracy of both CA and IA models in predicting the actual toxicity of binary mixtures of chemicals (from pesticides to pharmaceuticals) with 185 independent data sets including 98 different mixtures; they found that approximately half of the experimental toxicity could not be correctly described with either of the two models. They also concluded that when quantifying the maximal synergism and antagonism, none of them proved to be significantly better than the other. Therefore, we can conclude that currently there is a lack of a basic methodology to accurately predict the actual toxicity of about half of the possible mixture situations, since CA and IA models by definition ignore synergism.

3.4. Risk quotients assessment of antibiotic mixtures

The RQs for antibiotic mixtures were derived from dividing the MEC by the PNEC. The MEC used in this work were maximum actual environmental measured concentrations reported in surface waters and wastewater effluents (Table S1). The procedure aims to assess risk considering unfavorable situations (higher environmental concentrations). The PNEC were obtained by dividing the EC_{50} of the mixtures by a safety factor of 1000 (Von der Ohe et al., 2011). Table 2 shows the RQs of binary, four and five-antibiotic mixtures for both *Anabaena* CPB4337 and *P. subcapitata*. The RQs for individual antibiotics are also provided. As shown in the table, the TET + ERY combination was above the threshold value of 1 both in the green alga and the cyanobacterium for wastewater effluents. This mixture posed an ecological risk for both organisms considering today's pattern of antibiotic use, which is more pronounced for the green alga. As already mentioned, this mixture showed strong synergism at low effect levels in both organisms, emphasizing the danger associated with it. In fact, RQs for individual antibiotics (Table 2) also indicated a potentially high ecological risk of ERY for both organisms and of TET towards the green alga. For the cyanobacterium, the NOR-ERY and TET-LEV combinations in wastewater effluents presented values closer to 1, indicating a narrow safety margin for these mixtures. It should be noted that the multi-antibiotic mixtures RQs were below 1 in both organisms, indicating that, at actual environmental levels, these mixtures do not pose an ecological risk. As expected RQ values in effluent wastewater were significantly higher than those found in surface waters.

There are not many studies in the literature that report calculations of RQs for antibiotics/pharmaceuticals mixtures. Brain et al. (2004) evaluated the effect of eight pharmaceuticals including three antibiotics and found significant ecological risk for two aquatic macrophytes. In a subsequent study, Brain et al. (2005) reported that the mixture of four tetracyclines did pose a risk for the same macrophytes. For individual antibiotics, sulfamethoxazole, sulfathiazole, chlortetracycline, oxytetracycline and AMO were found to pose an ecological risk to aquatic ecosystems (Park and Choi, 2008). Gros et al. (2010) reported that ERY was hazardous for *Daphnia* and sulfamethoxazole and TET to algae. Isidori et al. (2005) also found that macrolides, which included ERY, was the antibiotic class most harmful to the environment. Our results and others indicate that ERY and TET both individually and in binary mixtures may pose a significant risk for aquatic ecosystems.

In this work, toxicological interactions of antibiotics were performed for the first time based on Combination Index (CI) analysis. This methodology allowed to identify a general tendency to synergism in complex antibiotic mixtures, and specially to identify tetracycline as a synergistic-inducer component. Correction of additivity predictions based on CA and IA with the information provided by the CI method allowed, for the first time to accurately predict the actual toxicity of synergistic mixtures. This study also identified the erythromycin and tetracycline combination as a potential ecological threat based on today's pattern of use of these antibiotics.

Table 2 – Risk quotients (RQs) based on MEC and PNEC for the five antibiotics for *Anabaena* sp. CPB4337 and *Pseudokirchneriella subcapitata*.

Components	Surface water	Wastewater effluent	*Anabaena* sp. CPB4337			*Pseudokirchneriella subcapitata*		
	MEC (μM)	MEC (μM)	PNEC (μM)	RQ surface water	RQ wastewater effluent	PNEC (μM)	RQ surface water	RQ wastewater effluent
AMO + ERY	0.001889[a,c]	0.009096[d,f]	1.1214	0.0016844	0.008111	–	–	–
NOR + ERY	0.003788[b,c]	0.013289[d,f]	0.0141	0.26854	0.9421	0.05683	0.06666	0.2338
NOR + AMO	0.005304[b,a]	0.011528[d,d]	0.09172	0.05783	0.1257	–	–	–
LEV + ERY	0.000429[c,c]	0.005601[c,f]	0.006766	0.06333	0.8279	–	–	–
LEV + AMO	0.001944[c,a]	0.003840[c,d]	0.06131	0.03171	0.06263	–	–	–
LEV + NOR	0.003843[c,b]	0.008034[c,d]	0.01285	0.299	0.62503	0.04587	0.08378	0.1751
TET + ERY	0.000434[d,c]	0.008579[d,f]	0.006674	0.06505	1.285	0.0005676	0.765	15.12
TET + AMO	0.001950[d,a]	0.006818[d,d]	0.01754	0.1112	0.3888	–	–	–
TET + NOR	0.003849[d,b]	0.011011[d,d]	0.01389	0.2772	0.7929	0.02861	0.1345	0.3848
TET + LEV	0.000489[d,c]	0.003323[d,c]	0.003904	0.1254	0.8512	0.01251	0.03914	0.2657
4 AB[g]	0.004278[b,c,d,c]	0.016612[d,c,d,f]	–	–	–	0.04857	0.08807	0.3420
5 AB[h]	0.005980[a,b,c,d,c]	0.020280[d,d,c,d,f]	0.03395	0.1761	0.5973	–	–	–
AMO	0.001702[a]	0.003667[e]	0.154	0.01104	0.0238	–	–	–
NOR	0.003602[b]	0.007861[e]	0.01753	0.2053	0.4482	0.04635[i]	0.0777	0.1696
LEV	0.000242[c]	0.000173[c]	0.01328	0.0182	0.013	0.01245[i]	0.01942	0.01387
TET	0.000248[d]	0.003150[e]	0.01395	0.01774	0.2258	0.0001362[i]	1.817	23.12
ERY	0.000187[c]	0.005429[f]	0.00003	6.227	181.1	0.0002568[i]	0.7269	21.138

a Kasprzyk-Hordern et al., 2008.
b Watkinson et al., 2009.
c Kim et al., 2009.
d Leung et al., 2011.
e Kolpin et al., 2002.
f Rodriguez-Gil et al., 2010.
g ERY + LEV + NOR + TET.
h ERY + LEV + NOR + TET + AMO.
i PNEC based on individual EC_{20}.

4. Conclusions

We performed individual and combined antibiotic toxicity tests in representative photosynthetic aquatic organisms. The cyanobacterium, due to its prokaryotic nature, was in general more sensitive than the green alga to the tested antibiotics. Toxicological interactions studies using the Combination Index (CI) method indicated that synergism was predominant in binary and multi-component mixtures under real scenarios of contamination. Especially remarkable was the synergism found in almost all mixtures which included tetracycline as a component. CI method proved to give a more accurate prediction of the actual toxicity of synergistic mixtures than that offered by CA and IA. When risk assessment of antibiotic mixtures was performed, the combination of erythromycin and tetracycline presented an RQ value which exceeded 1, posing a potential ecological risk for aquatic organisms with today's pattern of use of both antibiotics.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data related to this article can be found in the online version at http://dx.doi.org/10.1016/j.watres.2013.01.020.

REFERENCES

Altenburger, R., Walter, H., Grote, M., 2004. What contributes to the combined effect of a complex mixture? Environmental Science & Technology 38 (23), 6353–6362.

Allen, M.B., Arnon, D.I., 1955. Studies on nitrogen-fixing blue green algae. I Growth and nitrogen fixation by *Anabaena cylindrica* Lemm. Plant Physiology 30, 366–372.

Backhaus, T., Scholze, M., Grimme, L.H., 2000. The single substance and mixture toxicity of quinolones to the bioluminescent bacterium *Vibrio fischeri*. Aquatic Toxicology 49 (1–2), 49–61.

Baguer, A.J., Jensen, J., Krogh, P.H., 2000. Effects of the antibiotics oxytetracycline and tylosin on soil fauna. Chemosphere 40 (7), 751–757.

Batt, A.L., Bruce, I.B., Aga, D.S., 2006. Evaluating the vulnerability of surface waters to antibiotic contamination from varying

wastewater treatment plant discharges. Environmental Pollution 142 (2), 295–302.
Boltes, K., Rosal, R., García-Calvo, E., 2012. Toxicity of mixtures of perfluorooctane sulphonic acid with chlorinated chemicals and lipid regulators. Chemosphere 86 (1), 24–29.
Brain, R.A., Johnson, D.J., Richards, S.M., Hanson, M.L., Sanderson, H., Lam, M.W., Young, C., Mabury, S.A., Sibley, P.K., Solomon, K.R., 2004. Microcosm evaluation of the effects of an eight pharmaceutical mixture to the aquatic macrophytes *Lemna gibba* and *Myriophyllum sibiricum*. Aquatic Toxicology 70 (1), 23–40.
Brain, R.A., Wilson, C.J., Johnson, D.J., Sanderson, H., Bestari, K., Hanson, M.L., Sibley, P.K., Solomon, K.R., 2005. Effects of a mixture of tetracyclines to *Lemna gibba* and *Myriophyllum sibiricum* evaluated in aquatic microcosms. Environmental Pollution 138 (3), 425–442.
Cedergreen, N., Christensen, A.M., Kamper, A., Kudsk, P., Mathiassen, S.K., Streibig, J.C., et al., 2008. A review of independent action compared to concentration addition as reference models for mixtures of compounds with different molecular target sites. Environmental Toxicology and Chemistry 27, 1621–1632.
Cleuvers, M., 2003. Aquatic ecotoxicity of pharmaceuticals including the assessment of combination effects. Toxicology Letters 142 (3), 185–194.
Conte, S., Stevenson, D., Furner, I., Lloyd, A., 2009. Multiple antibiotic resistance in Arabidopsis is conferred by mutations in a chloroplast-localized transport protein. Plant Physiology 151 (2), 559–573.
Costanzo, S.D., Murby, J., Bates, J., 2005. Ecosystem response to antibiotics entering the aquatic environment. Marine Pollution Bulletin 51 (1–4), 218–223.
Chou, T.C., Talalay, P., 1984. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Advances in Enzyme Regulation 22, 27–55.
Chou, T.C., Martin, N., 2005. CompuSyn for Drug Combinations: PC Software and User's Guide: A Computer Program for Quantification of Synergism and Antagonism in Drug Combinations and the Determination of IC50 and ED50 and LD50 Values. ComboSyn, Inc., Paramus, NJ.
Chou, T.C., 2006. Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. Pharmacological Reviews 58 (3), 621–681.
Christensen, A.M., Ingerslev, F., Baun, A., 2006. Ecotoxicity of mixtures of antibiotics used in aquacultures. Environmental Toxicology and Chemistry 25 (8), 2208–2215.
Daughton, C.G., Ternes, T.A., 1999. Pharmaceuticals and personal care products in the environment: agents of subtle change? Environmental Health Perspectives 107, 907–938.
Dolliver, H., Kumar, K., Gupta, S., 2007. Sulfamethazine uptake by plants from manure-amended soil. Journal of Environmental Quality 36 (4), 1224–1230.
Ebringer, L., 1972. Are plastids derived from prokaryotic microorganism-action of antibiotics on chloroplasts of Euglena-gracilis. Journal of General Microbiology 71 (1), 35–52.
Eguchi, K., Nagase, H., Ozawa, M., Endoh, Y.S., Goto, K., Hirata, K., Miyamoto, K., Yoshimura, H., 2004. Evaluation of antimicrobial agents for veterinary use in the ecotoxicity test using microalgae. Chemosphere 57 (11), 1733–1738.
Elmund, G.K., Morrison, S.M., Grant, D.W., Nevins, M.P., 1971. Role of excreted chlortetracycline in modifying decomposition process in feedlot waste. Bulletin of Environmental Contamination and Toxicology 6 (2), 129–132.
EMEA, 2006. European Chemical Agency. Guideline on the Environmental Risk Assessment of Medicinal Products for Human Use. Doc ref. EMEA/CHMP/SWP/4447/00.
Faust, M., Altenburger, R., Backhaus, T., Blanck, H., Boedeker, W., Gramatica, P., Hamer, V., Scholze, M., Vighi, M., Grimme, L.H., 2001. Predicting the joint algal toxicity of multi-component s-triazine mixtures at low-effect concentrations of individual toxicants. Aquatic Toxicology 56 (1), 13–32.
Fernandez-Pinas, F., Wolk, C.P., 1994. Expression of luxCD-E in Anabaena sp. can replace the use of exogenous aldehyde for in vivo localization of transcription by luxAB. Gene 150 (1), 169–174.
Ginebreda, A., Muñoz, I., de Alda, M.L., Brix, R., López-Doval, J., Barceló, D., 2009. Environmental risk assessment of pharmaceuticals in rivers: relationships between hazard indexes and aquatic macroinvertebrate diversity indexes in the Llobregat River (NE Spain). Environment International 36 (2), 153–162.
Göbel, A., Thomsen, A., McArdell, C.S., Joss, A., Giger, W., 2005. Occurrence and sorption behavior of sulfonamides, macrolides, and trimethoprim in activated sludge treatment. Environmental Science & Technology 39 (11), 3981–3989.
Greenberg, B.M., Huang, X.D., Dixon, D.G., 1992. Applications of the aquatic higher plan *Lemna gibba* for ecotoxicological risk assessment. Journal of Aquatic Ecosystem Health 1, 147–155.
Gros, M., Petrovic, M., Ginebreda, A., Barceló, D., 2010. Removal of pharmaceuticals during wastewater treatment and environmental risk assessment using hazard indexes. Environment International 36 (1), 15–26.
Gulkowska, A., He, Y., So, M.K., Yeung, L.W.Y., Leung, H.W., Giesy, J.P., Lam, P.K.S., Martin, M., Richardson, B.J., 2007. The occurrence of selected antibiotics in Hong Kong coastal waters. Marine Pollution Bulletin 54 (8), 1287–1293.
Halling-Sørensen, B., Nors Nielsen, S., Lanzky, P.F., Ingerslev, F., Holten Lützhoft, H.C., Jorgensen, S.E., 1998. Occurrence, fate and effects of pharmaceutical substances in the environment – a review. Chemosphere 36 (2), 357–393.
Halling-Sørensen, B., Lutzhoft, H.C.H., Andersen, H.R., Ingerslev, F., 2000. Environmental risk assessment of antibiotics: comparison of mecillinam, trimethoprim and ciprofloxacin. Journal of Antimicrobial Chemotherapy 46, 53–58.
Harrass, M.C., Kindig, A.C., Taub, F.B., 1985. Responses of blue-green and green algae to streptomycin in unialgal and paired culture. Aquatic Toxicology 6 (1), 1–11.
Hektoen, H., Berge, J.A., Hormazabal, V., Yndestad, M., 1995. Persistence of antibacterial agents in marine-sediments. Aquaculture 133 (3–4), 175–184.
Hernando, M.D., Mezcua, M., Fernández-Alba, A.R., Barceló, D., 2006. Environmental risk assessment of pharmaceutical residues in wastewater effluents, surface waters and sediments. Talanta 69 (2), 334–342.
Hirsch, R., Ternes, T., Haberer, K., Kratz, K.-L., 1999. Occurrence of antibiotics in the aquatic environment. Science of The Total Environment 225 (1–2), 109–118.
Isidori, M., Lavorgna, M., Nardelli, A., Parrella, A., 2004. Integrated environmental assessment of Volturno river in South Italy. Science of The Total Environment 327 (1–3), 123–134.
Isidori, M., Lavorgna, M., Nardelli, A., Pascarella, L., Parrella, A., 2005. Toxic and genotoxic evaluation of six antibiotics on non-target organisms. Science of The Total Environment 346 (1–3), 87–98.
Jia, J., Zhu, F., Ma, X., Cao, Z.W., Li, Y.X., Chen, Y.Z., 2009. Mechanisms of drug combinations: interaction and network perspectives. Nature Reviews Drug Discovery 8 (2), 111–128.
Kasprzyk-Hordern, B., Dinsdale, R., Guwy, A., 2008. Multiresidue methods for the analysis of pharmaceuticals, personal care products and illicit drugs in surface water and wastewater by solid-phase extraction and ultra performance liquid chromatography–electrospray tandem mass spectrometry. Analytical and Bioanalytical Chemistry 391, 1293–1308.

Kerry, J., Slattery, M., Vaughan, S., Smith, P., 1996. The importance of bacterial multiplication in the selection, by oxytetracycline-HCl, of oxytetracycline-resistant bacteria in marine sediment microcosms. Aquaculture 144 (1–3), 103–119.

Kim, S.C., Carlson, K., 2007. Quantification of human and veterinary antibiotics in water and sediment using SPE/LC/MS/MS. Analytical and Bioanalytical Chemistry 387 (4), 1301–1315.

Kim, J.W., Jang, H.S., Kim, J.G., Ishibashi, H., Hirano, M., Nasu, K., et al., 2009. Occurrence of pharmaceutical and personal care products (PPCPs) in surface water from Mankyung river, South Korea. Journal of Health Science 55, 249–258.

Kolpin, D.W., Furlong, E.T., Meyer, M.T., Thurman, E.M., Zaugg, S.D., Barber, L.B., Buxton, H.T., 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in US streams, 1999–2000: a national reconnaissance. Environmental Science & Technology 36 (6), 1202–1211.

Kolpin, D.W., Skopec, M., Meyer, M.T., Furlong, E.T., Zaugg, S.D., 2004. Urban contribution of pharmaceuticals and other organic wastewater contaminants to streams during differing flow conditions. Science of The Total Environment 328 (1–3), 119–130.

Kong, W.D., Zhu, Y.G., Liang, Y.C., Zhang, J., Smith, F.A., Yang, A., 2007. Uptake of oxytetracycline and its phytotoxicity to alfalfa (*Medicago sativa* L.). Environmental Pollution 147 (1), 187–193.

Leung, H.W., Minh, T.B., Murphy, M.B., Lam, J.C.W., So, M.K., Martin, M., et al., 2011. Distribution, fate and risk assessment of antibiotics in sewage treatment plants in Hong Kong, South China. Environment International 42, 1–9.

Li, B., Zhang, T., Xu, Z., Fang, H.H.P., 2009. Rapid analysis of 21 antibiotics of multiple classes in municipal wastewater using ultra performance liquid chromatography-tandem mass spectrometry. Analytica Chimica Acta 645 (1–2), 64–72.

Lindsey, M.E., Meyer, M., Thurman, E.M., 2001. Analysis of trace levels of sulfonamide and tetracycline antimicrobials, in groundwater and surface water using solid-phase extraction and liquid chromatography/mass spectrometry. Analytical Chemistry 73 (19), 4640–4646.

Lutzhoft, H.C.H., Halling-Sorensen, B., Jorgensen, S.E., 1999. Algal toxicity of antibacterial agents applied in Danish fish farming. Archives of Environmental Contamination and Toxicology 36 (1), 1–6.

Maul, J.D., Schuler, L.J., Belden, J.B., Whiles, M.R., Lydy, M.J., 2006. Effects of the antibiotic ciprofloxacin on stream microbial communities and detritivorous macroinvertebrates. Environmental Toxicology and Chemistry 25 (6), 1598–1606.

McArdell, C.S., Molnar, E., Suter, M.J.F., Giger, W., 2003. Occurrence and fate of macrolide antibiotics in wastewater treatment plants and in the Glatt Valley Watershed, Switzerland. Environmental Science & Technology 37 (24), 5479–5486.

McFadden, G.I., Roos, D.S., 1999. Apicomplexan plastids as drug targets. Trends in Microbiology 7 (8), 328–333.

Migliore, L., Civitareale, C., Brambilla, G., DiDelupis, G.D., 1997. Toxicity of several important agricultural antibiotics to Artemia. Water Research 31 (7), 1801–1806.

Nicolas, P., 1981. Sensitivity of *Euglena gracilis* to chloroplast-inhibiting antibiotics, and properties of antibiotic-resistant mutants. Plant Science and Letters 22, 309–317.

OECD, 2006.) OECD/201 Guidelines for the Testing of Chemicals. Freshwater Algal and Cyanobacteria, Growth Inhibition Test.

OECD, 2008. Guidelines for the Testing of Chemicals, No.23: Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures. PDF Edition (ISSN 1607-310X), 18th Addendu.

Park, S., Choi, K., 2008. Hazard assessment of commonly used agricultural antibiotics on aquatic ecosystems. Ecotoxicology 17 (6), 526–538.

Perez-Parada, A., Aguera, A., Gomez-Ramos, M.D., Garcia-Reyes, J.F., Heinzen, H., Fernandez-Alba, A.R., 2011. Behavior of amoxicillin in wastewater and river water: identification of its main transformation products by liquid chromatography/electrospray quadrupole time-of-flight mass spectrometry. Rapid Communications in Mass Spectrometry 25 (6), 731–742.

Qin, L.-T., Liu, S.-S., Zhang, J., Xiao, Q.-F., 2010. A novel model integrated concentration addition with independent action for the prediction of toxicity of multi-component mixture. Toxicology 280 (3), 164–172.

Robinson, A.A., Belden, J.B., Lydy, M.J., 2005. Toxicity of fluoroquinolone antibiotics to aquatic organisms. Environmental Toxicology and Chemistry 24 (2), 423–430.

Rodea-Palomares, I., Fernández-Piñas, F., González-García, C., Leganes, F., 2009. Use of lux-marked cyanobacterial bioreporters for assessment of individual and combined toxicities of metals in aqueous samples. In: Handbook on Cyanobacteria: Biochemistry, Biotechnology and Applications. Nova Science Publishers, Inc, New York. USA.

Rodea-Palomares, I., Petre, A.L., Boltes, K., Leganés, F., Perdigón-Melón, J.A., Rosal, R., Fernández-Piñas, F., 2010. Application of the combination index (CI)-isobologram equation to study the toxicological interactions of lipid regulators in two aquatic bioluminescent organisms. Water Research 44 (2), 427–438.

Rodea-Palomares, I., Leganés, F., Rosal, R., Fernández-Piñas, F., 2012. Toxicological interactions of perfluorooctane sulfonic acid (PFOS) and perfluorooctanoic acid (PFOA) with selected pollutants. Journal of Hazardous Materials 30 (201–202), 209–218.

Rodriguez-Gil, J.L., Catalá, M., Alonso, S.G., Maroto, R.R., Valcárcel, Y., Segura, Y., Molina, R., Melero, J.A., Martínez, F., 2010. Heterogeneous photo-Fenton treatment for the reduction of pharmaceutical contamination in Madrid rivers and ecotoxicological evaluation by a miniaturized fern spores bioassay. Chemosphere 80 (4), 381–388.

Rosal, R., Rodea-Palomares, I., Boltes, K., Fernández-Piñas, F., Leganés, F., Petre, A., 2010a. Ecotoxicological assessment of surfactants in the aquatic environment: combined toxicity of docusate sodium with chlorinated pollutants. Chemosphere 81 (2), 288–293.

Rosal, R., Rodriguez, A., Perdigón-Melón, J.A., Petre, A., García-Calvo, E., Gómez, M.J., Agüera, A., Fernández-Alba, A.R., 2010b. Occurrence of emerging pollutants in urban wastewater and their removal through biological treatment followed by ozonation. Water Research 44 (2), 578–588.

Sacher, F., Lang, F.T., Brauch, H.J., Blankenhorn, I., 2001. Pharmaceuticals in groundwaters – analytical methods and results of a monitoring program in Baden-Wurttemberg, Germany. Journal of Chromatography A 938 (1–2), 199–210.

Samuelsen, O.B., 2006. Pharmacokinetics of quinolones in fish: a review. Aquaculture 255 (1â€“4), 55–75.

Sanderson, H., Johnson, D.J., Wilson, C.J., Brain, R.A., Solomon, K.R., 2003. Probabilistic hazard assessment of environmentally occurring pharmaceuticals toxicity to fish, daphnids and algae by ECOSAR screening. Toxicology Letters 144 (3), 383–395.

Sarmah, A.K., Meyer, M.T., Boxall, A.B.A., 2006. A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment. Chemosphere 65 (5), 725–759.

Schneider, J., 1994. Problems related to the usage of veterinary drugs in aquaculture – a review. Química Analítica 13 (Suppl. 1), S34–S42.

Suzuki, S., Hoa, P.T.P., 2012. Distribution of quinolones, sulfonamides, tetracyclines in aquatic environment and

antibiotic resistance in Indochina. Frontiers in Microbiology 3, 67.
Tamtam, F., Mercier, F., Eurin, J., Chevreuil, M., Le Bot, B., 2009. Ultra performance liquid chromatography tandem mass spectrometry performance evaluation for analysis of antibiotics in natural waters. Analytical and Bioanalytical Chemistry 393 (6–7), 1709–1718.
Teuschler, L.K., 2007. Deciding which chemical mixtures risk assessment methods work best for what mixtures. Toxicology and Applied Pharmacology 223 (2), 139–147.
USEPA, 1994. Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwaters Organism. U.S. Environmental Protection Agency.
USEPA, 2002. Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organism. United Estates Environmental Protection Agency.
Van Bambeke, F., Glupczynski, Y., PlÃ©siat, P., PechÃře, J.C., Tulkens, P.M., 2003a. Antibiotic efflux pumps in prokaryotic cells: occurrence, impact on resistance and strategies for the future of antimicrobial therapy. Journal of Antimicrobial Chemotherapy 51 (5), 1055–1065.
Van Bambeke, F., Michot, J.-M., Tulkens, P.M., 2003b. Antibiotic efflux pumps in eukaryotic cells: occurrence and impact on antibiotic cellular pharmacokinetics, pharmacodynamics and toxicodynamics. Journal of Antimicrobial Chemotherapy 51 (5), 1067–1077.
Von der Ohe, P.C., Dulio, V., Slobodnik, J., De Deckere, E., Kühne, R., Ebert, R.-U., Ginebreda, A., De Cooman, W., Schüürmann, G., Brack, W., 2011. A new risk assessment approach for the prioritization of 500 classical and emerging organic microcontaminants as potential river basin specific pollutants under the European Water Framework Directive. Science of the Total Environment 409 (11), 2064–2077.
Watkinson, A.J., Murby, E.J., Kolpin, D.W., Costanzo, S.D., 2009. The occurrence of antibiotics in an urban watershed: from wastewater to drinking water. Science of the Total Environment 407 (8), 2711–2723.
Xu, W.-H., Zhang, G., Zou, S.-C., Li, X.-D., Liu, Y.-C., 2007. Determination of selected antibiotics in the Victoria Harbour and the Pearl River, South China using high-performance liquid chromatography-electrospray ionization tandem mass spectrometry. Environmental Pollution 145 (3), 672–679.
Yang, L.H., Ying, G.G., Su, H.C., Stauber, J.L., Adams, M.S., Binet, M.T., 2008. Growth-inhibiting effects of 12 antibacterial agents and their mixtures on the freshwater microalga *Pseudokirchneriella subcapitata*. Environmental Toxicology and Chemistry 27 (5), 1201–1208.
Zuccato, E., Calamari, D., Natangelo, M., Fanelli, R., 2000. Presence of therapeutic drugs in the environment. The Lancet 355 (9217), 1789–1790.

WEB REFERENCES

<http://genome.kazusa.or.jp/cyanobase/> (accessed: 07.12.12).