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Influence of Select Antibiotics on *Vibrio fischeri* and *Desmodesmus subspicatus* at μg L^{-1} Concentrations

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Abstract The presence of pharmaceuticals in the aquatic environment is a contemporary reality and it is necessary to understand more about the effects of this presence on organisms. The purpose of this work was to assess the ecotoxicity of antibiotics metronidazole, nitrofurantoin, trimethoprim, and sulphamethoxazole (single and mixture) in *Vibrio fischeri* and *Desmodesmus subspicatus* at μg L^{-1} concentrations. The evaluation of the toxic effect of the antibiotics on *V. fischeri* and *D. subspicatus* was based on fluorescence and bioluminescence tests, respectively, using nominal concentrations. When tested individually, the four antibiotics gave rise to a toxic effect on the evaluated organisms. Sulphamethoxazole caused a higher toxic effect on *V. fischeri* and *D. subspicatus* from 7.81 to 500 μg L^{-1}. Trimethoprim and sulphamethoxazole showed hormesis for the concentrations, which ranged from 7.81 to 62.5 μg L^{-1}. The mixture of antibiotics induced a toxic effect on the *V. fischeri* and *D. subspicatus* organisms (from 0.03 to 1 μg L^{-1} concentrations) than when the antibiotics were evaluated individually. These results were significant since water quality problems are widespread all over the word, and emerging pollutants such as antibiotics have been detected in the aquatic environment in very low concentrations.

Keywords Environment · Environmental concentrations · Pharmaceuticals · Synergistic effects

Introduction

Several classes of pharmaceuticals have been identified and quantified in rivers and sewage treatment stations, including antibiotics (Castiglioni et al. 2004; Lin et al. 2008; Leung et al. 2012; Locatelli et al. 2011), analgesics and anti-inflammatory drugs (Bendz et al. 2005; Stackelberg et al. 2007; Gibbons et al. 2011), antihistamines (Stackelberg et al. 2007), stimulants (Lin et al. 2008; Locatelli et al. 2011), lipid regulators (Andreozzi et al. 2005; Lin et al. 2008), anti-hypertension drugs (Stackelberg et al. 2007), beta-blockers (Bendz et al. 2005; Lin et al. 2008; Andreozzi et al. 2005), and antipsychotic drugs (Bendz et al. 2005; Stackelberg et al. 2007; Lin et al. 2008; Loganathan et al. 2009).

The low levels of pharmaceuticals that have been found, varying from ng L^{-1} to μg L^{-1}, may interfere with biological systems. Some reported effects are: inducing genotoxic effects (Sponchiado et al. 2010; Ragugnetti et al. 2011) and endocrine disruption (Kim et al. 2012) in fish; effects on *Daphnia magna* population growth rate (Kim et al. 2012; Kim and Lee 2012; Liguoro et al. 2009; Garric et al. 2007; Flaherty and Dodson 2005); reduction in *Moina macrocopa* adult survival (Kim et al. 2012). Among these compounds, antibiotics attract added interest for their ability to induce bacterial resistance. Several strains of microorganisms that have been isolated from rivers and treatment stations have shown to be resistant to various antibiotics (Silva et al. 2010; Aleem et al. 2003; Dang et al. 2012;

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Koczura et al. 2012; Luczkiewicz et al. 2010; Costa et al. 2006; Threedeach et al. 2012; Middleton and Salierno 2013). Concern about the effect that antibiotics can cause to the environment goes beyond the induction of multi-resistance. Changes have also been observed in the ovaries of *Danio rerio* (Madureira et al. 2011) and in the liver of female and male *Danio rerio* when they were exposed to a mixture of non-steroidal drugs, including sulfamethoxazole (SMX), and trimethoprim (TMP) (Madureira et al. 2012). Among the organisms used to determine the potential ecotoxicological effects of pharmaceuticals in the environment are *Daphnia magna*, *Vibrio fischeri*, and *Desmodesmus subspicatus* (Ioele et al. 2016; You et al. 2016; Dang et al. 2012; Kim et al. 2012; Kim and Lee 2012; Liguoro et al. 2012; Gómez-Ramos et al. 2011). Despite of several organizations charged with biomonitoring and establishing aquatic chemical criteria incorporate these organisms into standard testing protocol guidelines, taking into account concentrations in $mg\,L^{-1}$ (OECD 2008, OECD 2011; ABNT 2011, 2012), further studies are lacking to environmental concentrations ($ng\,L^{-1}$ and $\mu g\,L^{-1}$) effects on these organisms. Effects like hormesis, which occurs at low concentrations, are not predicted by these methodologies. Zou et al. (2013) observed the effect of hormesis on *V. fischeri* in a mixture of antibiotics at low concentrations. Liguoro et al. (2010) observed that green algae had higher sensitivity to sulfonamides than did blue algae, which was predicted by the Committee for Medicinal Products for Veterinary Use (CVMP/VICH/790/03 2003).

V. fischeri has been used to evaluate the toxicity of pharmaceuticals. Trovó et al. (2011) used *V. fischeri* to evaluate the reduction in toxicity of amoxicillin after it was exposed to a photo-Fenton treatment. Similarly, Li et al. (2008) evaluated the reduction in toxicity of tetracycline after treatment with ozonation. Zou et al. (2013) studied the effect of hormesis for mixtures of antibiotics using *V. fischeri.*

Following this line of investigation, green microalgae have been similarly used (Liguoro et al. 2012; Lanzky and Halling-Sorensen 1997; Liguoro et al. 2010). Liguoro et al. (2012) reported that TMP may induce the CYP1A protein in various tissues of *Pseudokirchneriella subcapitata* (currently named Raphidocelis *subcapitata*). Lanzky and Halling-Sorensen (1997) observed acute toxicity of metronidazole (MET) on *Chlorella spp.* and *Selenastrum capricornutum* (currently named *Raphidocelis subcapitata*). Liguoro et al. (2010) showed that sulfaquinoxaline and sulfaguanidine are more toxic to green algae (*Pseudokirchneriella subcapitata*, *Scenedesmus dimophus* and *Synecococssus leopoliensis*).

SMX is an antimicrobial from the sulphonamide class; it competes with ρ-aminobenzoic acid, which is a precursor in the synthesis of folic acid, a key compound for the synthesis of DNA and RNA in bacteria. Sulpha drugs compete for the dihydropteroate synthetase enzyme to inhibit the synthesis of folate. TMP acts on the enzyme dihydrofolate reductase by deceiving it, since its structure is similar to part of folate's, a substance with which the enzyme interacts. This prevents the formation of tetrahydrofolic acid, which is a substance essential for bacterial and vegetable synthesis. Both drugs are administered together because TMP enhances the effect of SMX (Rang et al. 2011).

The compounds nitrofuranics, MET, and nitrofurantoin (NIT) activate as the nitro-group is reduced, and this leads to the formation of electrophilic species that can react with DNA. The result of this process is the breaking and destabilization of the DNA helix, which can be accelerated when adenine and thymine are present (Bosquesi et al. 2008).

In this study the antibiotics MET, NIT, TMP, and sulphamethoxazole (single and mixture) were tested for their adverse effects on *V. fischeri* and *D. subspicatus* to illustrate possible toxic or hormetic effects of pharmaceuticals in the aquatic environment.

Materials and Methods

The evaluation of the toxic effect of antibiotics on the bioindicators *V. fischeri* and *D. subspicatus* was based on the methods ISO 11348-1:2007 and ISO 8692 (2012). The toxicity tests were conducted at the Ecotoxicology Laboratory, SENAI-CIC (Serviço Nacional de Aprendizagem Industrial—Cidade Industrial de Curitiba) in Curitiba, Paraná, Brazil.

Standards and Solutions

The following antibiotics were used for the ecotoxicological tests: MET Sigma; NIT, Sigma; SMX Fluka and TMP Sigma-Aldrich. The antibiotic stock solutions (single and mixture) were prepared as following: 1 mg of each antibiotic (single and mixture) were dissolved in 10 mL systems of specific solvents according to the criteria of solubility (NIT in acetone; TMP in chloroform/ methanol, 1:1, v/v; SMX in methanol; and MET in ethanol/methanol 1:1, v/v). Finally the antibiotic stock solutions were completed with milliQ water until 1 L, resulting in an $1\,mg\,L^{-1}$ solution.

The antibiotic concentrations were equal or even higher than other authors (Supplementary Material S1) previously reported in rivers for compounds such as SMX (Gros et al. 2012; Vazquez-Roig et al. 2012; Na et al. 2013; Du et al. 2015), TMP (Locatelli et al. 2011; Gros et al. 2012; Vazquez-Roig et al. 2012; Na et al. 2013; Du et al. 2015) and hospital effluents (Santos et al. 2013). Therefore, the effects of these chemicals in aquatic organisms must be

analyzed, mainly concerning their significant potential for bioaccumulation (Caminada et al. 2006).

Acute Toxicity Testing

Vibrio fischeri

The test solutions of the single antibiotics were prepared in concentrations ranging from 3.91 to 500 $\mu g\,L^{-1}$ (nominal concentrations) for all the antibiotics in 2% aqueous sodium chloride solution. The concentration of the mixture ranged from 0.03 to 1 $\mu g\,L^{-1}$ (nominal concentrations) for each antibiotic.

The *V. fischeri* bacterium was grown in a solid culture medium for *Photobacterium* in the dark, at 22 °C, for a period of 72 h. Prior to use, the culture was observed in the dark to confirm the presence of luminescence. Bioluminescence was measured in a luminometer Lumistox 300 Dr. Langue, using the Lumissoft software. The luminescence inhibition percentage was calculated until 1%. Below 1% the results were considered as negative. The toxicity of the sample was corrected with the correction factor obtained from the non-toxic reference sample (2% NaCl solution).

The exposure time was 30 min (ISO 11348-1:2007) for the solutions containing the antibiotics (single and mixture). One solvent control and three treatments (replicates) were carried out for each concentration. The solvent control contained the same amount of solvent used to prepare the tested concentrations.

Desmodesmus subspicatus

D. subspicatus was chosen since it is a typical organism studied to analyze effects of substances on aquatic organisms, and other authors have observed the effects of antibiotics on this organism as well as on other species of algae.

For this test, the concentrations of aqueous single antibiotic solutions ranged from 7.81 to 1000 $\mu g\,L^{-1}$ (nominal concentrations) and the concentration of the mixtures ranged from 0.03 to 1 $\mu g\,L^{-1}$ (nominal concentrations) for each antibiotic.

The algae were incubated at 23 ± 2 °C under continuous fluorescent light (1120 $\mu Es^{-1}\,m^{-2}$) and were maintained in suspension by continuous stirring. One control and three treatments (replicates) were carried out for each concentration. The solvent control contained the same amount of solvent used to prepare the tested concentrations.

The results were quantified based on the measurements of chlorophyll by in vivo fluorimetry in a Turner Designs Trilogy fluorometer. The results of chlorophyll *a* measured in relative fluorescence units were converted to % inhibition using Eq. 1:

$$\%\,\text{inhibition} = [(\mu e - \mu c)/\mu c] \times 100, \quad (1)$$

where μc is the solvent control luminescence, μe the luminescence of solution with antibiotic.

Statistical Analysis

Statistical analysis was performed using the XLSTAT-2013, an add-on statistical package of Excel. The results were tested using the Shapiro–Wilk, Anderson–Darling, Lilliefors and Jarque-Bera tests. Since the data was not normally distributed, the non-parametric Wilcoxon test was used for the analysis of the results between two populations. For the comparisons between all the antibiotics in all concentrations for the same organism and the solvent control, the non-parametric Kruskal–Wallis test was performed. Differences were considered significant at $p < 0.05$.

Results and Discussion

Isolated Antibiotics

Vibrio fischeri

An inhibitory effect on *V. fischeri* by the four tested antibiotics was observed (Fig. 1). The effects of the inhibition of bioluminescence increased with an increase in concentration, $p < 0.0001$. There was no effect of the solvent controls into the measured bioluminescence.

At 3.91 $\mu g\,L^{-1}$ concentration, TMP had the lowest % inhibition (0.96%), followed by MET (3.21%), NIT (4.70%) and SMX (13.65%). NIT has not been reported in aquatic environments, however various species of bacteria that are resistant to this antibiotic, have been isolated from natural waters and effluents from sewage treatment plants (Aleem et al. 2003; Costa et al. 2006; Luczkiewicz et al. 2010; Dang et al. 2012; Koczura et al. 2012; Threedeach et al. 2012; Middleton and Salierno 2013).

The inhibition values were obtained using an exposure time of 30 min. Although the toxic effects for TMP and MET were much lower than observed for SMX and NIT (4.25 and 3.21 times respectively, Fig. 1), their influence on *V. fischeri* in field settings cannot be disregarded as it may involve longer contact times. 30 min has been used by other authors to test the effect of other antibiotics on *V. fischeri* in higher concentrations (Trovó et al. 2011; Li et al. 2008). Otherwise Froehner et al. (2000) noted that for other antibiotics (chloramphenicol, nalidixic acid, and streptomycin sulfate) an inhibition increasing when the test was carried out for 7 and 24 h. According to Froehner et al. (2000), two of those antibiotics had no effect in 30 min.

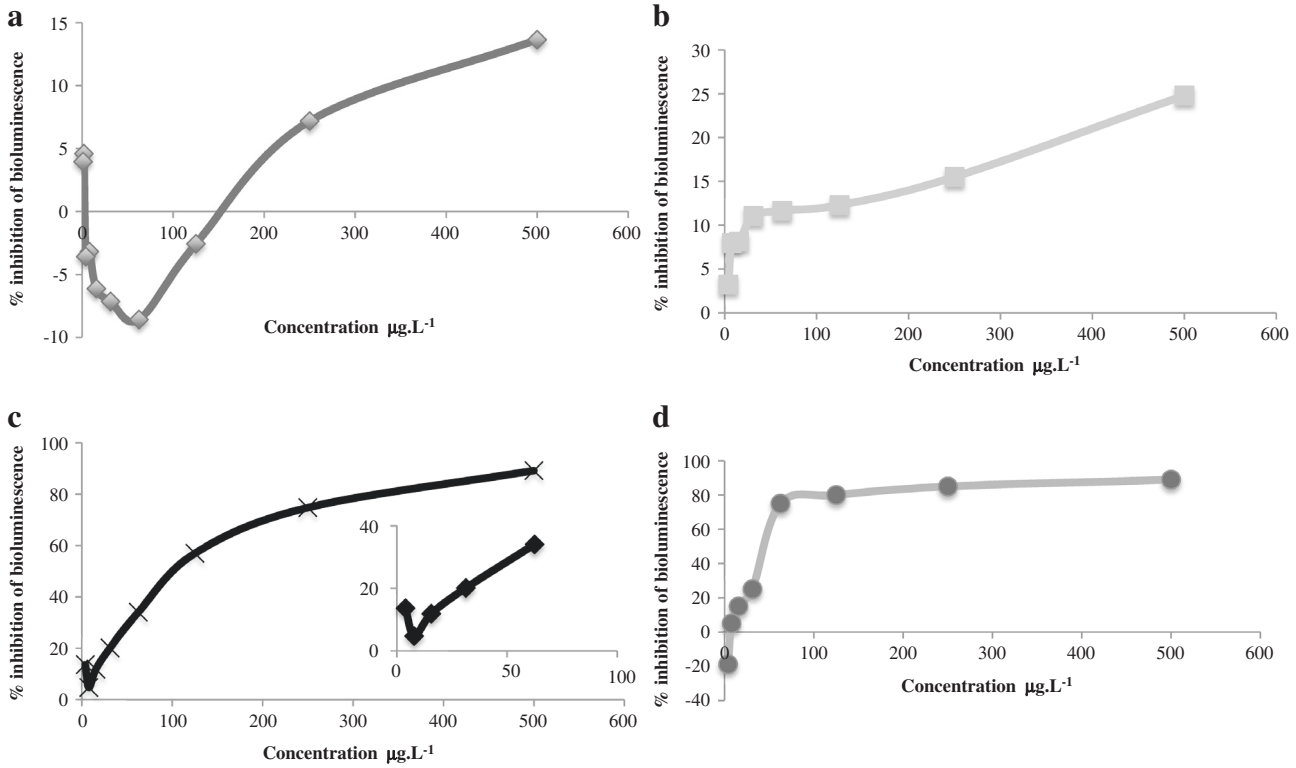

Fig. 1 Antibiotics effect on bioluminescence inhibition of *Vibrio fischeri* **a** effect of TMP, $p = 0.002$, **b** effect of MET, $p > 0.05$, **c** effect of SMX, highlighting the concentrations from 3.91 to 62.5 µg L^{-1}, $p = 0.01$ and **d** effect of NIT, $p > 0.05$

The concentrations that gave rise to active responses were higher than those measured in effluent from sewage treatment plants and river water: 1.09 µg L^{-1} of SMX in wastewater (Yang et al. 2005); 0.34 µg L^{-1} of TMP in effluent (Watkinson et al. 2007); and 0.314 µg L^{-1} of MET in river water (Leung et al. 2012). Still, further studies with longer testing times should be carried out to compare chronic effects.

There was an increase in inhibition with increased concentrations of the tested antibiotics for NIT and MET. Variations were observed for TMP (concentrations from 0.9 to 500 µg L^{-1}) and SMX (concentrations from 3.91 to 62.5 µg L^{-1}) (Fig. 1a and c). This variation was similar to that reported by Zou et al. (2013), which showed hormetic effects in a range from 253.28 to 25,000 µg L^{-1} for SMX ($p = 0.01$) and 29.03 to 2903.20 µg L^{-1} for TMP. Herein, an instance of hormesis was measured in lower concentrations. The toxicity effect was most evident for TMP, for 0.9 and 1.8 µg L^{-1} concentrations, with a negative effect from 3.9 to 125 µg L^{-1} and an increase in the effect from 250 to 500 µg L^{-1} ($p = 0.002$). Those toxic effects observed for SMX and TMP suggest the limitation of using only linear models, which determine the lowest observed effect concentrations (LOEC) and the no observable effect concentration (NOEC) to evaluate impacts of pollutants in aquatic environments. For example, Ioele et al. (2016) calculated LOEC for SMX as 95.05–97.03 µg mL^{-1} (95,050–97,030 µg L^{-1}) and NOEC as 93.01 µg mL^{-1} (93,010 µg L^{-1}), which do not represent the toxic behavior for this substance, according to the herein studies.

It is important to stress that the concentrations at which effects were observed for TMP and SMX in the present study were below the Predicted no-effect concentration (PNEC) values reported by Kümmerer and Henninger (2003), which were 1.0 and 20 µg L^{-1}, respectively. For MET, the concentrations which resulted in effects were higher than the PNEC values described by Lin et al. (2008) (1.3 µg L^{-1}). However, it should be emphasized that these molecules do not typically occur in an isolated manner in the aquatic environment and the effects of possible mixtures deserves attention.

Desmodesmus subspicatus

The percentage of inhibition of photosynthetic activity of *D. subspicatus* increased with greater concentrations of the four tested antibiotics (Fig. 2). NIT gave rise to the highest change in inhibition (4.82–89.28%) and SMX to the lowest (73.90–100%).

The concentrations of 250–1000 µg L^{-1} were considered to be high when compared to concentrations found in the surface water: 1.83 µg L^{-1} MET; 0.95 µg L^{-1} SMX; and

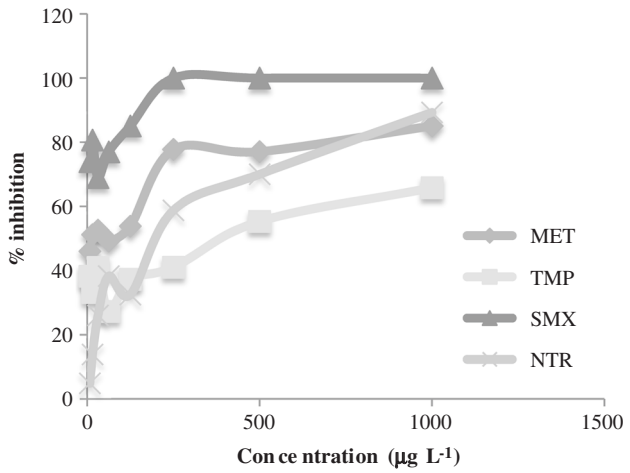

Fig. 2 Observed effect of the antibiotics on *Desmodesmus subspicatus*, $p = 0.02$

0.69 µg L^{-1} TMP (Valcárcel et al. 2011). For the % of inhibition observed for the lowest concentration, 7.81 µg L^{-1}, NIT gave rise to the lowest inhibition of photosynthetic activity (4.82%), followed by TMP (38.21%), MET (46%) and SMX (73.90%), which was a different result from that which was observed for the higher concentrations. Making such assessments requires considerations of these compounds' characteristics and their mechanisms of action. Even substances with similar structures and action mechanisms may present different results. Liguoro et al. (2010) evaluated the effect of two sulfonamides for veterinary use (sulphaquinoxaline and sulphaguanidine) in three types of algae and they found different percentage of inhibition for each compound, demonstrating the need to evaluate substances individually, even if they have similar actions.

Although the environmental concentrations of MET, TMP, and SMX described by Valcárcel et al. (2011) were lower than the inhibition concentrations here registered, further studies are needed to understand the chronic effects of the environmental concentrations.

The species of algae used to perform the test should also be considered. The same substance can show different results for different algae. Liguoro et al. (2010) observed that green algae had a higher sensitivity than the blue algae recommended by CVMP/VICH/790/03. In another study, the same authors achieved 20% growth inhibition of *P. subcapitata* for 6.25 mg L^{-1} of TMP. In the present study, 38.21% inhibition was observed for the same pharmaceutical, but for another species and at a lower concentration (7.81 µg L^{-1}). Consequently, comparisons of results between different species should be made with caution.

D. subspicatus showed higher sensitivity to MET, with 46% inhibition for the 7.81 µg L^{-1} concentration, compared to the test performed by Lanzky and Halling-Sorensen (1997) for *Selenastrum capricornutun*, which found 10% inhibition at a concentration between 19.9 and 21.7 mg L^{-1}. SMX induced a higher percentage of inhibition (Fig. 2), demonstrating the algaecide action of sulfonamides (Liguoro et al. 2010). The SMX algaecide action was higher than the NTR and MET nitro-group (Supplementary Material S2) effect, which could indicate that inhibition mechanism of folate prevails over the destabilization of DNA provoked through the reduction of the nitro-group (Bosquesi et al. 2008).

The changes in the percentage inhibition for the concentrations from 7.81 to 125 µg L^{-1} obtained for *V. fischeri* were also observed for *D. subspicatus* (Fig. 3), with significantly differences: p values equal to 0.04 (MET), 0.03 (TMP) and 0.04 (SMX). Such variations may suggest a similarity to the effect of hormesis (Calebrese and Baldwin 2002; Zou et al. 2013). The variation observed for NIT was not significantly different, $p = 0.16$.

Mixture of Antibiotics

The mixtures of antibiotics (0.03 to 1 µg L^{-1}) gave rise to higher toxic effects on *D. subspicatus* (average fluorescence inhibition: 76%) than single antibiotic solutions (at concentrations ranging from 7.81 to 1000 µg L^{-1}). When the antibiotics were tested individually, SMX had the highest effect, with 73.90% inhibition at a concentration of 7.8 µg L^{-1}. According to Liguoro et al. (2010), some sulfonamides may have an algaecide effect at concentrations in the order of µg L^{-1}, and this effect is more prevalent when they are in mixtures (Fig. 4).

The mixture also had an effect on *V. fischeri* but the variation occurred in a linear form, $R^2 = 0.98$, with an increase in the concentration. For concentrations below 0.125 µg L^{-1} no effect was observed, which is the lowest concentration at which an effect of the mixture on *V. fischeri* was observed. Considering that antibiotics are substances that are intended to interfere with microbial metabolisms, it is to be expected that bacterial communities will be the first aquatic organisms to suffer in the presence of these substances. The results observed in this study were the opposite because *V. fischeri* suffered less effect than *D. subspicatus*. This reinforces the algaecide effect of sulfonamides (Liguoro et al. 2010) and the influences of MET and NTR nitro-group (Bosquesi et al. 2008). It was not possible to identify if there was a differentiated effect from the antibiotics, when mixed, on the *V. fischeri.* The study herein indicated that antibiotic mixtures can elicit effects not observed by single exposures. Flaherty and Dodson (2005) reported the same effect on *Daphnia magna* for mixtures containing SMX, TMP and other antibiotics. The observed effects for the mixtures are of concern because, in nature,

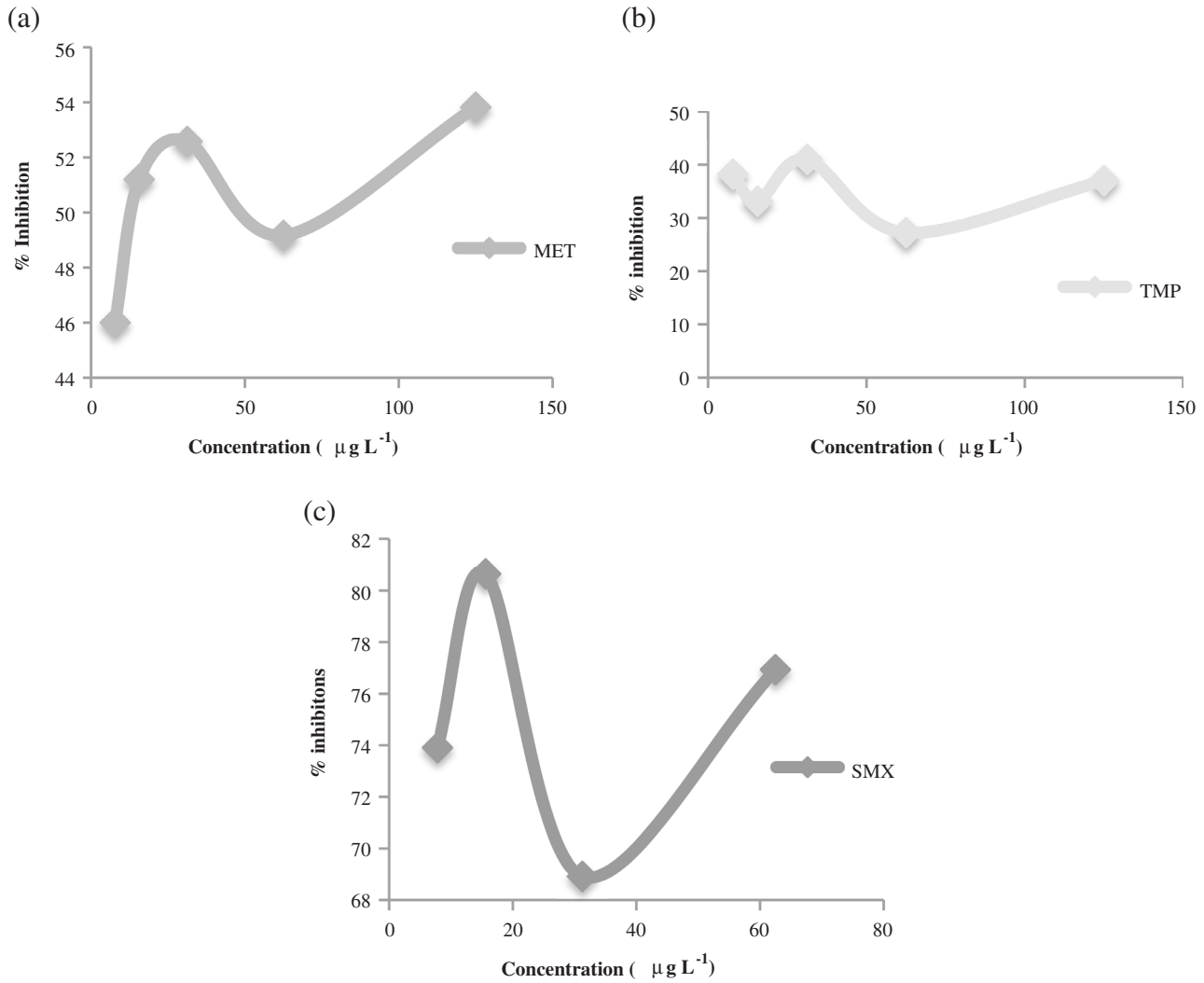

Fig. 3 Percent inhibition for the concentrations from 7.81 to 125 µg L^{-1} obtained for *Desmodesmus subspicatus*: **a** MET, $p = 0.04$, **b** TMP $p = 0.03$ and **c** SMX, $p = 0.04$

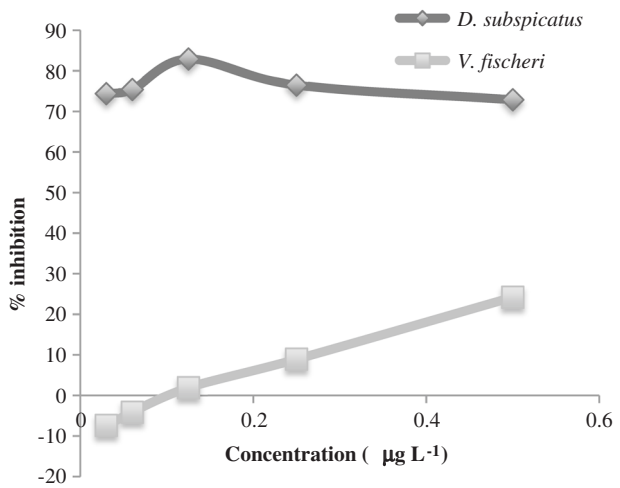

Fig. 4 Toxic effects observed from the mixture of antibiotics on the tested organisms for concentrations between 0.03 and 0.5 µg L^{-1}

these substances are mixed with others, increasing the possibility of synergistic effects.

Conclusion

The single tested antibiotics affected *V. fischeri* and *D. subspicatus* at low concentrations (3.91–500 and 7.81–1000 µg L^{-1}, respectively). SMX had the greatest effect on *D. subspicatus*, while NIT had on *V. fischeri*. At low concentrations (0.03–1 µg L^{-1}), the mixture caused a greater effect on *D. subspicatus*, than on *V. fischeri* compared to observations for the single antibiotics.

The results obtained allow for the identification of the aquatic toxicity of NIT, MET, SMX and TMP at concentration levels from µg L^{-1} to ng L^{-1}. It provides useful insights to the pharmaceutical industry for refining the

ecological information on the safety data sheet for these compounds. NIT ecotoxicological data are not available, and for MET, SMX, and TMP are reported at mg L^{-1}.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no competing interest.

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