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Diazepam, metformin, omeprazole and simvastatin: a full discussion of individual and mixture acute toxicity

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Abstract

High consumption of drugs, combined with their presence in the environment, raises concerns about its consequences. Even though researches are often engaged in analyzing substances separately, that is not the environmental reality. Therefore, the aim of this study was to investigate the acute toxicity of the pharmaceuticals simvastatin, metformin, omeprazole and diazepam, and all possible mixtures between them, to the organism *Aliivibrio fischeri*, verifying possible synergistic or antagonistic effects and assessing byproducts formation. In terms of individual toxicity, omeprazole is the most toxic of the active ingredients, followed by simvastatin, diazepam and, finally, metformin. When the toxicity of mixtures was tested, synergism, antagonism and hormesis were perceived, most probably generated due to byproducts formation. Moreover, it was observed that even when compounds are at concentrations below the non-observed effect concentration (NOEC), there may be toxicity to the mixture. Hence, this work points to the urgent need for more studies involving mixtures, since chemicals are subject to interactions and modifications, can mix, and potentiate or nullify the toxic effect of each other

Keywords Acute toxicity · *Aliivibrio fischeri* · Emerging contaminants · Mixtures toxicity · Pharmaceuticals

Introduction

The rapid improvement of analytical instrumentation and methods in the late twentieth century led to a large number of substances, previously undetectable, to emerge as environmental contaminants (Taylor and Senac 2014; Nilsen et al. 2019). Among this class of so-called emerging contaminants, there are drugs which are extensively used in human and veterinary medicine, considerably prolonging the life of living beings. A previous study (Reis et al. 2019) evaluated the occurrence of 28 pharmaceuticals in different drinking water treatment plants located in Brazil. These authors found that the occurrence in the environment are at trace concentration (ranging from ng/L up to µg/L).

Antipyretics, analgesics, lipid regulators, antibiotics, antidepressants, chemotherapy agents, antidiabetics, gastric pH regulators and contraceptive drugs are among the most consumed pharmaceuticals. In the present study, four different drugs were chosen based on their world wild consumption and previous monitoring researches. They are diazepam, metformin, omeprazole and simvastatin, which their main physicochemical properties are presented in Table 1. These drugs were recurrent in Brazilian waters according to previous studies (Foureaux et al. 2019; Reis et al. 2019) and composes the list of medicines provided free of charge by the Brazilian public health system (SUS), which corroborates with these drugs consumption and occurrence in the environment. These substances are not only present in the sewers, but also in water bodies in concentrations ranging from 8–6323 ng/L (Tambosi et al. 2010; Reis et al. 2019). Reis et al. (2019) found metformin in surface waters in Brazil in concentrations ranging from 8

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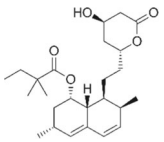
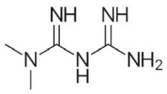
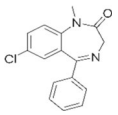
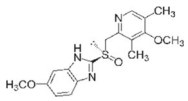
Table 1 Physicochemical properties of the studied drugs, diazepam, metformin, omeprazole and simvastatin

Pharmaceutical	Class	Chemical formula (Mol. wt. in g/mol)	KH (atm. m³/mol)	Molar volume (cm^3)	Structure	pKa	LogKow	Solubility (mg/mL)
Simvastatin	Lipid regulator	$C_{25}H_{28}O_5$ (418.574)	–	–		–	4.68	0.07
Metformin	Antidiabetic	$C_4H_{11}N_5$ (129.167)	3.46E−09	101		12.4	−2.64	1063.24
Diazepam	Antianxiety	$C_{16}H_{13}ClN_2O$ (284.743)	–	–		3.4	2.90	69.35
Omeprazole	Antacid	$C_{17}H_{19}N_3O_3S$ (345.417)	3.62E−06	252		1.2; 7.4	2.23	0.34

Reference: Patel et al. (2019)

to 203 ng/L. Diazepam was also detected in surface water in Brazil at concentrations up to 763 ng/L (Böger et al. 2018).

High medicines consumption, combined with their presence in the environment, raises concerns about its consequences since these compounds are bioactive and therefore capable of causing effects in living systems (Ginebreda et al. 2010; Nilsen et al. 2019; Patel et al. 2019; Peña-Guzmán et al. 2019). Moreover, many of these substances are designed to exhibit persistence in organisms (Fent et al. 2006; Patel et al. 2019), exacerbating the possible consequences of their environmental presence.

There are several organisms' classes that can be used to assess toxicity, but the use of *Aliivibrio fischeri*, a luminescent marine bacterium, is highlighted. Acute toxicity testing with this organism is considered an effective alternative due to the correlation with other bioassays using fish and invertebrates and the speed of results obtainment (Kaiser 1998). Correlation studies of *Aliivibrio fischeri* toxicity results and other aquatic organisms are quite numerous, which provides greater confidence in the use of this micro-organism in ecotoxicological tests (Kaiser and Palabrica 1991; Zhao et al. 1993; Kaiser et al. 1994; Dong et al. 2019; Baek et al. 2019; Zuriaga et al. 2019).

The evaluation of mixtures toxicity in the environment is important because although researches usually analyze a substance separately, that is not the environmental reality (Gomez-Eyles et al. 2009; Lindim et al. 2019; Ukić et al. 2019). Chemicals are subject to interactions and modifications, they can mix, and are able to potentiate or nullify the toxic effect of another.

Researches on toxicity of mixtures have shown increasing reach in the scientific community. These studies include in vitro and in vivo studies (Cedergreen et al. 2012; Coors et al. 2012; Boyd et al. 2013), evaluation of toxicity using combined effects models (Moser et al. 2012; Crépet et al. 2013; Hertzberg et al. 2013; Webster 2013), environmental impacts analysis (Løkke 2010; Allan et al. 2012), risk assessment studies (Johnson et al. 2013; Løkke et al. 2013; Meek 2013) and examination of chemical reactivity in the context of complex mixtures (Goel et al. 2013). While the specifics of these surveys vary, their common goal is to improve our ability to predict the effects of exposure to chemicals' mixtures.

According to Cleuvers (2005), two different concepts are used to predict the mixtures toxicity, called concentration addition (CA) and independent action (IA) models. The CA model assumes that components of a chemical mixture share a common action mechanism, i.e.: each component has the same specific interaction with a molecular target in the test organism (Berenbaum 1985). By contrast, the IA model assumes different, not similar, action mechanisms among the mixture components, i.e.: the toxics interact with different molecular targets, resulting in a common toxicological response by different reactions chains in an organism (Faust et al. 2003; Cleuvers 2003). Such concepts, for Faust et al. (2003), represent different hypotheses about the functional relationship between the toxicity of substances in individual action and combined action, and may lead to possible synergism and antagonisms.

A previous work exemplified the synergistic and antagonistic toxicity impact of drugs. Yang et al. (2008) tested

toxicity of twelve antibiotics: triclosan, triclocarban, roxithromycin, clarithromycin, tylosin, tetracycline, chlortetracycline, norfloxacin, sulfamethoxazole, ciprofloxacin, sulfamethazine and trimethoprim; here arranged in ascending toxicity order. Analyzes were performed with the algae *Pseudokirchneriella subcapitata* in order to assess growth inhibition. Among the results, it was noted the important antagonistic effect of the triclosan and norfloxacin combination, where the mixture was less toxic than its own components individually considered.

Cleuvers (2003) conducted tests with *Daphnia magna*, *Desmodesmus subspicatus* and *Lemna minor* organisms exposed to clofibric acid, carbamazepine, propranolol, metoprolol, ibuprofen, diclofenac, naproxen, captopril and metformin. In this research, analysis with various pharmaceuticals' combinations revealed more toxic effects than expected by only measuring the drugs individual toxicity (synergism), leading to the fact that it is difficult to predict toxicity of mixtures from single compound toxicity data.

Given this scenario, this research aimed at the investigation of the acute toxicity of simvastatin, metformin, diazepam, omeprazole and all their possible mixtures, to *Aliivibrio fischeri*. It is also intended to verify if the mixture of the aforementioned substances generates synergistic or antagonistic toxic effects. It should be mentioned that discussion in this article will also happen based in by-products formation in mixtures, which might modify samples' toxicity.

Materials and methods

Materials

Solutions containing the studied drugs diluted in Milli-Q water were prepared. The active ingredients were purchased from Sigma-Aldrich and have purity levels higher than 99%. It should be noted that the second and third stages (corresponding to mixture toxicity assays; see "Toxicity tests") were performed in triplicate.

Individual pharmaceuticals analysis

The objective at this stage was to prepare saturated solutions (initial concentration: simvastatin: 0.07 mg/mL; metformin: 1063.24 mg/mL; diazepam: 69.35 mg/mL; and omeprazole: 0.34 mg/mL; corresponding to their maximum solubility in water at 25 °C) of each drug in Milli-Q water and immediately make dilutions in series (from solubility up to 0.001 µg/mL; dilution factor: 1:2) that would correspond to the effective concentration (EC) or toxicity values. The pharmaceutical compounds were quantified after analysis with a HPLC-MS system (see "HPLC-MS analysis"). The stock solutions were stored in the absence of light, at <-20 °C and their concentrations were verified prior to each toxicity test in order to assure their preservation.

Analysis with mixtures of pharmaceuticals

Aiming to test possible synergistic or antagonistic effects between the drugs mixtures, ecotoxicological tests with all possible mixtures of the active ingredients studied were performed. There were 11 possible mixtures to be formed between the four active ingredients evaluated, corresponding to six possibilities of two-drugs mixtures, four possibilities of three-drugs mixtures and a unique possibility to mix the four-drugs together (all combinations represented in "Toxicity of the pharmaceutical's mixtures," Table 4).

Possible antagonism or synergism phenomena were assessed taking into account both the active ingredient individual EC_{10} (concentration capable of causing effect in 10% of tested population; established as described in "Individual pharmaceuticals analysis") and the number of compounds in the mixture (Cleuvers 2005). That means, that to establish the mixture concentration between two drugs, $EC_{10}/2$ corresponding to each of those drugs were used for the test organism. The same occurred with mixtures of more compounds, in which the concentration adopted for mixing was $EC_{10}/3$, in the case of three components mixture, and $EC_{10}/4$ in case when all four compounds were used. Following the concept of concentration addition, the mixture effect should add up to a total effect 10%. As described previously, dilutions were performed in series using Milli-Q water and EC_{10} determined by linear concentration-effect curves.

Furthermore, to adequately assess the effect of the mixtures, toxicological tests were carried out with the substances separately in their $EC_{10}/2$, $EC_{10}/3$ and $EC_{10}/4$ concentrations.

Toxicity tests

Acute ecotoxicological tests were performed with the luminescent marine bacterium *Aliivibrio fischeri* provided by SDI using a MICROTOX® model 500 Analyzer (SDI), following the ABNT NBR 15411-3 (ABNT 2012) and MICROTOX® Omni Software standard protocols. Analyzes were divided into three steps: the first one was designed to meet the toxicity of the drugs individually to the bacteria, the second was with equitoxic mixtures containing each of the drugs in their $EC_{10}/2$, $EC_{10}/3$ and $EC_{10}/4$ and the last with all possible mixtures of compounds. Firstly, acute toxicity was determined from nine dilutions of the initial solution in measurements of bacteria luminescence in 30 min. To determine toxic effect, the software performs a comparison based on emitted sample light from its various

dilutions and the control solution. In the second and third step, the procedure was different, since the objective was not to find a effective concentration, but the effect caused to the bacteria in certain individual or mixture concentrations. In this case, there was an adaptation of the recommended procedure by NBR 15411-3 (ABNT 2012), using the three following equations. The first one was used to calculate the correction factor (f_{kt}) from the measured light output, correcting the initial values of all samples, before using them as reference values to determine the decrease in luminescence caused by the sample, as follows:

$$f_{kt} = I_{kt}/I_0 \tag{1}$$

I_{kt} means the luminescence intensity of the control after the exposure period and I_0 is the intensity of control luminescence, immediately before addition of the diluent, in relative luminescence units. The second equation ensures the usage of the corrected values for each reading, as follows:

$$I_{ct} = I_{kt} * \overline{f_{kt}} \tag{2}$$

$\overline{f_{kt}}$ is the average of the controls' f_{kt} and I_{ct} represents the corrected values of I_0. Finally, the sample effect on the bacteria's luminescence can be calculated from the following equation:

$$E_t = (I_{ct} - I_{kt}/I_{ct}) * 100 \tag{3}$$

E_t is the inhibitory effect of the bacteria suspension after the exposure period, expressed as a percentage (%). The $EC_{x\%}$ for each compound was determined through concentration-effect curves.

In all the above steps, before carrying out the tests, the samples were subjected to pH adjustment between an acceptable range of 6.5–7.5 with HCl or NaOH and dilution with a 2% NaCl solution, called diluent. These possible dilutions of the compounds were considered in the effect's calculations.

The salinity of the samples was checked with a high-resolution refractometer (RTS-101ATC, Instrutherm). Those samples which have salinity values below 22% must receive the addition of osmotic-adjusting solution for test execution. In this study, all samples showed salinity values below 22%.

Mixtures' effect: synergism and antagonism

The two models used in this study to predict mixtures toxicity were concentration addition (CA) and independent action (IA). The concept of CA can be described mathematically by Eq. (4) (Berenbaum 1985):

$$\sum_{(i=1)}^{n} c_i/EC_{x,i} = 1 \tag{4}$$

c_i is the concentration of each substance from the mixture and $EC_{x,i}$ is the substance concentration that causes x% effect on the tested population.

Contrastingly, the equation that describes the combined effect that acts upon the independent action model for a mixture is given by:

$$E_{C,Mix} = 1 - \prod_{i=1}^{n} 1 - E_{Ci} \tag{5}$$

E_{Ci} is the effects of the individual substances and $E_{C,Mix}$ is the total mixture effect.

The described models provide an initial mathematical basis for the prediction of toxicity effects of the mixtures, but interactions among compounds may occur, which may result with deviations of mixture toxicity from the model(s) applied. If mixture results in toxicity greater than the one predicted by the model, the mixture components act synergistically. On the other hand, if the combined action of mixture components s result with toxicity smaller than the one predicted by the model, the components acts antagonistically. The methodology for identifying possible synergisms and antagonisms was proposed by Cedergreen et al. (2007).

Finally, in order to compare deviation of observed mixtures toxicities from the applied models, the method of effect residual ratio (*ERR*), proposed by Wang et al. (2010) was used. The equation that describes the ERR model is:

$$ERR = \left(E_{prd} - Eobs/Eobs\right) * 100 \tag{6}$$

E_{prd} and E_{obs} are, respectively, the effect values predicted by the IA or CA models and the observed effects values to a given concentration level.

HPLC-MS analysis

The analytical determinations were performed using an HPLC (LC 20A, Shimadzu) coupled to a mass spectrometer MicroTOF QII (Bruker), with an electronic electrospray ionization (ESI), at a resolution of 12,000 *m/z*. The chromatographic separation was achieved by a reverse phase C_{18} column (Shim-pack XR-ODS). The mobile phases used were water (A), methanol (B) and formic acid. Furthermore, the solvent composition started with 10% of B, increased to 70% in 3 min, then again increased to 95% over 6 min and remaining stable for 7 min. After, it was decreased to 10% of B in 10 min, and finally remained at 10% of B for 15 min. The injected sample volume was 20 µL at 20 °C.

Table 2 Acute toxicity to *Aliivibrio fischeri* (30 min) of omeprazole, simvastatin, diazepam and metformin

	EC_{10}	EC_{20}	EC_{50}
Metformin	870.79 mg/L	NA[a]	NA[a]
Diazepam	8.69 mg/L	28.40 mg/L	NA[a]
Simvastatin	9.30 µg/L	29.00 µg/L	NA[a]
Omeprazole	5.30 µg/L	6.50 µg/L	15.00 µg/L

[a]Not available: there was no value for this sample, which means that the toxicity found was lower than this parameter

The drugs quantification consisted in the concoction of an external calibration curve from the active ingredients, using different concentrations (0.50, 1.00, 2.00, 10.00 and 20.00 mg/L) in methanol and water in a 1:1 ratio. The peak area generated by the sample was compared to the external calibration curve to determine the exact drug concentration.

Results and discussion

Individual toxicity of pharmaceuticals

Toxicity was observed for all drugs tested in a 30-min test, as shown in Table 2.

Omeprazole was observed to be the most toxic among the active ingredients, followed by simvastatin, diazepam and finally metformin. Furthermore, the only effective concentration identified in all drugs was EC_{10}. For this reason, this parameter was selected as the basis for the next stage, analysis of mixtures toxicity. It should be mentioned that only one of the drugs has the EC_{50} value, which is a more commonly referred parameter in the literature. This pharmaceutical is omeprazole, with a value of EC_{50} of 0.015 mg/L. The disparity in the acute toxicity observed among the pharmaceutical compounds is most probably caused by the effects derived from the different substituents in their molecule structure. The following order of contribution to toxicity was reported Dong et al. (2019): $-NO_2$ > -Cl > -CH3 > $-NH_2$ > -OH, most of them possible to be formed by omeprazole decomposition.

Ortiz de Garcia et al. (2014) reported that the toxicity of omeprazole (EC_{50}) for *Aliivibrio* fischeri was 1.76 mg/L in the 30-min test. Another study (Zuriaga et al. 2019), reported that the omeprazole EC_{50} for *Aliivibrio fischeri* was 3.7 mg/L (30-min test). It is noteworthy that although both of these papers have observed high toxicity of the mentioned compound for *Aliivibrio fischeri*, the values are different probably due to omeprazole speciation. Both standard procedures adopted by Ortiz de Garcia et al. (2014) and Zuriaga et al. (2019) are different from this study, and the difference in the test pH directly affects the omeprazole speciation and bioavailability. Under higher pH conditions, the pharmaceutical is considered more bioavailable (Prichard et al. 1985) and different ionic species may be presented, leading to higher inhibitory effects.

The second most toxic to *Aliivibrio fischeri* was simvastatin, and although there was no EC_{50} value for this compound in the current study, some researches discuss the consequences of this anti-hypertensive for the reproduction and survival of amphibians (Neuparth et al. 2014), damage caused to fish cells (Ellesat et al. 2011; Ribeiro et al. 2015) and chronic toxicity to copepods (Dahl et al. 2006).

In contrast, about diazepam, some articles identify the impact on living beings' life cycle (Muñoz et al. 2008) and acute toxicity to cnidarian (Pascoe et al. 2003). According to Nunes et al. (2005), diazepam has EC_{50} of 12.7, 12.2 and 16.5 mg/L for *Gambusia holbrooki*, *Artemia parthenogenetica* and *Tetraselmis chuii*, respectively. It is noteworthy that these are higher toxicity values than those identified for *Aliivibrio fischeri* in this article.

It is also noteworthy that hydrophobic compounds pose a higher toxicity compared to hydrophilic compounds, as they present higher propensity for interacting with the cell membrane. A similar trend was noticed in this study, in which metformin, considered a hydrophilic compound, presented the lowest luminescence inhibition. Furthermore, excluding simvastatin, a significant (p value < 0.05) increase in toxic effect (lower EC_{10}) was observed when increasing the logKow. The most hydrophobic compound (simvastatin) did not show the highest luminescence inhibition. This may be due to the other factors such as drugs physical-chemical characteristics, mode of action, functional groups among others. Baillie (2008) showed that an interplay of drug-metabolizing enzymes and drug transporters can represent a critical determinant of drug disposition, drug interactions, and toxicity in animals and humans.

Finally, it should be mentioned a relevant research that dealt with acute toxicity of metformin combined with other drugs for three test organisms (Cleuvers 2003). In this study, metformin hydrochloride showed EC_{50} 64, 320 and 110 mg/L for *Daphnia magna*, *Desmodesmus subspicatus* and *Lemna minor*, respectively. Again, these are higher values than those identified for *Aliivibrio fischeri* in this article.

Toxicity of the pharmaceutical's mixtures

The results of mixtures toxicity, regarding $EC_{10}/2$, $EC_{10}/3$ $EC_{10}/4$, are presented in Table 3.

Some of the tests indicated a phenomenon called hormesis, which means a positive deviation observed in the test organism in presence of the contaminant. Furthermore, hormesis can be characterized by low-dose stimulation and high-dose inhibition. Several reports have shown phenomenon of hormesis in toxicity assays as well as in the natural

environment (Calabrese and Baldwin 2003; Calabrese 2008), especially when organisms are exposed to mixtures, rather than single chemicals. It is emphasized that hormesis detection is not a sign that the contaminant is beneficial to the organism, on the contrary, it could be a case of a contaminant that is toxic in chronic toxicity tests or an acute toxicity tests in higher concentration. Also according to Calabrese (2008), hormesis is considered an evolutionary mechanism, because it is a compensation or an adaptive response of organisms to overcome some imbalance in order to prevent the extinction of the species.

After the preliminary tests were completed, it was possible to develop the mixtures analyzes, identify the responses provided by CA and IA models, as well as verify any discrepancies between the models and reality (ERR). The results are compiled in Table 4.

Table 3 Observed effect (%) for *Aliivibrio fischeri* organism in a 30-min test at various concentrations

	Concentration	Effect (%)	Concentration	Effect (%)
	EC_{10}		$EC_{10}/2$	
Metformin	870.79 mg/L	10.0	435.40 mg/L	Hormesis
Diazepam	8.69 mg/L	10.0	4.35 mg/L	6.5
Simvastatin	9.30 µg/L	10.0	5.00 µg/L	2.7
Omeprazole	5.30 µg/L	10.0	3.00 µg/L	8.3
	$EC_{10}/3$		$EC_{10}/4$	
Metformin	290.26 mg/L	Hormesis	217.70 mg/L	Hormesis
Diazepam	2.90 mg/L	4.4	2.17 mg/L	Hormesis
Simvastatin	3.10 µg/L	Hormesis	2.30 µg/L	Hormesis
Omeprazole	1.80 µg/L	5.2	1.30 µg/L	Hormesis

Regarding the binary mixtures, all of those containing metformin generate the phenomenon called hormesis, discussed above. Furthermore, this pharmaceutical alone, at $EC_{10}/2$, promotes the same effect when in binary mixtures. In this sense, because the result is below the predicted by mathematical models CA and IA, it is said that there is antagonism. Godoy et al. (2015) observed that another binary mixture with pharmaceuticals, propranolol hydrochloride and losartan potassium, behaved similarly in tests with the macrophyte *Lemna minor*.

The other binary mixtures (simvastatin + omeprazole, simvastatin + diazepam and diazepam + omeprazole) show toxicity, and in a higher value than expected by both CA and IA models, featuring synergism. Similarly, Zou et al. (2012) found a synergistic effect when testing mixtures of sulfonamides in contact with the luminescent bacterium *Photobacterium phosphoreum*. It must be emphasized that the mixture of simvastatin and diazepam is the one that generated the highest toxicity effects on *Aliivibrio fischeri* in this work.

About the ternary mixtures, all are toxic, with effective concentration close to those predicted by the CA model. It should be noted that the mixture of metformin + simvastatin + diazepam has the highest value among the ternary mixtures, again bringing out specifically the interaction between simvastatin and diazepam, which in a binary mixture generated a high toxicity effect. The referred ternary mixture, as well as the sample containing metformin, simvastatin and diazepam, present a synergistic behavior for both models. Regarding the combination between metformin, simvastatin and omeprazole, it is synergistic to the IA model and corresponds to the CA model prediction. The last ternary mixture, simvastatin + omeprazole + diazepam, behaves similarly to that provided by both mathematical models. As in this article, Phyu et al. (2011), tested the toxicity of pesticide mixtures for *Ceriodaphnia*

Table 4 Identified effect on acute toxicity tests with *Aliivibrio fischeri* (30 min) with pharmaceutical mixing samples, inhibition provided by the CA and IA models and values of residual ratios effect (% ERR) calculated for differences between the real effects and those predicted by the CA and IA model

Mixtures	Effect observed in the toxicity test (%)	Predicted effect by CA model (%)	ERR (%) from the CA model	Predicted effect by IA model (%)	ERR (%) from the IA model
Metformin + simvastatin	Hormesis	10.0	100.0	2.7	100.0
Metformin + diazepam	Hormesis	10.0	100.0	6.5	100.0
Metformin + omeprazole	Hormesis	10.0	100.0	8.3	100.0
Simvastatin + diazepam	17.1	10.0	41.6	9.0	47.5
Simvastatin + omeprazole	11.4	10.0	12.7	10.8	6.0
Diazepam + omeprazole	14.9	10.0	33.1	14.3	4.7
Metformin + simvastatin + omeprazole	9.7	10.0	2.5	5.2	4.6
Simvastatin + omeprazole + diazepam	9.9	10.0	0.8	9.4	5.5
Metformin + omeprazole + diazepam	10.7	10.0	7.0	9.4	12.9
Metformin + simvastatin + diazepam	14.0	10.0	28.8	4.4	68.7
Simvastatin + omeprazole + diazepam + metformin	16.2	10.0	38.3	0.0	100.0

dubia, founding better match to the CA model compared to IA. Other than that, the referred authors encouraged the use of this method whenever there is similarity of action of the compounds tested.

The quaternary mixtures generated one of the highest toxicity effects (16.23%—Table 4), even though the four compounds, individually, are in concentrations below the one where there is no effect (NOEC). While this mixture generates a synergistic effect compared to both models, this behavior was predicted by Cleuvers (2005) when discussing the conditions of the CA model and reports that substances applied below its concentration of no observed effect may, however, contribute to the total effect of the mixture. A similar result was found by Backhaus et al. (2011), when investigating the toxic potential for periphyton of the pharmaceuticals fluoxetine, propranolol, triclosan, zinc pyrithione, clotrimazole and its mixtures. In the cited study, clear effects were identified on the organism when in contact with the mixture of pharmaceuticals, even when all five components were present at non-observed effect concentration (NOEC).

About the adequacy of the findings to those provided by CA and IA models (ERR), the values are in most cases close to the predicted values, except those results where the mixture generates hormesis or where the mixture is quaternary. Data analysis clarifies that the clear majority of the values presents a discrepancy of 0.8 to 12.92% to that suggested by CA and AI models. This denotes a closer proximity to these models than those found by other authors, such as Godoy et al. (2015), where ERR reaches 228%. It can also be said that the data in this article is closer to the predictions of the CA model compared to IA. The CA model proposes that the components of a chemical mixture share a common mechanism of action, which means that each component has the same specific interaction with a molecular target in the test organism (Berenbaum 1985).

Still, it must be noted that the models' adjustment to reality is not precise, which means that it is difficult to accurately predict the toxicity of mixtures without actually performing tests with real samples. That is because other factors, such as the byproducts formation, can affect the mixtures toxicity, as described in the literature (Cleuvers 2003; Yang et al. 2008; Godoy et al. 2015) and will be discussed below.

Identification of transformation products (TPs) formation in the mixtures and their relationship with toxicity

The analysis of transformation products was carried out right after the drugs mixture. If not, the mixture was stored in 1.5 mL amber vials at <−20 °C to avoid degradation. The formation of these new products could be due to the interactions that can be established between the drugs molecules, for example hydrogen bonding interactions between hydroxyl and amino groups, as well as many other intermolecular interactions at lower energies (Zuriaga et al. 2019). In Table S1 are shown the transformation products found after mixing the pharmaceuticals. It was observed the existence of byproducts in the mixtures under analysis, many of these compounds are common to several mixtures. Examples are $C_9H_{20}NO$, $C_6H_{11}N_6O$, $C_{16}H_9N_2O$, $C_4H_7N_{12}$, and especially $C_6H_{18}N_3O_3$ and $C_{14}H_{42}N_3O_2$. This similarity is expected, since the precursors of the eleven mixtures were the same four drugs. Based on this result, it is expected homogeneous toxicity results, which did not happen.

One possible explanation for the highly heterogeneous toxicity results are the presence of infrequent TPs, which occurred in only one or two samples. As can be seen, it was found 13 compounds that did not replicate in any of the other samples. For example, it was observed that the mixtures simvastatin + diazepam and metformin + omeprazole + diazepam presented an increase in their toxicity compared to the isolated substances. This may be due to the presence of TP $C_{13}H_{25}N_2O$, which showed significative presence in both samples and only in them.

As already discussed, the mixture containing metformin + simvastatin + diazepam showed the highest toxicity results. In this sample, it was found the compounds C_8H_{13}, C_8H_{15}, $C_6H_{11}N_6O$ and $C_{16}H_9N_2O$. It should also be highlighted that the degradation product C_8H_{13} was only found in this mixture, suggesting that the reported toxicity is related to the presence of these TPs.

Finally, it is discussed the quaternary mixture, which has one of the highest toxic effects (16.23%). In this mixture, it was noted a large quantity of TP $C_9H_{11}N_6O$, which was only identified in this sample. Therefore, it is believed that this byproduct may be related to the toxicity of the mentioned mixture.

It is known that more tests are necessary for the effective confirmation of the reasons why some mixtures are more toxic than others. Despite this, the identification of byproducts confirmed by this work clarifies that when two chemicals are combined, other compounds are generated, which interferes in the survival of living organisms. Other authors (De Souza Santos et al. 2014; Hassold and Backhaus 2014; Long et al. 2016) do the same discussion, pointing to the toxic effects of byproducts formed in mixtures or by degradation processes.

Conclusions

Acute toxicity effects of diazepam, metformin, omeprazole and simvastatin were measured for the organism *Aliivibrio*

fischeri, both individually and in mixtures. In terms of individual toxicity, omeprazole (EC_{10}: 0.0053 mg/L) shows the higher toxicity among the active ingredients, followed by simvastatin (EC_{10}: 0.0093 mg/L), diazepam (EC_{10}: 8.69 mg/L) and, finally, metformin (EC_{10}: 870.79 mg/L). It was also noticed a significant increase (p value < 0.05) in toxicity effect while increasing the compound hydrophobicity, as they present higher propensity for interacting with the cell membrane. Synergism, antagonism, and hormesis phenomena were found when mixture toxicity was tested. It was emphasized that hormesis detection is not a sign that the contaminant is beneficial to the organism, on the contrary, it could be a case of a contaminant that is toxic in chronic toxicity tests or an acute toxicity tests in higher concentration. Furthermore, a series of transformation products was found in these solutions, which may directly affect their toxicities. These results indicate that although mathematical models which predict mixture toxicity are important tools for environmental management, they may fail to address many possible phenomena. This work points to the urgent need for more studies about mixtures' behavior, since chemicals are subject to interactions and modifications, can mix and are able to potentiate or nullify the toxic effects of each other.

Data availability

The research data for this article is not available. Data can be made available on request.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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References

ABNT (2012) Aquatic ecotoxicology — Determination of the inhibitory effect of aqueous samples on the light emission of Vibrio fischeri (luminescent bacteria test) Part 2: Method using liquid-dried bacteria (ABNT NBR 15411-2:2012). 2nd edn. 21 pp. Avaliable at https://www.abntcatalogo.com.br/norma.aspx?ID=148920

Allan SE, Smith BW, Tanguay RL, Anderson KA (2012) Bridging environmental mixtures and toxic effects. Environ Toxicol Chem 31:2877–2887. https://doi.org/10.1002/etc.2018

Backhaus T, Porsbring T, Arrhenius Å et al. (2011) Single-substance and mixture toxicity of five pharmaceuticals and personal care products to marine periphyton communities. Environ Toxicol Chem 30:2030–2040. https://doi.org/10.1002/etc.586

Baek I-H, Kim Y, Baik S, Kim J (2019) Investigation of the synergistic toxicity of binary mixtures of pesticides and pharmaceuticals on *Aliivibrio fischeri* in Major River Basins in South Korea. Int J Environ Res Public Health 16:208. https://doi.org/10.3390/ijerph16020208

Baillie TA (2008) Metabolism and toxicity of drugs. Two decades of progress in industrial drug metabolism. Chem Res Toxicol 21:129–137. https://doi.org/10.1021/tx7002273

Berenbaum MC (1985) The expected effect of a combination of agents: the general solution. J Theor Biol 114:413–431. https://doi.org/10.1016/S0022-5193(85)80176-4

Böger B, Do Amaral B, Estevão PL, Da S et al. (2018) Determination of carbamazepine and diazepam by SPE-HPLC-DAD in Belém River water, Curitiba-PR/Brazil. Ambient e Agua 13:1. https://doi.org/10.4136/ambi-agua.2196

Boyd J, Vrana JA, Williams HN (2013) In vitro approach to predict post-translational phosphorylation response to mixtures. Toxicology 313:113–121. https://doi.org/10.1016/j.tox.2012.10.010

Calabrese EJ (2008) Hormesis: why it is important to toxicology and toxicologists. Environ Toxicol Chem 27:1451–1474. https://doi.org/10.1897/07-541.1

Calabrese EJ, Baldwin LA (2003) Toxicology rethinks its central belief. Nature 421:691–692. https://doi.org/10.1038/421691a

Cedergreen N, Kudsk P, Mathiassen SK et al. (2007) Reproducibility of binary-mixture toxicity studies. Environ Toxicol Chem 26:149–156. https://doi.org/10.1897/06-196R.1

Cedergreen N, Sørensen H, Svendsen C (2012) Can the joint effect of ternary mixtures be predicted from binary mixture toxicity results? Sci Total Environ 427–428:229–237. https://doi.org/10.1016/j.scitotenv.2012.03.086

Cleuvers M (2005) Initial risk assessment for three β-blockers found in the aquatic environment. Chemosphere 59:199–205. https://doi.org/10.1016/j.chemosphere.2004.11.090

Cleuvers M (2003) Aquatic ecotoxicity of pharmaceuticals including the assessment of combination effects. Toxicol Lett 142:185–194. https://doi.org/10.1016/S0378-4274(03)00068-7

Coors A, Dobrick J, Möder M, Kehrer A (2012) Mixture toxicity of wood preservative products in the fish embryo toxicity test. Environ Toxicol Chem 31:1239–1248. https://doi.org/10.1002/etc.1827

Crépet A, Héraud F, Béchaux C et al. (2013) The PERICLES research program: an integrated approach to characterize the combined effects of mixtures of pesticide residues to which the French population is exposed. Toxicology 313:83–93. https://doi.org/10.1016/j.tox.2013.04.005

Dahl U, Gorokhova E, Breitholtz M (2006) Application of growth-related sublethal endpoints in ecotoxicological assessments using a harpacticoid copepod. Aquat Toxicol 77:433–438. https://doi.org/10.1016/j.aquatox.2006.01.014

De Souza Santos LV, Teixeira DC, Jacob RS et al. (2014) Evaluation of the aerobic and anaerobic biodegradability of the antibiotic norfloxacin. Water Sci Technol 70:265–271. https://doi.org/10.2166/wst.2014.214

Dong Y, Fang Z, Xu Y et al. (2019) The toxic effects of three active pharmaceutical ingredients (APIs) with different efficacy to Vibrio fischeri. Emerg Contam 5:297–302. https://doi.org/10.1016/j.emcon.2019.08.004

Ellesat KS, Yazdani M, Holth TF, Hylland K (2011) Species-dependent sensitivity to contaminants: an approach using primary hepatocyte cultures with three marine fish species. Mar Environ Res 72:216–224. https://doi.org/10.1016/j.marenvres.2011.09.003

Faust M, Altenburger R, Backhaus T et al. (2003) Joint algal toxicity of 16 dissimilarly acting chemicals is predictable by the concept of independent action. Aquat Toxicol 63:43–63. https://doi.org/10.1016/S0166-445X(02)00133-9

Fent K, Weston AA, Caminada D (2006) Ecotoxicology of human pharmaceuticals. Aquat Toxicol 76:122–159. https://doi.org/10.1016/j.aquatox.2005.09.009

Foureaux AFS, Reis EO, Lebron Y et al. (2019) Rejection of pharmaceutical compounds from surface water by nanofiltration and reverse osmosis. Sep Purif Technol 212:171–179. https://doi.org/10.1016/j.seppur.2018.11.018

Ginebreda A, Muñoz I, de Alda ML et al. (2010) Environmental risk assessment of pharmaceuticals in rivers: relationships between hazard indexes and aquatic macroinvertebrate diversity indexes in the Llobregat River (NE Spain). Environ Int 36:153–162. https://doi.org/10.1016/j.envint.2009.10.003

Godoy AA, Kummrow F, Pamplin PAZ (2015) Ecotoxicological evaluation of propranolol hydrochloride and losartan potassium to *Lemna minor* L. (1753) individually and in binary mixtures. Ecotoxicology 24:1112–1123. https://doi.org/10.1007/s10646-015-1455-3

Goel S, Evans-Johnson JA, Georgieva NI, Boysen G (2013) Exposure profiling of reactive compounds in complex mixtures. Toxicology 313:145–150. https://doi.org/10.1016/j.tox.2012.11.012

Gomez-Eyles JL, Svendsen C, Lister L et al. (2009) Measuring and modelling mixture toxicity of imidacloprid and thiacloprid on *Caenorhabditis* elegans and *Eisenia fetida*. Ecotoxicol Environ Saf 72:71–79. https://doi.org/10.1016/j.ecoenv.2008.07.006

Hassold E, Backhaus T (2014) The predictability of mixture toxicity of demethylase inhibiting fungicides to *Daphnia* magna depends on life-cycle parameters. Aquat Toxicol 152:205–214. https://doi.org/10.1016/j.aquatox.2014.04.009

Hertzberg RC, Pan Y, Li R et al. (2013) A four-step approach to evaluate mixtures for consistency with dose addition. Toxicology 313:134–144. https://doi.org/10.1016/j.tox.2012.10.016

Johnson LA, Welch B, Whitfield SM (2013) Interactive effects of pesticide mixtures, predators, and environmental regimes on the toxicity of two pesticides to red-eyed tree frog larvae. Environ Toxicol Chem 32:2379–2386. https://doi.org/10.1002/etc.2319

Kaiser KLE (1998) Correlations of Vibrio fischeri bacteria test data with bioassay data for other organisms. Environ Health Perspect 106:583–591. https://doi.org/10.2307/3433809

Kaiser KLE, McKinnon MB, Fort FL (1994) Interspecies toxicity correlations of rat, mouse and *Photobacterium phosphoreum*. Environ Toxicol Chem 13:1599–1606. https://doi.org/10.1002/etc.5620131008

Kaiser KLE, Palabrica VS (1991) *Photobacterium phosphoreum* toxicity data index. Water Pollut Res J Can 26:361–431. https://doi.org/10.2166/wqrj.1991.017

Lindim C, de Zwart D, Cousins IT et al. (2019) Exposure and ecotoxicological risk assessment of mixtures of top prescribed pharmaceuticals in Swedish freshwaters. Chemosphere 220:344–352. https://doi.org/10.1016/j.chemosphere.2018.12.118

Løkke H (2010) Novel methods for integrated risk assessment of cumulative stressors—results from the NoMiracle project. Sci Total Environ 408:3719–3724. https://doi.org/10.1016/j.scitotenv.2010.05.009

Løkke H, Ragas AMJ, Holmstrup M (2013) Tools and perspectives for assessing chemical mixtures and multiple stressors. Toxicology 313:73–82. https://doi.org/10.1016/j.tox.2012.11.009

Long X, Wang D, Lin Z et al. (2016) The mixture toxicity of environmental contaminants containing sulfonamides and other antibiotics in *Escherichia coli*: differences in both the special target proteins of individual chemicals and their effective combined concentration. Chemosphere 158:193–203. https://doi.org/10.1016/j.chemosphere.2016.05.048

Meek MEB (2013) International experience in addressing combined exposures: increasing the efficiency of assessment. Toxicology 313:185–189. https://doi.org/10.1016/j.tox.2012.09.015

Moser VC, Padilla S, Simmons JE et al. (2012) Impact of chemical proportions on the acute neurotoxicity of a mixture of seven carbamates in preweanling and adult rats. Toxicol Sci 129:126–134. https://doi.org/10.1093/toxsci/kfs190

Muñoz I, José Gómez M, Molina-Díaz A et al. (2008) Ranking potential impacts of priority and emerging pollutants in urban wastewater through life cycle impact assessment. Chemosphere 74:37–44. https://doi.org/10.1016/j.chemosphere.2008.09.029

Neuparth T, Martins C, Santos CBdl et al. (2014) Hypocholesterolaemic pharmaceutical simvastatin disrupts reproduction and population growth of the amphipod *Gammarus locusta* at the ng/L range. Aquat Toxicol 155:337–347. https://doi.org/10.1016/j.aquatox.2014.07.009

Nilsen E, Smalling KL, Ahrens L et al. (2019) Critical review: grand challenges in assessing the adverse effects of contaminants of emerging concern on aquatic food webs. Environ Toxicol Chem 38:46–60. https://doi.org/10.1002/etc.4290

Nunes B, Carvalho F, Guilhermino L (2005) Acute toxicity of widely used pharmaceuticals in aquatic species: *Gambusia holbrooki*, *Artemia parthenogenetica* and *Tetraselmis chuii*. Ecotoxicol Environ Saf 61:413–419. https://doi.org/10.1016/j.ecoenv.2004.08.010

Ortiz de García SA, Pinto Pinto G, García-Encina PA, Irusta-Mata R (2014) Ecotoxicity and environmental risk assessment of pharmaceuticals and personal care products in aquatic environments and wastewater treatment plants. Ecotoxicology 23:1517–1533. https://doi.org/10.1007/s10646-014-1293-8

Pascoe D, Karntanut W, Müller CT (2003) Do pharmaceuticals affect freshwater invertebrates? A study with the cnidarian *Hydra vulgaris*. Chemosphere 51:521–528. https://doi.org/10.1016/S0045-6535(02)00860-3

Patel M, Kumar R, Kishor K et al. (2019) Pharmaceuticals of emerging concern in aquatic systems: chemistry, occurrence, effects, and removal methods. Chem Rev 119:3510–3673. https://doi.org/10.1021/acs.chemrev.8b00299

Peña-Guzmán C, Ulloa-Sánchez S, Mora K et al. (2019) Emerging pollutants in the urban water cycle in Latin America: a review of the current literature. J Environ Manag 237:408–423. https://doi.org/10.1016/j.jenvman.2019.02.100

Phyu YL, Palmer CG, Warne MSJ et al. (2011) A comparison of mixture toxicity assessment: examining the chronic toxicity of atrazine, permethrin and chlorothalonil in mixtures to *Ceriodaphnia* cf. Dubia. Chemosphere 85:1568–1573. https://doi.org/10.1016/j.chemosphere.2011.07.061

Prichard PJ, Yeomans ND, Mihaly GW et al. (1985) Omeprazole: a study of its inhibition of gastric pH and oral pharmacokinetics after morning or evening dosage. Gastroenterology 88:64–69. https://doi.org/10.1016/S0016-5085(85)80133-5

Reis EO, Foureaux AFS, Rodrigues JS et al. (2019) Occurrence, removal and seasonal variation of pharmaceuticals in Brasilian drinking water treatment plants. Environ Pollut 250:773–781. https://doi.org/10.1016/j.envpol.2019.04.102

Ribeiro S, Torres T, Martins R, Santos MM (2015) Toxicity screening of diclofenac, propranolol, sertraline and simvastatin using danio rerio and paracentrotus lividus embryo bioassays. Ecotoxicol Environ Saf 114:67–74. https://doi.org/10.1016/j.ecoenv.2015.01.008

Tambosi JL, de Sena RF, Favier M et al. (2010) Removal of pharmaceutical compounds in membrane bioreactors (MBR) applying submerged membranes. Desalination 261:148–156. https://doi.org/10.1016/j.desal.2010.05.014

Taylor D, Senac T (2014) Human pharmaceutical products in the environment—the "problem" in perspective. Chemosphere 115:95–99. https://doi.org/10.1016/j.chemosphere.2014.01.011

Ukić Š, Sigurnjak M, Cvetnić M et al. (2019) Toxicity of pharmaceuticals in binary mixtures: assessment by additive and non-additive toxicity models. Ecotoxicol Environ Saf 185:109696. https://doi.org/10.1016/j.ecoenv.2019.109696

Wang LJ, Liu SS, Zhang J, Li WY (2010) A new effect residual ratio (ERR) method for the validation of the concentration addition and independent action models. Environ Sci Pollut Res 17:1080–1089. https://doi.org/10.1007/s11356-009-0265-7

Webster TF (2013) Mixtures of endocrine disruptors: how similar must mechanisms be for concentration addition to apply? Toxicology 313:129–133. https://doi.org/10.1016/j.tox.2013.01.009

Yang LH, Ying GG, Su HC et al. (2008) Growth-inhibiting effects of 12 antibacterial agents and their mixtures on the freshwater microalga *Pseudokirchneriella subcapitata*. Environ Toxicol Chem 27:1201–1208. https://doi.org/10.1897/07-471.1

Zhao YH, Wang LS, Gao H, Zhang Z (1993) Quantitative structure-activity relationships—relationship between toxicity of organic chemicals to fish and to *Photobacterium phosphoreum*. Chemosphere 26:1971–1979. https://doi.org/10.1016/0045-6535(93)90024-Y

Zou X, Lin Z, Deng Z et al. (2012) The joint effects of sulfonamides and their potentiator on *Photobacterium phosphoreum*: differences between the acute and chronic mixture toxicity mechanisms. Chemosphere 86:30–35. https://doi.org/10.1016/j.chemosphere.2011.08.046

Zuriaga E, Lomba L, German B et al. (2019) Ecotoxicity in *Aliivibrio fischeri* of ibuprofen, omeprazole and their mixtures. Chem Ecol 35:102–114. https://doi.org/10.1080/02757540.2018.1540608