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Original Article

Effects of Non-steroidal Anti-inflammatory Drugs (Ibuprofen and Diclofenac) and Their Mixtures on the Growth of Three Green Algae

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Abstract

Ibuprofen (IBU) and diclofenac (DFN) are the most widely consumed non-steroidal anti-inflammatory (NSAIDs) worldwide. Since they are not completely metabolized in the human body, the unaltered active ingredients can reach aquatic ecosystems through domestic effluents. The aim of this study was to analyse the toxic effects of IBU and DFN, individually and in mixtures, on three freshwater green algae strains, the international standard strain *Raphidocelis subcapitata* and two native strains, *Ankistrodesmus fusiformis* and *Tetradesmus obliquus*. Bioassays were carried out according to the 72-h algal growth inhibition test. The concentration-response curve of the mixture was compared to predicted effects based on both the concentration addition (CA) and the independent action (IA) models. The two drugs tested individually were toxic to all three algal strains, with EC_{50} values <100 mg L^{-1}. According to these values, the most sensitive strain was *R. subcapitata* (IBU = 20 mg L^{-1} and DFN = 8 mg L^{-1}). The most resistant strain was *T. obliquus* (IBU = 74.1 mg L^{-1} and DFN = 92.7 mg L^{-1}) and the *A. fusiformis* strain showed intermediate sensitivity (IBU = 26.7 mg L^{-1} and DFN = 14.6 mg L^{-1}). The mixtures showed a synergistic effect on *R. subcapitata*, an additive effect on *A. fusiformis*, and an antagonistic effect on *T. obliquus*. Neither the CA nor the IA model was appropriate for predicting the toxicity of the mixtures. In conclusion, IBU and DFN showed toxic effects on the algae growth at the concentrations tested. Individual and mixed toxicity was different according to the species. These differences could be species-specific, showing the importance of including native strains in ecotoxicological studies to evaluate the potential effects of pollutants in regional aquatic environments.

Keywords: ecotoxicity, emerging pollutants, *Raphidocelis subcapitata*, *Ankistrodesmus fusiformis*, *Tetradesmus obliquus*

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INTRODUCTION

Pharmaceuticals consist of a large set of chemical substances widely used in human and veterinary medicine. Most of the drugs are not fully metabolized in the human body, so they are excreted in urine and faeces as a parent compound or its active metabolites. These residues tend to reach the aquatic environment through wastewater and sewage sludge (Jjemba, 2006). Developed countries have specific technologies for wastewater treatment that can reduce the pollutant load to the environment. However, those technologies are not fully effective in removing pharmaceuticals (Santos *et al*., 2010). Besides, most developing countries do not have adequate wastewater treatment technology. Pharmaceuticals and their metabolites are diluted in large freshwater bodies and, consequently, their concentration decreases several times. However, these compounds constantly enter and contaminate freshwater ecosystems worldwide (Zuccato *et al*., 2006; Kümmerer, 2009; Hejna *et al*., 2022), so that non-target aquatic organisms are subjected to the chronic effects of these biologically active products (Grzesiuk *et al*., 2018).

Ibuprofen (IBU) and diclofenac (DFN) are non-steroidal anti-inflammatory drugs (NSAIDs), often purchased in pharmacies or shops through free sale (Li, 2014). The mode of action of IBU and DFN is the non-selective inhibition of cyclooxygenase (COX), an enzyme involved in prostaglandin synthesis (Geiger *et al*., 2016). These drugs usually contain functional groups (aromatic rings, -F, -Cl, $-CF_3$, etc.) highly resistant to metabolism, which increases their half-life in the human body and prolongs the degradation time in the environment (Bácsi *et al*., 2016). Thus, they are stable molecules that can persist in natural water bodies for periods of time ranging from days to months (Benotti & Brownawell, 2009; Yamamoto *et al*., 2009). IBU has been found in European surface waters in concentrations of 1.53 up to 9.89 µg L^{-1} (Helenkár *et al*., 2010; Ginebreda *et al*., 2010), and DFN in concentrations of 0.24 up to 15 µg L^{-1} (Jux *et al*., 2002; Ashton *et al*., 2004; Helenkár *et al*., 2010). On the other hand, IBU and DFN have been found in Latin America surface waters in concentrations of 0.96-785.28 and 0.05-759.06 µg L^{-1}, respectively (Elorriaga *et al*., 2013; Ferreira do Nascimento, 2023).

Several studies have shown that IBU and DFN can produce adverse effects on the growth and metabolism of algae and plants. For example, growth inhibition in *Chlorella vulgaris* (Geiger *et al*., 2016), changes of biochemical parameters in *Chlamydomonas reinhardtii* (Seoane *et al*., 2023), and growth inhibition in the duckweed *Lemna minor* (Pomati *et al*., 2004) were reported. DFN produces growth inhibition in *Desmodesmus subspicatus* (EC_{50} = 60.44 mg L^{-1} (Doležalová Weissmannová, 2018) and changes in biochemical parameters of *Lemna minor* (Alkimin *et al*., 2019). A recent review summarized studies on the impact of NSAIDs on aquatic animals (Świacka *et al*., 2021). For example, IBU concentrations higher than 10 µg L^{-1} resulted in decreased hatching and growth rates in the zebrafish *Danio rerio*, as well as anomalies and malformation during the development of its embryos (Anuradha & Pancharatna, 2009). Chronic exposure of the microcrustacean *Daphnia magna* to IBU (up to 80 mg L^{-1}) significantly reduced population growth rate (Heckmann et al., 2007). DFN in water induce oxidative stress in the mussel *Mytilus galloprovincialis* (Gonzalez-Rey & Bebianno, 2014), inmobilization in the zebra fish *Danio rerio* (Bio & Nunes, 2020; Cleuvers, 2004) and changes in the defense system of the crustaceans *Daphnia magna* (Nkoom *et al*., 2019) and *Ceriodaphnia dubia* (Russo *et al*., 2023).

The role of phytoplankton in aquatic ecosystems is crucial, as it provides oxygen and food for other organisms, including fish and invertebrates. As primary producers in aquatic ecosystems, algae are particularly important for assessing the ecotoxicity of pollutants and their effects on water quality (Hu *et al*., 2017). Toxicity and risk assessment of chemicals on algae is usually performed using the algal growth inhibition test (OECD, 2006). The freshwater algae *Raphidocelis subcapitata* (= *Pseudokirchneriella subcapitata*) has been used as a model organism in several studies as it can be easily cultured, exhibits rapid reproduction and it is sensitive to xenobiotic pollutants (USEPA, 2002; Environmental Canada, 2007; ISO, 2019). Moreover, indigenous algae species representative of certain aquatic ecosystems may be more sensitive or tolerant to contaminants than the standard strain (Magdaleno *et al*., 2014; Carusso *et al*., 2018). In this way, the species isolated from natural environments could provide additional information on the effects of xenobiotics present in a certain region.

In 2012, around 70 million units of IBU and DFN, among others, were sold at the outpatient level in Argentina (Prozzi *et al*., 2018) and these two compounds were also detected simultaneously in some aquatic ecosystems of this country (Elorriaga *et al*., 2013). Therefore, surface waters are more likely to be polluted by different pharmaceuticals than single substances and these mixtures can possess additive, antagonistic or synergistic effects. So, the effects of mixtures need to be considered during ecotoxicological tests (Magdaleno *et al*., 2015; Carusso *et al*., 2018). Two different concepts are usually used for the prediction of toxicity of mixtures, the concentration addition (CA) and the independent action (IA) (Altenburger *et al*., 2004). CA is based on the proposal that the two compounds in a binary mixture have a similar site and mode of action within an organism, meaning that the effect of a mixture remains constant if one component is exchanged for an amount of the other component that causes an equal effect. On the other hand, the concept of IA assumes that the substances act independently and have different sites of actions. This concept implies that the effect of a single substance would not change in the presence of another substance (Brezovšek *et al*., 2014).

The aim of the present study was to analyse the adverse effects of the NSAIDs IBU and DFN, individually and in mixtures, on three distinct species of freshwater algae: the standard strain of the species *Raphidocelis subcapitata*, and two native strains of the species *Ankistrodesmus fusiformis*

and *Tetradesmus obliquus* from Argentina. The ecotoxicity of the binary mixtures was analysed using the CA and IA models for predictive approaches and the results were compared with the experimental data.

MATERIALS AND METHODS

Test chemicals

IBU (CAS no. 15687-27-1) and DFN (CAS no. 15307-86-5), with a purity grade > 98%, were purchased from Stanton (Química Córdoba S.A., Ciudad de Buenos Aires, Argentina). The stock solutions of IBU and DFN (1 g L^{-1}) used for bioassays were prepared below the water solubility limits of each compound using algal assay medium. For IBU, a concentrated solution (10 g L^{-1}) was previously prepared dissolving 0.01 g in 10 mL NaOH 4% p/v and then diluting 10 times in algal assay medium. Chemicals used for the High Performance Liquid Chromatography-Ultraviolet (HPLC-UV) were as follows: LC grade methanol, purchased from Sintorgan S.A. (BA, Argentina), and analytical grade formic acid, obtained from J.T. Baker (New Jersey, USA). The ultrapure water (conductivity of 0.055 µS cm^{-1}) used to prepare mobile phases and solutions was obtained from an EASY pure™ RF equipment (Barnstead, Dubuque, IA, USA).

HPLC-UV analysis

IBU and DFN concentrations were quantified in HPLC Spectra System SCM1000 (Thermo Scientific, Waltham, MA, USA) provided with Syncronis™ C18 column (150 × 4.6 mm, 5 µm-particle diameter, Thermo Scientific), and quaternary pump, P4000 degasser, AS3000 autosampler and UV2000 Dual λ absorbance detector. The mobile phase consisted of a mixture of methanol and water with 0.1% v/v formic acid (80:20 v/v) in isocratic mode. The flow rate was set at 1.0 mL min^{-1} and the column temperature was set at 35°C. Detection was performed at 220 nm wavelength. Chromatograms were processed using the ChromQuest Chromatography Data System software. Concentrations of each test solution were measured at the beginning and end of the experiments (72-h exposure) in order to evaluate the stability of drugs under bioassay conditions (i.e. losses due to volatilization, photo-oxidation or adsorption on the test vessel during the experiment). Analyses were performed in the absence of cells.

Strains, culturing conditions and laboratory experimental setup

The strains of *Raphidocelis subcapitata* (Korshikov) Nygaard, Komárek, J.Kristiansen and O.M. Skulberg 1987, *Ankistrodesmus fusiformis* Corda 1838 and *Tetradesmus obliquus* (Turpin) M.J.Wynne 2016 (Sphaeropleales, Chlorophyta) were obtained from the Culture Collection of the Protists Biology laboratory, Department of Biodiversity and Experimental Biology, belonging to the Genetic Resources Centre of the Faculty of Exact and Natural Sciences, Universidad de Buenos Aires. The native Argentine strains *A. fusiformis* (BAFC CA 11) and *T. obliquus* (BAFC CA 14) were isolated from polluted rivers in Buenos Aires province (Magdaleno *et al*., 2014; Afione Di Cristofano *et al*., 2021). The cultures were maintained under axenic growth conditions in Bold's Basal Medium (BBM) (Archibald & Bold, 1970), prepared in ultrapure water obtained from a Millipore Milli-Q system and maintained at pH 6.8. This medium contains macro (0.27 g L^{-1} $NaNO_3$, 0.075 g L^{-1} $MgSO_4.7H_2O$, 0.025 g L^{-1} NaCl, 0.075 g L^{-1} K_2HPO_4, 0.175 g L^{-1} KH_2PO_4, 0.025 g L^{-1} $CaCl_2.2H_2O$), and micronutrients salts (8.82 mg L^{-1} $ZnSO_4.7H_2O$, 1.44 mg L^{-1} $MnCl_2.4H_2O$, 0.71 mg L^{-1} MoO_3, 1.57 mg L^{-1} $CuSO_4.5H_2O$, 0.49 mg L^{-1} $Co(NO_3)_2.6H_2O$, 11.40 mg L^{-1} H_3BO_4, 50 mg L^{-1} Na_2EDTA, 31 mg L^{-1} KOH, 4.98 mg L^{-1} $FeSO_4.7H_2O$).

The bioassays were conducted in 125 mL flasks containing 20 mL of BBM medium with six different concentrations of IBU (from 10 to 250 mg L^{-1}) or DFN (from 5 to 250 mg L^{-1}), with an initial cell density of 1×10^5 cells mL^{-1}. Control cultures were performed under the same conditions without any drugs. Cells from exponential phase culture were used as inoculum. The flasks were incubated in a shaker thermal bath at 22±2 ºC and 210 rpm, with continuous cool white fluorescent light illumination (80 µmol photons m^{-2} s^{-1}). The experimental treatments were performed according to the standard algal growth inhibition test (USEPA, 2002). The pH values of the growth medium were measured with a Hanna pH-meter at the beginning and end of the bioassays. After 72-h, the cell number was evaluated by indirect measurement methods (absorbance 750 nm). The experiments were carried out in triplicate, with a minimum of five trials. Calibration curves of cell density (number of cells mL^{-1}) versus optical density (750 nm) were performed prior to the assays. Cell counts were carried out under an optical microscope using a Neubauer chamber. Binary mixtures were tested using combinations of the effective concentrations 20, 50 and 80 (EC_{20}, EC_{50} and EC_{80}) obtained for each individual drug.

Data analysis

The EC_{20}, EC_{50}, and EC_{80} values for each drug were determined by creating dose-response curves, which represented the percentage of algal growth inhibition in relation to the control at various concentrations. These curve data were fitted to a classical sigmoidal equation as follows:

$$Y = A_2 + [(A_1 - A_2)/(1 + (x/x_0)^d)] \qquad (1)$$

where d represents the slope parameter, x_0 is the midpoint of the curve, and A_1 and A_2 correspond to the upper and lower asymptotes, respectively.

Both graphics and equations were generated using the OrigenPro 8 program (OrigenLab Corporation, Northampton, Massachusetts, United States). One-way analysis of variance (ANOVA), followed by a Dunnett´s post hoc test, was performed to evaluate significant differences between each drug concentration and the control. A *p* value less than 0.05 was considered statistically significant. The NOEC of each drug was calculated using Dunnett's test. After the determination of single toxicity, mixture toxicity tests were performed to assess the effects of interactions between these drugs in mixtures of each of the EC_{20}, EC_{50} and EC_{80} combinations.

Mixture toxicity predictions based on CA and IA equations

Experimental toxicity of the binary mixtures was computed based on the predictive CA and IA additivity equations (Altenburger *et al*., 2004). The CA equation (1) was as follows:

$$EC_{x,\,mix} = \left(\sum_{i=1}^{n} \frac{C_i}{EC_{xi}}\right)^{-1} = 1 \qquad (2)$$

where *Ci* represents the individual concentrations of the single components present in a mixture with a total effect of *x%*, and *ECxi* are those concentrations of the single components that would alone cause the same effect *x* as observed for the mixture. According to Eq. (2), the effect of the mixture remains constant when one component is replaced by an equal fraction of an equally effective concentration of another.

The equation applied for the IA model (3) was as follows:

$$E_{(Cmix)} = 1 - [(1 - E(c_1))(1 - E(c_2))] \qquad (3)$$

where $E_{(Cmix)}$ is the effect caused by the total mixture at concentrations c_1 and c_2, and $E(c_1)$ and $E(c_2)$ are the effects that each individual component would cause if applied singly at the concentration at which they are present in the mixture.

The ECmix obtained for each binary mixture (combinations of the EC_{20}, EC_{50} and EC_{80} of each individual drug) was compared with the experimental results.

RESULTS

Single toxicity assessments

The HPLC-UV analysis revealed that none of the test concentrations varied more than 10% during the bioassays (Fig.1). Differences between nominal and measured concentrations were less than 5% at the beginning of the tests (Table 1). Nominal concentrations were used for data analyses.

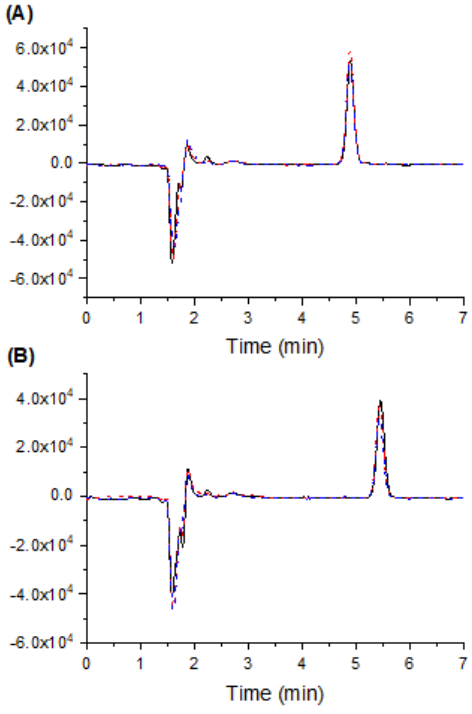

Figure 1. HPLC chromatograms: (A) Diclofenac and (B) Ibuprofen. Standard solution at 10 mg L^{-1} (___), test solution at the beginning (- - -) and at 72 hours (- - -) exposure.

Table 1: Ibuprofen and diclofenac content expressed as mean ± SD, n=3 and n=6 for T_0 (at the beginning of the test) and T_f(at 72-h exposure), respectively.

	Ibuprofen		Diclofenac	
	Nominal concentration (µg mL^{-1})	**Experimental concentration (µg mL^{-1})**	**Nominal concentration (µg mL^{-1})**	**Experimental concentration (µg mL^{-1})**
T_0	10.0	10.8 ± 0.2	10.0	11.30 ± 0.03
T_f		9.7 ± 0.3		9.9 ± 0.6
T_0	100.0	99.41 ± 0.04	100.0	98.2 ± 0.8
T_f		99 ± 2		104 ± 5

After 72 hours of growth, the controls reached a cell density of 3.41 ± 0.37 x10^6 cells mL^{-1} (*R. subcapitata*), 1.48 ± 0.54 x10^6 cells mL^{-1} (*A. fusiformis*), and 3.04 ± 1.56 cells mL^{-1} (*T. obliquus*). A clear dose-response effect was observed in algae exposed to increasing concentrations of IBU and DFN (Fig. 2). In the case of *R. subcapitata*, the IBU curve reached a plateau at nearly 60 mg L^{-1}and the DFN curve at nearly 20 mg L^{-1}, showing that DFN was more toxic than IBU for this strain (Fig. 2a). The EC_{20}, EC_{50} and EC_{80} values obtained from the sigmoidal equation are shown in Table 2. The EC_{50} for IBU was higher than for DFN. In the case of *A. fusiformis*, growth inhibition by exposure to the two NSAIDs was similar to that obtained for *R. subcapitata*, The IBU and DFN curves reached a plateau at nearly 60 and 40 mg L^{-1}, respectively, showing that DFN was more toxic than IBU for this strain (Fig. 2b). The EC_{50} values for IBU and DFN were 26.67 ± 2.75 and 14.57 ± 1.89 mg L^{-1}, respectively (Table 2). *T. obliquus* showed a different pattern of growth inhibition. The IBU curve reached a plateau at nearly 100 mg L^{-1}, and DFN at nearly 160 mg L^{-1}, showing that IBU was more toxic than DFN for this strain (Fig. 2c). According to the EC_{50} values (Table 2), *T. obliquus* was much more resistant to both NSAIDs than the previous strain (IBU = 74.09 ± 8.16 mg L^{-1} and DFN = 92.71 ± 6.07 mg L^{-1}).

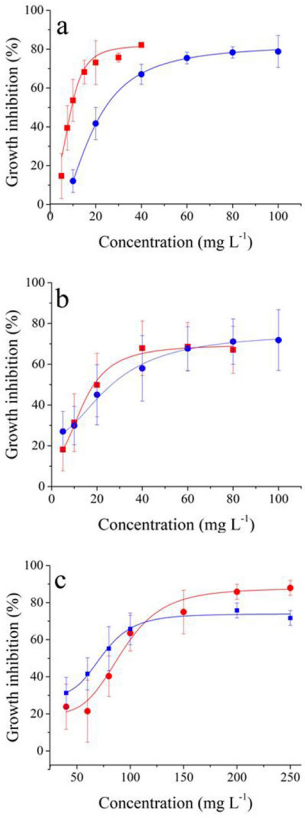

Figure 2. Dose-response curves of ibuprofen (—●—) and diclofenac (—■—) for R. *subcapitata* (a), A. *fusiformis* (b), and T. *obliquus* (c). Results are expressed as means and error bars represent the standard deviation.

Table 2. Effective concentrations of ibuprofen and diclofenac in *R. subcapitata*, *A. fusiformis*, and *T. obliquus*. EC values corresponds to the mean of five tests with the standard deviation (n=5). No Observed Effect Concentrations (NOEC) of each drug was calculated using Dunnett's test.

	EC_{20} (mg L^{-1})	EC_{50} (mg L^{-1})	EC_{80} (mg L^{-1})	NOEC (mg L^{-1})
Ibuprofen				
R. subcapitata	11.78 ± 2.39	20.05 ± 5.14	34.26 ± 10.94	10
A. fusiformis	13.43 ± 1.87	26.67 ± 2.75	52.96 ± 10.31	10
S. acutus	56.52 ± 9.29	74.09 ± 8.16	97.13 ± 11.38	40
Diclofenac				
R. subcapitata	5.78 ± 1.45	8.03 ± 1.5	11.28 ± 1.76	7,5
A. fusiformis	8.31 ± 2.08	14.57 ± 1.89	25.88 ± 4.63	10
S. acutus	69.63 ± 9.24	92.71 ± 6.07	123.43 ± 9.98	80

Mixture toxicity assessments

To analyse the joint effect of both compounds, the three strains of green algae were exposed to EC_{20}, EC_{50} and EC_{80} of IBU and DFN simultaneously. In the case of *R. subcapitata*, the toxicity of the IBU+DFN mixture was significantly higher (p<0.05) than that obtained for each individual compound (Fig. 3a). This suggests a synergistic effect at all three concentrations tested. On the other hand, only in *A. fusiformis* was the EC_{20} mixture significantly higher (p<0.05) than that obtained for IBU or DFN individually (Fig. 3b). Thus, a synergistic effect would only occur at low concentrations. On the contrary, the toxicity of the EC_{50} and EC_{80} mixtures was significantly lower (p<0.05) than that obtained for each individual compound in *S. acutus* (Fig. 3c). This suggests an antagonistic effect at high concentrations in this strain.

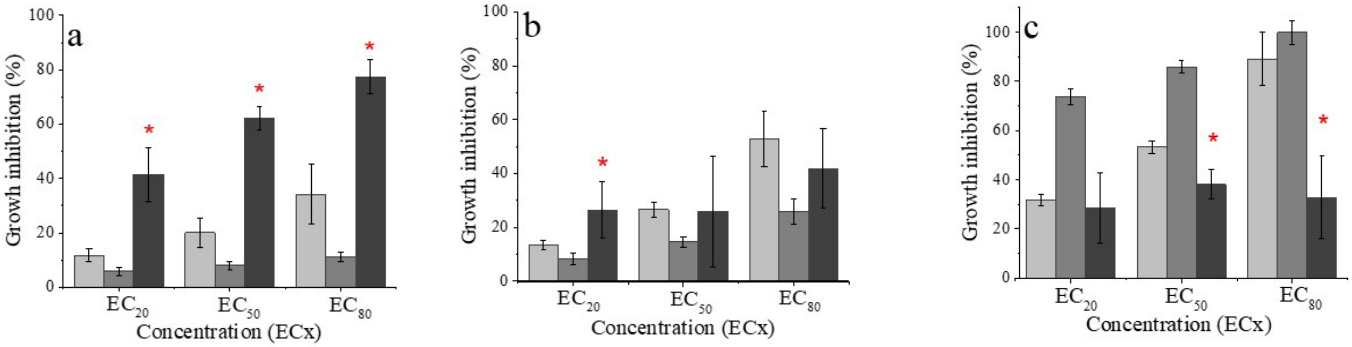

Figure 3. Toxicity of single ibuprofen) and diclofenac), and their binary mixtures) at EC_{20}, EC_{50} and EC_{80}, in R. *subcapitata* (a), A. *fusiformis* (b), and T. *obliquus* (c). Results are expressed as means and error bars represent the standard deviation. *p<0.05.

The predictive concentration-response based on the CA and IA models was compared to the concentration-response values obtained experimentally (Fig. 4). Neither the CA nor the IA model was suitable for predicting the joint effect of the three mixtures on *R. subcapitata* and *T. obliquus*. In the first case, the two models underestimated toxicity by more than 40%, whereas in the second case the two models overestimated toxicity by more than 50% at EC_{50} and EC_{80} (Fig.4a,b). In the case of *A. fusiformis*, both models were able to predict the results obtained experimentally by mixing the EC_{50} of each drug. However, only the IA model was able to predict the experimental joint effect of the EC_{20} mixture, while the CA model could predict to the EC_{80} mixture (Fig.4c).

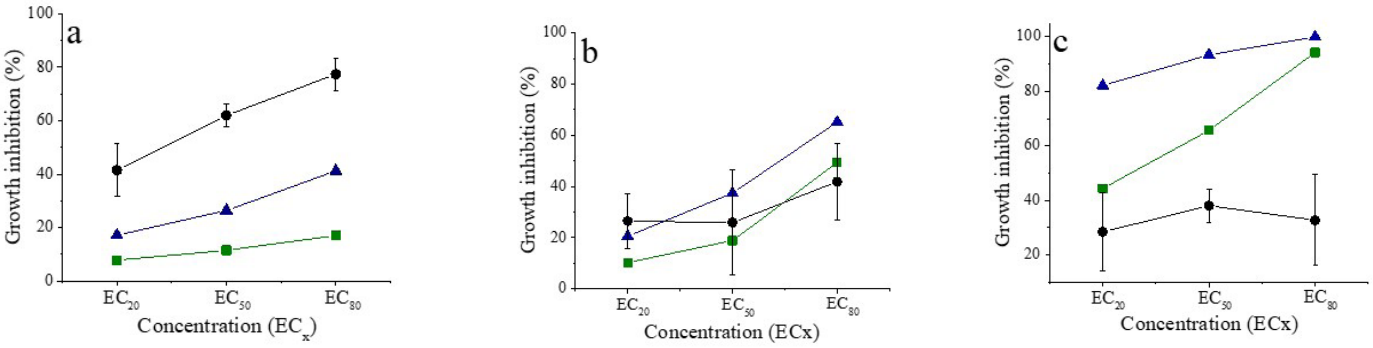

Figure 4. Toxicity of ibuprofen+diclofenac mixtures in *R. subcapitata* (a), *A. fusiformis* (b), and *T. obliquus* (c). The curves represent the experimental (Exp) measurements (—●—), the concentration addition model (—■—), and independent action model (—▲—). Exp = mean ± SD, n=5.

DISCUSSION

Single toxicity assessments

In the present study, IBU and DFN showed different toxicity to the three selected representative species of freshwater phytoplankton. As expected, the standard species *R. subcapitata* was the most sensitive species to exposure to the two drugs (Table 2). In the traditional toxicity test, EC_{50} was widely used as the endpoint to evaluate the algal growth inhibition effects and compare their toxicity sensitivity (Yang *et al*., 2008). Therefore, the EC_{50} values obtained for the three microalgae exposed to IBU (20 − 74 mg L^{-1}) were lower than that obtained by Blaise *et al*. (2006) for *R. subcapitata* after 72-h exposure (90.5 mg L^{-1}). On the other hand, *T. obliquus* was the most resistant strain to IBU, according to the three EC_x values (EC_{20}=56.5, EC_{50}=74.1, and EC_{80}=97.1 mg L^{-1}). These results were even lower than those obtained by Cleuvers (2004) for the species *Desmodesmus subspicatus*, whose values were EC_{20}=155.5, EC_{50}=342.2, and EC_{80}=753.2 mg L^{-1}.

In the case of DFN, the most sensitive strain was *R. subcapitata*, followed by *A. fusiformis* and *T. obliquus*, in that order (Table 2). The EC_{50} obtained for these three strains (8.0, 14.5, and 92.7 mg L^{-1}, respectively) were even lower than those obtained by Wimmerova *et al*. (2022) for *R. subcapitata* at 72 h exposure (177.68 mg L^{-1}), and Majewska *et al*. (2018) for *Chlamydomonas reinhardtii* (135.0 mg L^{-1}). The three EC_x values for *T. obliquus* (EC_{20}=69.6, EC_{50}=92.7, and EC_{80}=123.4 mg L^{-1}) were higher than for *D. Subspicatus*: EC_{20}=56.1, EC_{50}=71.9, EC_{80}=92.2 mg L^{-1} (Cleuvers, 2004) and EC_{20}=30.9, EC_{50}=60.4 mg L^{-1} (Doležalová Weissmannová, 2018). On the other hand, according to Cleuvers (2004), DFN was more toxic than IBU for *D. subcapitatus*, as were the results obtained for *R. subcapitata* and *A. fusiformis* in this study. On the contrary, IBU was more toxic than DFN for *T. obliquus*. These results demonstrate the existence of interspecific variations regarding the response to contaminants.

According to the classification of the toxicity level of substances by the EU Directive 67/548/EEC for aquatic organisms and the EC_{50} values obtained for the algal strains, IBU could be classified as a harmful substance (EC_{50}, 10−100 mg L^{-1}), whereas DFN could be classified as a toxic (EC_{50}, 1−10 mg L^{-1}) or harmful substance (Table 2). This classification is made based on experimental data for acute aquatic toxicity (short-term responses with acute effects on test organisms). The sensitivity of the native strain *A. fusiformis* was very similar to that obtained for *R. subcapitata*, considering the EC_{20} and EC_{50} values (Table 2). This strain, isolated from a highly polluted Argentine river, was also the most sensitive strain among different species of native green algal when exposed to increasing concentrations of the heavy metals cadmium, copper and zinc (Magdaleno *et al*., 2014). On the other hand, the NOEC values (IBU: 10 – 40 mg L^{-1}; DFN: 7.5 – 80 mg L^{-1}) (Table 2) showed that *S. acutus* was the most resistant strain to the NSAIDs exposition. The NOEC values could be considered a long-term result with chronic effect (Ferrari *et al*., 2003).

The three algal strains showed different sensitivities to the two NSAIDs tested. These results show that pharmaceutical effects are species-specific and, hence, some species such as *T. obliquus* may develop recovery abilities under a certain stress. Perales-Vela *et al*. (2006) demonstrated that some of those abilities could depend on various factors, such as acclimatization or genetical adaptation to the new environmental conditions. Although the results were obtained under laboratory conditions, they could serve as a reference to evaluate the possible effects of these drugs on aquatic ecosystems, altering their natural balance. Direct effects of pharmaceuticals on species may, in turn, indirectly affect other species in the ecosystem, e.g., through toxic effects on growth and survival, inhibitory effects on organism physiology, disruption of chemical communication within and between species (van Donk *et al*., 2015) or by crossing trophic levels and moving up the food chain (Grzesiuk *et al*., 2018). According to the EC_x and NOEC values (Table 2), the three microalgae could tolerate concentrations much higher than those reported so far in Latin American surface waters (up to 0.78 and 0.76 mg L^{-1}, respectively) (Ferreira do Nascimento, 2023). In any case, the toxicity data highlight the risk to which aquatic ecosystems are exposed, for example, at times of effluent discharges containing a high concentration of drugs.

Binary mixture toxicity

The predominant regulatory approach for managing chemical compounds is the assessment of individual substance toxicity (EC, 2006). Nonetheless, in aquatic environments, organisms are typically exposed to a blend of various chemicals simultaneously. In the case of NSAIDs, both IBU and DFN have been detected simultaneously in surface waters (Elorriaga *et al.,* 2013; Ferreira do Nascimento, 2023). Therefore, binary mixtures of IBU+DFN were tested in order to obtain more realistic information on the possible effects of these mixtures on algae. In this study, an experimental approach combining the two drugs in equal ratios of their individual EC_{20}, EC_{50} and EC_{80} was used for testing the predictive values of a mixture toxicity model. The IBU+DFN mixtures exerted a synergistic effect on *R. subcapitata* (Fig. 3a). However, neither the CA model nor the IA model could predict these experimental results (Fig. 4a). This synergistic effect could be due to two different forms of action: (1) one drug could enhance the activity or level of another drug in the mixture, or (2) the two drugs could act on the same target at different sites, at overlapping sites or on different targets of the same pathway (González-Pleiter *et al*., 2013).

On the contrary, the IBU+DFN mixtures exerted toxicity on *T. obliquus* compatible with an antagonistic effect between their components (Fig. 3c). These experimental results showed lower algal growth inhibition than the CA and IA model predictions (Fig. 4c). This means that the presence of one drug reduces the action of the other in the mixture. According to the results obtained in the present study, *T. obliquus* was the most resistant strain when it was exposed to individual action of the drugs. At the same time, the joint action of IBU+DFN was significantly reduced, which contributes to the drug resistance of this strain. In the case of *A. fusiformis*, only the EC_{20} mixture showed a synergistic effect (Fig. 3b), which could be explained by the IA model (Fig. 4b). On the other hand, the algal growth obtained in the EC_{50} mixture could be predicted by both the CA and IA models, whereas in the EC_{80} mixture by the CA model. Since these trials were carried out under high nutrient concentrations (BBM medium), the growth inhibition effects on algae were only due to variations in drug concentrations. These results again show that pharmaceuticals effects are species-specific. Some species may develop recovery abilities under certain stress, and/or may have physiological characteristics that result in them having greater resistance to stress.

Among the three strains used in this study, *T. obliquus* showed the highest resistance and *R. subcapitata* the highest sensitivity to IBU and DFN. The *T. obliquus* resistance could be due to certain species-specific mechanisms that allow it to grow in environments with adverse conditions. It has also been proposed that the difference in the sensitivity of the different species/strains of microalgae may be due to morphological and structural characteristics such as size, cell volume and the presence or absence of a cell wall, and its chemical composition (Xin *et al*., 2021). According to the taxonomic morphometric descriptions, *R. subcapitata* is smaller in size and volume (2.1 to 32.9 μm^3) than *T. obliquus* (14.6 to 735.9 μm^3) and the later, is smaller than *A. fusiformis* (149.2 to 1003.5 μm^3). These characteristics could explain, in part, the lowest resistance of *R. subcapitata* to IBU and DFN. However, *A. fusiformis* showed greater sensitivity than *T. obliquus*. The species in this genus are characterized by highly resistant cell walls composed of the hydrophobic polymer "algaenan" (Soeder & Hegewald, 1988, Schiariti *et al*., 2004), which could confer certain resistance to the entry of contaminants inside the cell.

CONCLUSION

IBU and DFN are toxic to the three strains belonging to different species of freshwater algae. Both the standard strain *R. subcapitata* and the native strain *A. fusiformis* showed higher sensitivity to IBU and DFN than the native strain *T. obliquus*. These differences could be due to species-specific variations and different morphological and physiological mechanisms of tolerance to contaminants. The three algal strains also showed different responses to binary mixtures of IBU+DFN. These mixtures showed a synergistic effect on the growth of *R. subcapitata*, while they showed an antagonistic effect on *T. obliquus*. The CA and IA models could not predict the results obtained experimentally for these two strains. In *A. fusiformis*, the effect of IBU+DFN mixtures is mainly additive, which can be explained by the CA and IA models. These results also show the importance of analysing responses of native strains in ecotoxicological studies to obtain more information about the potential effects of pollutants in regional aquatic environments.

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AUTHOR STATEMENT

No potential conflict of interest was reported by the author(s).

The authors confirm that the data supporting the findings of this study are available within the article.

AUTHORS CONTRIBUTIONS

All authors contributed to the study conception and design.

M. T.: Conceptualization, Methodology, Investigation. **M. M.:** Conceptualization, Methodology, Supervision. **V. T.:** Supervision, Funding acquisition. **A.B. J.:** Writing-Reviewing and Editing. Anahí Magdaleno: Conceptualization, Data curation, Writing- Original draft preparation.

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